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Extracellular acidity and increased exosome release as key phenotypes of malignant tumors

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Abstract
The tumor milieu is characteristically acidic as a consequence of the fermentative metabolism of glucose that results in massive accumulation of lactic acid within the cytoplasm. Tumor cells get rid of excessive protons through exchangers that are responsible for the extracellular acidification that selects cellular clones that are more apt at surviving in this challenging and culling environment. Extracellular vesicles (EVs) are vesicles with diameters ranging from nm to μm that are released from the cells to deliver nucleic acids, proteins, and lipids to adjacent or distant cells. EVs are involved in a plethora of biological events that promote tumor progression including unrestricted proliferation, angiogenesis, migration, local invasion, preparation of the metastatic niche, metastasis, downregulation or hijacking of the immune system, and drug resistance. There is evidence that the release of specific exosomes is increased many folds in cancer patients, as shown by many techniques aimed at evaluating "liquid biopsies". The quality of the exosomal contents has been shown to vary at the different moments of tumor life such as local invasion or metastasis. *In vitro* studies have recently pointed out that cancer acidity is a major determinant in inducing increased exosome release by human cancer cells, by showing that exosomal release was increased as the pH moved from 7.4 pH to the typical pH of cancer that is 6.5. In this review, we emphasize the recent evidence that tumor acidity and exosomes levels are strictly related and strongly contribute to the malignant tumor phenotypes.

Keywords Acidity · Exosomes · Liquid biopsy · Microenvironment · NTA (nanoparticle tracking analysis) · Biomarkers

1 Introduction

Tumors are characterized by unique properties including loss of contact inhibition, uncontrolled growth, irregular angiogenesis, insufficient oxygen and nutrient supply, local invasion, and distant metastasis [1–10]. These characteristics lead to the generation of a very competitive environment that is frequently lethal to normal cells [1–10]. When tumor mass exceeds a certain given volume, due to the irregular arborization of blood vessels, whole areas of neoplastic tissue do not receive adequate supply of oxygen and nutrients, thus dying and becoming necrotic [11–14]. The cells at the periphery of these areas revert to anaerobic metabolism of glucose to cope with the lack of nutrients and to generate ATP. Interestingly, this phenomenon is not restricted to the so-called hypoxic areas of cancer, but actually, this metabolic shift happens at a very initial stage of tumor development [2–5]. Cancer cells metabolize greater quantities of glucose, lactate, pyruvate, hydroxybutyrate, acetate, glutamine, and fatty acids than do normal cells [1–3]. This conversion despite deceptively appearing to be energetically inefficient, nonetheless, allows cancer cells to synthetize ATP molecules while simultaneously maintaining the redox balance and leaving a significant amount of substances to anabolic processes central to survival, proliferation, and dissemination [15, 16]. The anomalous anaerobic metabolism of tumor results in massive generation and release of lactate that ultimately leads to extracellular acidification (Warburg effect) (Fig. 1) [1–10, 15, 16]. Tumor adaptation to hypoxia within its microenvironment involves a switch of the transcription factor hypoxia-inducible factor-1a (HIF-1a) [17]. Affecting nearly all aspects of cancer cell phenotype, HIF-1a is also a pivotal controller of cancer cell metabolism [18, 19]. Lactate dehydrogenase isoform A (LDHA), a key HIF-1a target, promotes the reduction of pyruvate to lactate and supports the endurance of hypoxic cells counterbalancing for the decrease in oxidative mitochondrial

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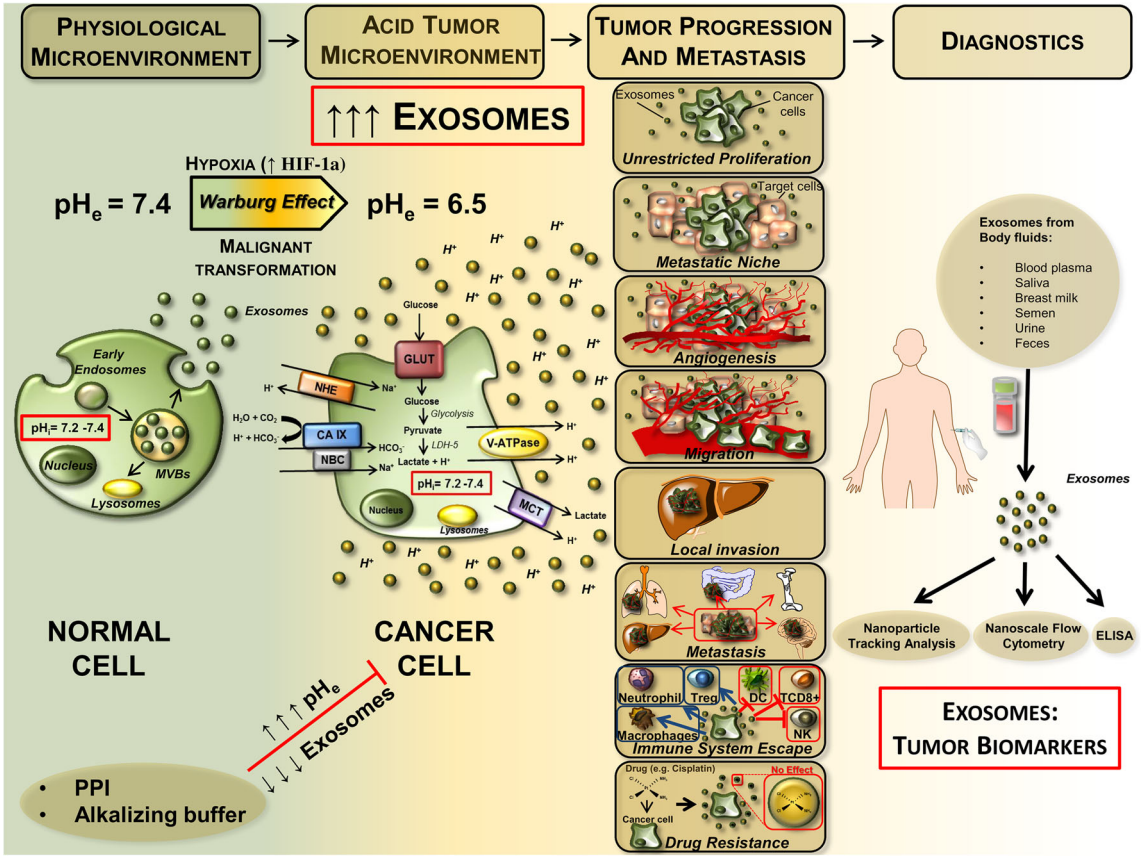

Fig. 1 Effect of acid tumor microenvironment on the release of exosomes by cancer cells and the role of exosomes in tumor progression and diagnostics. Tumor acidity is a phenotype common to almost all metastatic tumors as a consequence of the anaerobic metabolism of glucose, triggered by hypoxia, with consequent massive accumulation of lactate within the cytoplasm (Warburg effect). Cancer cells extrude excess protons through exchangers such as Vacuolar ATPase (V-ATPase), Na+/H+ exchanger (NHE), monocarboxylate transporters (MCTs), cotransporter sodium bicarbonate (NBC), and carbonic anhydrase 9 (CA IX) that are responsible for extracellular acidification around pH 6.5. Acid microenvironment selects the cell clones that are best suited to survive in this hostile environment. Results of this environmental pressure lead to the increase in exosome release and chemoresistance by cancer cells. Exosomes are extracellular vesicles of 40–180 nm secreted by all cells both in physiological and in pathological conditions. Exosomes originate from the early endosomes that mature in multivesicular bodies (MVBs) which, merging with the plasma membrane, release the exosomes in the extracellular space. Increased levels of exosomes by cancer cells play a key role in tumor progression promoting tumor growth and metastasis, through unrestricted proliferation, preparation of the metastatic niche, angiogenesis, migration, local invasion, metastasis, immune system escape, and drug resistance. Increased exosome release may well be included in the list of phenotypes common to all cancers. Therefore, measurements of exosomes plasmatic levels by nanoparticle tracking analysis, nanoscale flow cytometry, and ELISA may represent a new and non-invasive diagnostic tool with a potentially high clinical impact in terms of early diagnosis and ease of response monitoring collecting diagnostic samples from body fluids including blood, saliva, cerebrospinal fluid, breast milk, and urine. An antiacidic approach, through proton pump inhibitors (PPIs) and buffers, increasing the extracellular pH induces a reduction in the release of exosomes. It can represent a winning strategy against tumors both in prevention and treatment

effectiveness [20]. As above reported, while the metabolic shift of hypoxic cells towards anaerobiosis is easily understandable, the cancer cells' tendency to ferment glucose, even in normoxic conditions, a phenomenon generally known as "the Warburg effect," remains largely unraveled. The biological evidence of the connection between oncogenic mechanisms and the Warburg effect was provided by the discovery that the oncogene c-MYC is involved in the transcriptional regulation of LDHA under normoxic conditions [21]. A major feature of the Warburg effect is the generation and release of large volumes of H+ into the extracellular compartment, resulting in its acidification [1–10]. Additional protons are generated and released by oxygenated cancer cells due the high levels of carbonic dioxide produced during mitochondrial respiration. The biochemical complete conversion of one molecule of glucose into carbonic dioxide yields 6 HCO3− and 6 H+, generating a three times greater amount of H+ than anaerobic glycolysis [15, 16]. However, this may represent a further feature of cancers that may distinguish them from the normal tissues; in fact, this condition may reduce the available protons for ATP in turn generating an entirely deranged metabolism [22]. One fascinating theory is that the highly competitive microenvironment generated by acidification culls the weakest cells leaving only those more adept at surviving [23].

In order to prosper in such this unique aggressive milieu, tumor cells must overexpress large amounts of proton extruders [1–5, 15, 16]. These mechanisms mainly include V-ATPase, Na+/H+ exchanger (NHE), monocarboxylate transporters (MCTs), and carbonic anhydrase 9 (Fig. 1) [1–5, 15, 16, 24] that actively extrude excess protons, avoiding intracellular accumulation of toxic molecules, thus showing similarities with unicellular microorganisms. We have shown that cancer cells to survive and propagate in a very hostile microenvironment behave like unicellular microorganisms, sharing with amoebae a transmembrane protein TM9SF4 [25, 26]. TM9SF4 is involved to the cannibal behavior of these cells [26]. In fact, cancer cells feed by "cannibalizing" other cells, either dead or alive above all in low nutrient supply or low pH conditions, suggesting its key survival option for malignant cancers [25–28].

Recent investigations pointed out that cancer cells can pervert the functions of normal cells and transform the extracellular matrix (ECM) to suit their purposes through several pathways that generate the tumor microenvironment [29–34]. This process happens through the secretion of soluble mediators such as growth factors, cytokines, proteins, and metabolites that together create a "tumor niche" promoting tumor proliferation and diffusion [31–34]. Among these mediators, the release of extracellular vesicles (EVs), and in particular exosomes, has recently been recognized as a parallel messaging system within the tumor niche [31–35]. Exosomes are natural nanoparticles (40–180 nm) which are released from all cell types within the body both under normal and pathological conditions [31–34, 36]. Their cargo is made up by different molecules including nucleic acids, proteins, and lipids [37, 38]. Exosomes are part of a broader array of extracellular vesicles (EVs) together with microvesicles (from 180 nm to 1 μm) and apoptotic bodies (from 1 to 5 μm), each one with its own peculiar biophysical, biochemical, and molecular composition [37–42]. The lipid bilayer surrounding the exosomes is particularly rich in cholesterol and sphingomyelin, usually copious in lipid rafts [43], in ceramide, important for the loading of microRNAs into exosomes [44–46] and in Bis (monoacylglycero) phosphate (BMP) [36, 47]. Exosomes also transport different types of molecular cargos (lipids, proteins, DNA, mRNA, micro RNA), to their ultimate cellular destination, thus affecting physiological or pathological processes, including tumor progression [48–56]. Besides their delivery functions, exosomes work as an additional framework within organs and compartments [38]. Matrix cells in the tumor milieu interact with their neoplastic counterparts through exosomes and are deeply involved in tumor evolution and progression [35, 57–60]. The lipid component of tumor exosomes can facilitate miRNA release into the tumor microenvironment [61, 62]. Specifically, tumor released exosomes and their cargo are strongly involved in local invasion, evasion from the immune surveillance, angiogenesis, preparation of the metastatic niche, and metastasis (Fig. 1) [25, 29, 35, 63]. As previously reported, the tumor microenvironment is typically acidic and extracellular acidity is a common phenotype of almost all tumors [2–5]. The current orientation is to consider the acidic extracellular microenvironment a distinct independent feature of malignancy, which cannot be isolated from the intrinsic phenotypic and molecular characteristics of cancer cells [2–5]. Tumor pH may range from 6.0 to 6.8, and the level of acidity is directly associated to the tumor level of malignancy [1–5, 64–68]. The extracellular acidity exerts a culling action selecting cancer cells that are able to survive in such a hostile microenvironment. Results of this environmental pressure lead to the increase in exosome release [36, 43, 63, 69] and chemoresistance [63]. Several features of the tumor microenvironment could be the cornerstone of the paracrine regulation of exosome traffic within the tumor mass. For example, hypoxia and acidity are key features in cancer that could affect exosome release, promoting and sustaining local invasion and metastasis and thus acting as a mechanism of neoplastic paracrine diffusion [35, 42–44]. For instance, *in vitro* experiments have shown that melanoma cells cultured at pH 6.5 release a consistently larger amount of exosomes, than when cultured at pH 7.4 [36, 43, 69], mimicking what plausibly happens to cancer patients [36, 69]. These data have been recently confirmed by a study that clearly showed a marked increase of released exosomes under acidic pH (6.5) as compared to a physiological pH (7.4), independently of the tumor histotype (metastatic prostate carcinoma LNCaP, Metastatic melanoma Me30966, SaOS2 osteosarcoma, SKBR3 metastatic breast adenocarcinoma, and HCT116 colorectal carcinoma) [36]. These experiments were performed using the NTA technology (nanoparticle tracking analysis) together with the nanoscale flow cytometry [36, 70–72], to quantify the released exosomes, thus providing clear experimental evidence on the crucial role of tumor pH in regulating exosome production and release.

2 Role of acidity in exosome release and metastasis progression

These investigations have underscored the crucial function of tumor acidity as a regulator of exosomal trafficking in tumors, as well as autophagy and chemoresistance [26–28, 36, 43, 73, 74]. Exosomes play a key role in tumor growth and metastasis through the formation of "tumor niches" in target organs, inducing malignant transformation in resident mesenchymal stem cells (Fig. 1) [29, 35]. Recent evidence suggests that tumor exosomes have the power to transform mesenchymal stem cells into tumor-like cells [75], suggesting that these nanovesicles may have an additional role in the development

of metastasis in target organs, than merely generating a pre-metastatic niche [35].

Therefore, exosomes may contribute in transforming the microenvironment of primary tumors to favoring the selection of cancer cells with a metastatic behavior [29]. For example, it has been shown that microenvironmental acidity not only enhances the release of exosomes by human melanoma cells but also promotes significant modifications in the lipid component of the same particles [43]. The quantitative releases of exosomes were significantly downgraded by selectively countering acidity [36, 43]. Recent reports describe an increased level of exosomes in cancer patients compared to non-tumor bearing individuals [63, 69, 76–88]. However, reports in melanoma and in prostate carcinoma patients have been provided convincing number while using different techniques [69, 89]. More in details, prostatic carcinoma release of PSA-carrying particles has been analyzed by three different methods, such as NTA, nanoscale flow cytometry, and immunocapture-based ELISA, all showing a significantly greater content in exosomes from cancer patients [69]. This condition was strictly dependent on acidic milieu *in vitro* and was also measured in the plasma of prostate cancer patients [69]. This pilot clinical study has become a clinical study, and the results of this investigation will be published within 2019. However, in Logozzi et al. [69], it appears clear that the extracellular acidity of malignant tumor may represent a pivotal factor in inducing an huge amount of exosomes that from the tumor site are continuously spilled over the blood stream. To support this consideration in a previous study in melanoma, a preclinical experiment in xenograft models has shown that the exosome plasmatic levels are significantly related with the tumor mass [89], and this data was consistent with the clinical numbers in melanoma patients [89].

The experimental evidence supports a key role of the acidic tumor milieu in boosting exosome release from malignant cells. It appears conceivable that the exosome hyperproduction may be induced by the toxic microenvironment that probably select cells that use vesicles release as a primeval function, i.e., to eliminate toxins or to avoid intracellular accumulation of toxins, including H^+. The paracrine accumulation of exosomes within tumors leads to the exosome dissemination through the blood stream, thus contributing to both tumor progression and metastasis. The exploitation of this condition for diagnostic/prognostic purposes as well as its therapeutic reversal will represent one of the most intriguing and promising frontiers in cancer medicine [36, 43, 76–88, 90].

3 Discussion

A common feature of almost all tumors is the extracellular acidity secondary to the H^+ buildup caused by sugar fermentation and proton exchangers activity [1–10]. Recent reports have shown that the extracellular acidity of the tumor microenvironment increased the release of exosomes [36, 43, 69, 91, 92], while also hypoxia may contribute in triggering exosome release by tumor cells [93]. This represents a major switch towards uncontrolled growth, local invasion, and metastasis that mark the clinical progression. In fact, tumor-released exosomes have shown to contribute not only to the formation of the so-called metastatic or pre-metastatic niche, but to induce a tumor-like transformation in resident cells of metastatic organs, e.g., mesenchymal stem cells [41, 75, 94]. Increased exosome release induced by extracellular acidity may represent an adaptive mechanism of cancers to elude the immune system [95–97] and currently adopted anti-cancer therapies [63, 98] . The ability of thriving in anaerobic conditions is one of the main advantages of cancer cells, having a rapid production of energy combined with acidification of the extracellular milieu. As a consequence, the metabolic *tumor microenvironment* (TME) is characterized by severe hypoxia (in certain districts even anoxia), extracellular acidosis, considerably increased adenosine (ADO) and lactate concentrations, and nutrient deprivation [11–15]. Tumor hypoxia and acidity crucially contribute to genetic instability, tumor cell heterogeneity, local invasion, tumor stem cell maintenance, neoangiogenesis, chemo and radioresistance, and metabolic reprogramming, but also to tumor immune escape [1–15, 99]. There is clear evidence that tumor acidity promotes an increase in exosome release in human tumor cells of different histotypes including prostate cancer, melanoma, osteosarcoma, and colon and breast cancer [36]. We want to emphasize here that the same human tumor cell lines released higher amount of exosomes when cultured at pH of 6.5 as compared to the same cells at 7.4. Moreover, the exosomes released at low pH showed homogenously a lower size as compared to those released at high pH, which showed a more heterogeneous size [36]. These results not only support the importance of the acidic microenvironment in increasing exosome release: It actually generates some problems in accepting all the data collected in the last decades using human tumor cell lines cultured at the pH of 7.4, which is a non-physiological pH for cancers. The mechanism underlying the increased release of nanovesicles in acidic condition is not known; however, one of the most important and recognized function of exosome is also to eliminate toxics [36, 63, 100] including chemotherapeutics [63]. It is highly likely that low pH may prompt an amplified exosome extrusion with detoxifying goals. The results of *in vitro* studies suggest that the selection made by the acidic microenvironment may result in an abnormal release of exosomes expressing different cargoes including some known tumor biomarkers such as PSA [69], CEA [101, 102], or MART-1 [103]. The intriguing hypothesis is that the increased exosome levels, including those expressing tumor biomarkers, may result from the microenvironmental pressure due to the low pH [69]. More

recently, the expression and activity in tumor exosomes of surrogate tumor markers, such as Carbonic Anhydrase IX, have been shown to be increased under acidic conditions [104]. Furthermore, it has been demonstrated in a xenograft model that plasma levels of extracellular vesicles were related to the tumor size [89], thus further supporting the use of a plasma test based on exosome characterization and quantification as a useful new tool in clinical oncology [105]. Several clinical studies have suggested that measurements of plasma levels of exosomes may represent a new and non-invasive diagnostic tool in the laboratory follow-up of patients with tumors of different histologies [89, 101–103, 106]. However, exosomes have been purified from the vast majority of body fluid including not only blood but also saliva, cerebrospinal fluid, breast milk, and urine [76–90, 101–103, 106, 107], thus providing a potential source of new biomarkers for many diseases including cancer. An antiacidic approach can represent a winning strategy against tumors both in prevention and treatment [5, 24]. This may be achieved with proton pump inhibitors (PPIs) or buffers, wherein neutralization of tumor acidity could be monitored *via* a reduction in the release of exosomes [5].

Indeed, the alkalization of tumor cell medium with buffers results in a drastic reduction of exosome release [36]. Several investigations have proven that buffering the tumor milieu or blocking the deprotonation mechanisms slows tumor growth of xenografts in laboratory animals and increases response to chemotherapy in human and veterinary patients [108, 109]. The potent anti-tumor effect of PPI, through inhibition of proton pumps in tumor cells, is due to their characteristic of prodrugs needing acidity to be activated into the active molecule unlike the vast majority of drugs that are neutralized by protonation [25]. It was shown that *in vivo* treatment with proton pump inhibitors similarly reduced the plasmatic exosome levels in murine models suggesting that exosome modulation might be another exploitable strategy to achieve tumor control [36, 63]. Furthermore, according to their physical functions, exosomes may represent the ideal vehicle for delivering of drugs in cancer therapy increasing efficacy of cancer chemotherapy, with low immunogenicity and toxicity [37, 105, 110]. Our group showed that an extended drug delivery time of exosomes was loaded with Acridine Orange to melanoma cells as compared to free Acridine Orange, improving the cytotoxicity of AO and suggesting a potential role of exosomes in theranostics [110].

4 Conclusions

The bulk of the current literature infers that (i) tumor acidity increases the release of exosomes; (ii) either buffering, alkalinization, or anti-acidic treatment reduce the exosomes release by tumors; and (iii) the exosomes plasma levels are a measure of the tumor progression with a potentially high clinical impact in terms of early diagnosis and ease of response monitoring. Therapeutic strategies counteracting the immunesuppressive and tumor promoting activities of exosomes are currently being actively pursued. Nevertheless, an alkalizing approach to cancer is a promising strategy to treat the constellation of diseases known as cancer.

A last thought is that increased exosome release, being a feature of virtually all tumor histotypes, may well be included in the list of phenotypes ("Hallmarks") common to all cancers, together with hypoxia, uncontrolled growth, low blood, nutrient supply, and extracellular acidity.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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