Energy Sources for Exosome Communication in Cancer Microenvironment

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Exosomes are crucial extracellular vesicles (EVs) with a diameter of approximately 30–200 nm. They are released by most cell types in their extracellular milieu and carry various biomolecules, including proteins and nucleic acids. Exosomes are increasingly studied in various diseases, including cancer, due to their role in local and distant cell–cell communication in which they can promote tumor growth, cancer progression, and metastasis. Interestingly, a tremendous number of exosomes is released by malignant cancer cells, and these are then taken up by autologous and heterologous recipient stromal cells such as immune cells, cancer stem cells, and endothelial cells. All these events demand an enormous amount of energy and require that exosomes remain stable while having the capacity to reach distant sites and cross physical barriers. Nevertheless, there is a dearth of research pertaining to the energy sources of exosomes, and questions remain about how they maintain their motility in the tumor microenvironment (TME) and beyond. Moreover, exosomes can produce adenosine triphosphate (ATP), an important energy molecule required by all cells, and mitochondria have been identified as one of the exosomal cargoes. These findings strengthen the prospect of exosomal communication via transfer of mitochondria and the bioenergetics of target recipient cells. In the TME, the accumulation of ATP and lactate may facilitate the entry of exosomes into cancer cells to promote metastasis, as well as help to target cancer cells at the tumor site.

Keywords: exosome ; extracellular vesicle ; cancer ; energy metabolism

1. Introduction

Exosomes are nano-sized extracellular vesicles (EVs) with a diameter of approximately 30–200 nm. They carry various kinds of cargo including mRNA, proteins, lipids, long non-coding RNAs (lncRNAs) and small RNA. Exosomes are released from many different cell types following the endosomal–lysosomal pathway and are present in various biofluids including blood, cerebrospinal fluid (CSF), and urine [1][2]. It is believed that exosomes originate from multivesicular bodies (MVBs) inside the cells. The biogenesis of exosomes begins with the inward budding of the endosomal membrane, with consequent formation of MVBs. Some MVBs then fuse with the plasma membrane and release nanovesicles into the extracellular space. Apart from exosomes, there are other types of heterogenous populations of EVs, such as microvesicles (MVs) and apoptotic bodies (ABs). They can be distinguished based on their size, biogenesis pathway, and surface markers, although their sizes often overlap. MVs are around 50–1000 nm and originate directly from the cell membrane, whereas ABs are around 50–2000 nm, and are released via blebbing and fragmentation of cells undergoing apoptosis [3]. Detailed classification, as well as the characteristics of these EVs, have been discussed elsewhere [4][5].

Exosomes play a crucial role in various physiological and pathological events [6][7][8]. Importantly, their release is dramatically augmented under disease conditions, indicating a vital phenomenon during disease progression [9][10]. They participate in cell–cell communication by travelling to both adjacent and distant sites. However, the means by which exosomes obtain sufficient energy for communication is unclear [6]. As noted above, exosomes are released in the extracellular milieu after the fusion of MVBs with the plasma membrane, and they are required in order to overcome various energy barriers. During this event, several protein–protein and protein–lipid interactions take place that lower the energy barriers to facilitate the fusion of MVBs to the plasma membrane; for example, tethering factors, Rabs, soluble *N*-ethylmaleimide-sensitive factor attachment-protein receptors (SNAREs), and other Rab GTPases have been reported to play important roles in these interactions [11]. The role of lipids has also been investigated in the context of exosomal release. For example, hexadecylglycerol, an ether lipid precursor, stimulates the release and alters the constituents of PC-3 cell-derived exosomes, signifying that augmented cellular ether lipids are linked to the altered release of exosomes and their composition [12]. Strauss et al. demonstrated that the release of exosomes could ameliorate lysosomal storage of cholesterol in Niemann–Pick type C (NPC) disease. Free cholesterol was found to be released by exosomes and dependent on flotillin. In the presence of an over-supply of cholesterol, exosomal cholesterol release was increased in

NPC disease, and could be a potential mechanism by which to overcome the cytotoxic accumulation of cholesterol in late endosome/lysosomes. This suggests that exosomes and flotillin play a role in the regulation of cholesterol homeostasis [13]. Llorente et al. revealed that when cholesterol levels were reduced in PC-3 cells by adding methyl-β-cyclodextrin (a cholesterol-sequestering agent) or by metabolically inhibiting its formation, the secretion of various exosomal proteins was augmented [14]. It is therefore clear that various proteins and lipids, and cholesterol, play crucial roles in facilitating the release of exosomes.

Accumulating evidence of glycolytic enzymes as exosomal constituents supports their potential role in providing energy to exosomes. A recent study utilizing proteomic analysis demonstrated the presence of enzymes of the glycolytic chain in exosomes and prostasomes isolated from prostate cancer cells (PC3), and both EVs could produce ATP when presented with substrates. In particular, overall extracellular ATP generation was increased for PC3-derived exosomes due to their low ATPase activity, while the production of extracellular ATP by prostasomes was relatively lower because of their high ATPase activity. The uptake of prostasomes by recipient cells, normal prostate epithelial cells (CRL2221), and PC3 cells, was glycolytic-flux-dependent and involved the generation of extracellular ATP via EVs or the production of intracellular ATP from recipient cells. Therefore, the internalization of EVs by the recipient cells can be considered an energy-demanding process, suggesting the need for an active ATPase. The ability of EVs to release extracellular ATP might play a crucial role during this process [15]. In another recent study by Ludwig et al., simultaneous inhibition of glycolysis and oxidative phosphorylation prompted a copious surge in the release of exosomes, possibly via 2′,3′-cAMP [16]. The release of exosomes from cells in the extracellular space is a complex and inadequately understood process, because until recently, they were widely dismissed as cellular waste products. Studies associated with exosomes can be facilitated via methods that enhance their generation and release. Interestingly, the treatment of cultured cells with sodium iodoacetate (IAA; glycolysis inhibitor) plus 2,4-dinitrophenol (DNP; oxidative phosphorylation inhibitor) (up to 10 µM each) has been found to augment the release of exosomes 3- to 16-fold. Moreover, in vivo injection of IAA/DNP increased the concentration of circulating exosomes and decreased the level of ATP in cells. Notably, a cell-membrane-permeable form of 2′,3′-cAMP and 3′-AMP mimicked the effects of IAA/DNP on exosome release. The effects of IAA/DNP on exosome release were augmented in cells that lacked 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase), an enzyme that metabolizes 2′,3′-cAMP into 2′-AMP. Therefore, the combined effect of IAA/DNP is a potent stimulus for exosome release, partly arbitrated by 2′,3′-cAMP [16]. Consequently, the role of glycolytic enzymes is believed to be crucial to the energy producing processes, including glycolysis. The roles of exosomal glycolytic enzymes warrant further investigation.

The physical properties of exosomes may also contribute to how they communicate, travel, and acquire specificity towards target cells. One of the contributing properties is the surface charge of exosomes, mostly represented by zeta potential (ZP), a more specific electrokinetic potential that regulates nano-particle delivery [17]. In particular, the ZP of exosomes is unique because of their type, and this impacts exosome–exosome interactions. Interestingly, changes to ZP regulate how exosomes behave. For example, the tendency of exosomal aggregation can be predicted by the value of ZP, which also represents the stability and efficiency of exosomes. Additionally, the ZP of exosomes varies in different tissues and body fluids, as well as in different disease states. In the latter case, it suggests dysfunctional potential in diseases such as cancer, where exosomes are sometimes critical to disease progression [18].

2. Zeta Potential of Exosomes: How does Surface Charge Regulate Exosomal Behavior?

The stability of exosomes plays a crucial role in cell–cell communication, controlled by the surface charge of exosomes and often referred to as zeta potential (ZP). The ZP of dispersal systems, including emulsions, suspensions, and colloidal dispersions, is a quantitative parameter of charge stability that influences the interaction between particles. Interestingly, like other materials, exosomes acquire electrical charge on their surface upon interaction with a polar medium. Notably, a negative charge on their surface is developed when they encounter hydrophilic buffers via a mechanism involving a cascade of steps, such as differences in the electron affinity of the two phases, ionization of exosomal membrane, and the differential ion adsorption from electrolyte solutions. Importantly, the higher value of ZP yields stronger electrostatic repulsion between particles, and thereby reduces their aggregation behavior. For example, the ZP of EVs varying from −30 to +30 mV shows aggregation, even though the specific threshold of stability depends on the type of particle [18]. During exosomal communication, there is fusion of exosomes with the plasma membrane of recipient cells, with consequent expulsion of exosomal constituents. This signifies that exosome can operate as a transporter for ligands, such as the lipophilic Wnt proteins, that need a lipid environment for transport. Exosomes serve as an optimum vehicle for carrying Wnt proteins [19]. During endocytosis or micropinocytosis-based communication, exosomal surface proteins interact with recipient cells. Eventually, exosomal surface proteins become lysed by proteases in recipient cells, producing protein fragments that may act as ligands specific to the receptors on the surface of recipient cells [20][21]. Therefore, it is evident that the specificity of exosomal communication is accomplished via interaction between the ligands and the

receptors of vesicles and those of recipient cells, respectively. Notably, exosomes remain a suspension of colloid in various media, including cultured condition medium, buffer, and biofluids such as urine and serum. To safeguard their functionality, it is necessary to guarantee the dispersal of exosomes homogeneously. This will also augment their stability throughout the storage process [22]. In the present context, the value of ZP may play a crucial role in understanding and distinguishing between specific exosomal interactions and exosomal aggregation.

Conventionally, the characterization of ZP is based on a combination of measurements including streaming potentiometry, phase analysis light scattering, and Doppler velocimetry. All these methods can help to determine the electrophoretic mobility of particles in suspension. Nevertheless, the adoption of this combined approach when dealing with polydisperse samples, which could cover a wide range of ZPs, has posed a challenge. Deblois et al. demonstrated single-particle electrokinetic measurements of nanoparticles by resistive pulse sensing, based on the velocity and electrophoretic mobility of the particles [23]. Recently, tunable resistive pule sensing (TRPS) has been demonstrated to measure the ZP based on the time duration of resistive pulse signals for individual particles [24][25]. Importantly, the measurement of ZP by TRPS is robust and reproducible, as it emphasizes the duration of displacement of nanoparticles as a function of both voltage and pressure. Moreover, the ability of TRPS to measure size and ZP simultaneously depicts a novel methodology for examining the heterogenous properties of dispersed particles [23].

3. Bioenergetics of Exosome Release and Uptake in TME

Donor cell release of exosomes requires a huge expenditure of both energy and biosynthetic materials. In particular, cancer cells release a tremendous number of exosomes compared with normal cells and regulate the immune response [26]. The generation of ATP by exosomes can be attributed to various needs such as immunoregulation and communication with stromal cells in the TME. This leads to several advantages, including the production of extracellular ATP, the formation of a lactate induced TME, the establishment of a seed-and-feed system in the TME, and the creation of a long-range chemotactic gradient system or “find me” signal. ATP generated from tumor exosomes may have various effects in cancer, including regulation of the immune system. The augmented amount of ATP in the TME enhances the production of extracellular adenosine, a strong immunoregulator, and facilitates the immune evasion process in tumors [27]. The extracellular ATP has also been found to play roles in the uptake of exosomes in the TME and the communication of cancers cells with stromal cells via exosomes [15][20]. In the TME, the production of glycolytic ATP can reduce the glucose level while enhancing the lactate level, because exosomes have been found to contain lactate dehydrogenase (LDH) which facilitates the transformation of glycolytic end product pyruvate to lactate [28][29]. Another aspect of the role of extracellular ATP is that it can be taken up by tumor cells, which is energetically favorable, leading to augmented intracellular levels of ATP. This suggests that the release of ATP-generating tumor exosomes can lay the foundation for a seed-and-feed system, where cancer cells can partially outsource their energy outside the cell [30]. Extracellular ATP has also been reported as a potent “find me” signal that is employed by apoptotic bodies (a category of EVs) to attract phagocytes, leading to the formation of a chemotactic gradient in the TME [31]. In the context of cancer metastasis, the extracellular ATP produced by exosomes has also been found to create a local inflammatory state where metastasizing cells can accumulate [32][33].

4. Adenosine Triphosphate as an Energy Source for EVs in Cancer Progression

Adenosine triphosphate (ATP) is deemed the molecular basis of currency in living eukaryotes, providing massive amounts of energy for cells through oxidative phosphorylation. Generated by mitochondria, ATP is synthesized when adenosine diphosphate (ADP) is turned into ATP by the addition of a phosphate molecule, driven by the electron transport chain [34].

Glycolysis and oxidative phosphorylation (OXPHOS) are the two foremost metabolic pathways that provide energy to cells. Glycolysis is the process of breaking down glucose into pyruvate, but is otherwise less efficient than OXPHOS at producing energy [35]. Interestingly, inflammatory immune cells utilize glycolysis more often to gain energy, a phenomenon dubbed the “Warburg Effect” [36]. This assumption is based on the knowledge that tumor cells exist in a hypoxic environment that results in a dramatic increase in glucose uptake. In this regard, although it has been believed that cancer cells almost exclusively utilize glycolysis to thrive, it has recently been suggested that they can also utilize neighboring cancer cells symbiotically. One theory posits that a cell can consume glucose and produce lactate, and a neighboring cell can obtain the secreted lactate and use it to produce ATP through OXPHOS [37]. Research is increasingly moving away from focusing only on the “Warburg effect” to looking at other co-existing energy sources such as oxidative phosphorylation [36][37]. A summary of cancer cell energy metabolism requirements from various tumor types can be found elsewhere [34].

Both glucose and lactate energy precursors in tumor cells make them heterogeneous and contribute to the complex question of how cancer cells stay metabolically active. The TME is the ecosystem of malignant tumors, and is composed of many cell types and dependent upon intercellular crosstalk [38]. Enzymes such as cycloozygenase-2 (COX-2) have been shown to be upregulated in multiple cancer types and promote metastatic progression and chemotherapy evasion [38][39]. More interestingly, increased extracellular ATP in the TME leads to increased expression of COX-2, resulting in increased invasiveness of cancer cells [39]. The TME facilitates cancer progression and metastasis by releasing circulating tumor cells (CTCs) as well as exosomes that travel from the initial tumor site; large amounts of circulating CTCs are correlated with a poor prognosis in patients [40]. Exosomes provide a paradoxical relationship within the TME, as their secretion can be initiated by both increased and decreased levels of ATP [16][41]. EVs have also been shown to provide ATP for donor cells in vitro, under both natural physiological and engineered conditions [42][43]. The release of exosomes is stimulated by P2X7 receptors that have been shown to be involved in enhancing the invasive properties of metastasizing melanoma cells to the lung, at least partially through supply of ATP [44]. This suggests an important role of ATP in the TME in the release of exosomes to instigate their journey. Kumar S et al. chemically engineered the membrane proteins of exosomes with a moiety of catechol, facilitating fusion via supramolecular complexation to bridge the membranes. Notably, this approach enabled the encapsulation of various enzymes and set up the minimal electron transport chain, consequently augmenting the catalytic reaction, enough for the synthesis of ATP. Interestingly, it was found to be functional for several hours after being internalized by recipient live cells, which showed repair of damaged regions by providing ATP. This demonstrated an excellent advancement in exosomal engineering to produce programmed exosomes as a potential energy source[43].

Although the travel of exosomes is largely passive through body fluids, they contain crucial energy packages that can be delivered to nearby or target cells. Exosomes do not go through replication, a common cellular process that utilizes ATP [45], but they have been shown to contain mitochondria [46][47]. Mitochondria generate power for the cell via the electron transport chain, producing the ATP needed for cell survival [48]. It is not clear if these mitochondria are required for exosomes per se, or are merely a package transfer from the cells of origin, although the latter seems more likely. Defined recently, ‘mitovesicles’ have been extracted from brain regions and appear to be mitochondria-derived small vesicles [47]. Mitochondrial dysfunction has been implicated most commonly in aging but also in cancer, where the epithelial–mesenchymal transition (EMT) is induced from this dysregulation [49]. EVs are found to contain mitochondria and mitochondrial dysfunction has been associated with these disease conditions. Neverheless, it is important to note that isolation methods vary for exosomes with different filtration sizes, so varying studies may have isolated entire mitochondria or only partial sequences. Further investigation with more cohesive isolation methods is required.

P2X7R, an extracellular ATP receptor, is abundant in inflammatory immune cells and requires high ATP levels for activation [41]. This receptor induces the release of massive numbers of EVs in microglial cells [50]. More recently, P2X7R has been shown to be highly expressed in malignant cancer patients, correlating with a poor prognosis. This showed that EV miRNA content was highly dependent on P2X7R expression, which was altered by varying ATP levels [44]. In both mouse organ cultures and EC cultures, lower ATP levels caused by inhibited glycolysis enhanced the secretion of EVs [16]. Taken together, this shows a complicated, dynamic but paradoxical relationship of ATP with EVs. Applying this hypothesis, the different states of the cells of origin may account for the up- and down-regulation of ATP in EV secretion. Overall, it also suggests that EVs do not explicitly need ATP to be activated, but further detailed investigation is needed before any conclusions can be drawn.

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