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Review

Extracellular vesicles and energy metabolism

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ABSTRACT

Glycolytic enzymes are among the most frequently identified proteins in proteomics of exosomes/extracellular vesicles. This review brings up the possibility that exosomes/extracellular vesicles during their life-time in bodily fluids power important energy-consuming functions by glycolytic conversion of glucose or fructose into ATP. It was seen that prostasomes (exosomes of the prostate) could produce ATP by glycolysis and that the produced ATP quickly was consumed by adjacent prostasomal ATPases. The glycolytic ATP production appeared to be coupled to self-sustaining energy requirements. It will also be discussed how a failure in this machinery (lowered activity of ATPases) with a resultant polluting leakage of extracellular ATP could affect cancer development.

1. Extracellular vesicles – exosomes and prostasomes

Extracellular vesicles have important roles in cell-cell communication [1,2]. They are produced by most cells in the body and can be recovered from bodily fluids or cultured cell-lines [1,2].They can be classified into three main groups: Shedded vesicles-vesicles that originate directly from the plasma membrane; Apoptotic bodies-vesicles that originate from disintegrating cells during controlled cell-death; and Exosomes-vesicles that originate from multivesicular organelles/multivesicular bodies inside cells. The creation of exosomes begins with inward buddings of the limiting endosomal membrane giving rise to a multivesicular organelle [3,4]. Next, some of these multivesicular organelles fuse with the plasma membrane and exocytose the internal vesicles extracellularly as exosomes [5]. Observations made by electron microscopy and devices based on dynamic light scattering have shown that the majority of exosomes are in the size-range of 30–200 nm in diameter [6,7]. Exosomes are produced by most cells of the body and can be recovered in bodily fluids [1]. Prostasomes can be seen as a subgroup of exosomes and are formed in endosomes of prostate epithelial cells and therewith secreted into semen [3,8].

2. Environmental challenges and keeping shape in circulation

Exosomes encounter changing environments while circulating in bodily fluids. The half-life of extracellular vesicles in vivo in athymic nude mice has been estimated to 30 min and most extracellular vesicles were cleared in 6 h after intra venous injection [9]. It is more difficult to estimate the half-life of human exosomes in blood. However, the entire blood volume of humans will be pumped around the body in 60 s, calculated on pumping volume of each stroke, resting pulse, and total blood volume. It can be anticipated that blood borne human exosomes encounter different compartments in the body with changing conditions during their lifetime. In order to preserve and protect their cargo, they have to adjust to these varying conditions so that they in the end can deliver functional cargo to the designated location. Prostasomes populate a different bodily fluid. Their main task in semen is to find and adhere to sperm. Those which adhere to a sperm will promote sperm fitness and sperm motility in the competition to reach the egg first. [10,11]. The others may have roles in reducing immune reactions towards sperm or evoke female genital cells to emit factors for a successful fertilization.

In order for exosomes to fulfil their tasks and adapt to shifting conditions, they most likely bring a tool kit of essential and fluid specific active surface enzymes. The exosomal cytosol is tightly packed with cargo (proteins, RNA, DNA) [7,12,13]. To protect and to avoid aggregation of packed material, a flexible lipid bilayer and an intravesicular environment of chaperons, pH-homeostasis, and the right ion composition must be preserved. The adaption of the lipid bilayer and the maintenance of a suitable environment for packed material may involve ATP-driven phospholipid transporters and ion-pumps, working at the membrane surface and the cytosolic side. In fact, several ATPases can be found in the proteomic lists of exosomes and among these, the ligand-gated cation channel P2X4, which is controlled by extracellular ATP [14,15]. This purinergic receptor was also identified in

Abbreviations: ATP, adenosine triphosphate; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; NAD, nicotinamide adenine dinucleotide; MSC, mesenchymal stem cell

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prostasomal lipid rafts [16].

3. Extracellular ATP in blood and semen

The extracellular ATP-level in semen is too low to measure due to rapid breakdown by various ATPases and phosphatases [17]. Extracellular ATP-level in blood plasma is also low [18]. Because of the low levels of ATP in these bodily fluids, ATP-dependent proteins identified in prostasomes and exosomes have no fuel. Nevertheless, seminal vesicles contribute with fructose to semen [19] and stored glycogen is converted to glucose by the liver and skeletal muscles to keep a tightly regulated glucose level in blood [20]. The availability of fructose in semen and glucose in blood, may power circulating exosomes and prostasomes, sustaining a self-supportive glycolytic production of ATP to maintain important energy consuming functions.

4. Glycolytic enzymes in exosomes

The frequent appearance of glycolytic enzymes in proteomics on exosomes and prostasomes may lead to speculate that the most important maintenance functions for the right cargo environment e.g. energy-driven ion-pumps and phospholipid transporters may be interconnected with their own energy supply, in possible glycolytic metabolons. The hypothesis of glycolytic metabolons is not new [21]. In the metabolon the glycolytic enzymes will complex in proximity of each other and thereby be able to deliver the catabolite from one enzyme to the next in an ordered way. Several glycolytic enzymes have been seen to complex with band 3 protein in erythrocytes to form glycolytic complexes in the membrane [22]. Additionally, a search for a glycolytic metabolon in muscle cells, to support the need of muscle cells for fast energy, showed that some glycolytic enzymes adsorbed to F-actin–-tropomyosin–troponin complexes [23]. Multienzyme metabolic complexes, that are not only handling glucose catabolism but also the complete metabolic processing of glucose, have been found inside living cells [24]. The full set of glycolytic enzymes (with an exception for 6-phosphofructokinase that was missing in bull and dog prostasomes) was identified in prostasomes across 4 animal species (Table 1) [25].

There are initiatives to characterise exosomes. Two of these are EVpedia [26] and ExoCarta [27]. At these webdomains, researchers in the field are able to upload proteomic lists of identified proteins of the exosomes they are working with. For each new uploaded list, identified proteins in that list can be added to those already existing in the database. This gives an opportunity to create a listing of the most frequently identified proteins generally found in exosomes. It has for a long time been known that glycolytic enzymes are commonly found in proteomic profiling of exosomes of different origins. In fact, a majority of the glycolytic enzymes can be found among the 100 most frequently identified proteins in exosomes (Table 1). Several A majority of the glycolytic enzymes have as well been identified as components of lipid rafts (Table 1) [16]. These membrane domains exhibit an enhanced ordering of their constituting lipids [28]. The lipid constituents of lipid raft domains display a high ratio of saturated fatty acid chains providing space for intercalating cholesterol molecules and this combination confers highly ordered lipid domains [28]. It is known that the high ordering of these domains attract lipid anchors such as glycosylphosphatidylinositol-anchor that can attach proteins, which do not have hydrophobic membrane spanning peptides, to membranes [29]. Moreover, the domains also provide the grounds for protein clustering at the surface of membranes [29]. This could explain why cytosolic enzymes of the glycolytic pathway are recovered in purified prostasomal lipid rafts (Table 1). Another possible mechanism for attachment of glycolytic enzymes to the surface is by so called tetraspanin-enriched microdomains [30]. In these domains tetraspanins build platforms where proteins adhere to form functional complexes. Tetraspanins are commonly found in proteomics of exosomes and CD9, CD63 and CD81 are considered as markers for exosomes [31]. The creation of exosomes and prostasomes as intraluminal vesicles in the endosome cytosol provides a possibility for the attachment of non-membranous proteins (in the endosomal cytosol) to membrane anchors on exosomes´ outer surface.

Noteworthy is that, the identified glycolytic enzymes in prostasomal lipid rafts included fructose-bisphosphate aldolase (the forth enzyme in the glycolytic chain) and all of the subsequent enzymes in the glycolytic pathway catalyzing the 3 carbon atom intermediates (Table 1 and Table 2). These enzymes (except phosphoglycerate mutase) are among the top 20 most frequently identified exosomal proteins in EVpedia and among the top 27 most frequently identified proteins in ExoCarta (Table 1). It is important to remember that 6-phosphofructokinase (the third enzyme in the glycolytic chain) is considered the committed step to continuing glycolysis, and that this enzyme together with the first (hexokinase) are the two ATP-consuming steps in glycolysis (Table 2). The two glycolytic enzymes (hexokinase and 6-phosphofructokinase, the two ATP consuming steps), which are not found in prostasomal lipid raft domains may either be localized to the membrane surrounding lipid raft domains or may be part of the complex in the presence of ATP.

Table 1
Glycolytic enzymes and occurrence in exosomes and prostasomes.

Enzyme	Evpedia Top 100	ExoCarta Top 100	Prostasomal lipid raft	Specie
1. Hexokinase			No	H, D, S, B
2. Glucose-6-phosphate isomerase	(63)		Yes	H, D, S, B
3. 6-Phosphofructokinase (PFKL)[a]			No	H, S
4. Fructose-bisphosphate aldolase	(12)	(18)	Yes	H, D, S, B
5. Triosephosphate isomerase	(20)	(27)	Yes	H, D, S, B
6. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	(1)	(4)	Yes	H, D, S, B
7. Phosphoglycerate kinase (PGK)	(16)	(16)	Yes	H, D, S, B
8. Phosphoglycerate mutase (PGM)	(87)		Yes	H, D, S, B
9. Enolase	(2)	(9)	Yes	H, D, S, B
10. Pyruvate kinase	(3)	(12)	Yes	H, D, S, B
Lactate dehydrogenase-A (LDH-A)	(7)	(13)	Yes	H, D, S, B

From left, Enzymes in the glycolytic pathway and their frequent appearance in exosomes. Number in parenthesis refers to their placement in the top 100 most frequently identified proteins of exosomes in Evpedia and ExoCarta. Glycolytic proteins identified in purified lipid raft domains of human prostasomes and in intact prostasomes of different species. H = Human; D = Dog; S = Stallion; B = Bull.
Proteomic data was retrieved 2018-10-16 from:
http://evpedia.info (Evpedia website).
www.exocarta.org (ExoCarta website).
Dubois L et al. Mol Cell Proteomics. [16], doi: https://doi.org/10.1074/mcp.M114.047530, Supplementary Table 1.
Ronquist KG et al. Biochim Biophys Acta. [25], doi: https://doi.org/10.1016/j.bbagen.2013.05.019, Supplementary Tables 1–4.
[a] 6-Phosphofructokinase (PFKL) is the committed step to continuing glycolysis.

Table 2
Overview of steps in the glycolytic pathway.

Intermediate		Enzyme	ATP	NAD+	No. of carbon atoms in intermediate
Glucose					6C
↓		1. Hexokinase (HK)	−1 ATP		
Glucose 6-phosphate					6C
↑↓		2. Glucose-6-phosphate isomerase (GPI)			
Fructose 6-phosphate					6C
↓		3. 6-Phosphofructokinase (PFKL)[a]	−1 ATP		
Fructose 1,6-bisphosphate					6C
↑↓	↑↓	4. Fructose-bisphosphate aldolase (ALDO)			
Dihydroxyacetone phosphate (DHAP)	Glyceraldehyde 3-phosphate (GAP)	5. Triosephosphate isomerase converts DHAP to GAP (TPI)			2 × 3C
↑↓		6. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)		−2 NAD+ +2 NADH	
1,3-Bisphosphoglycerate					2 × 3C
↑↓		7. Phosphoglycerate kinase (PGK)	+2 ATP		
3-Phosphoglycerate					2 × 3C
↑↓		8. Phosphoglycerate mutase (PGAM)			
2-Phosphoglycerate					2 × 3C
↑↓		9. Enolase (ENO)			
Phosphoenolpyruvate (PEP)					2 × 3C
↓		10. Pyruvate kinase (PKM)	+ 2ATP		
Pyruvate					2 × 3C
Lactate		Lactate dehydrogenase-A (LDHA)		−2 NADH +2 NAD+	

From left, intermediates of the glycolytic pathway and enzymes involved in catalyzing the intermediates. The fifth enzyme (triosephosphate isomerase) converts DHAP to GAP which is further catabolized in the pathway. In bottom of the table, pyruvate is converted into lactate by lactate dehydrogenase A. This step is not part of the glycolytic pathway even though it provides NAD+ for the next circuit. Steps involving ATP and NAD+ conversion are tabulated and in the last column, the number of carbon atoms in intermediates.

[a] 6-Phosphofructokinase (PFKL) is the committed step to continuing glycolysis.

Notably, lactate dehydrogenase-A, which is not a glycolytic enzyme, but converts the end product of the glycolytic chain (pyruvate) to lactate by oxidation of NADH to NAD+, and thereby recycles NAD+ to prevent shortage in the next glycolytic cycle, is on place 7 in EVpedia and 13 in ExoCarta among the most frequently identified proteins in exosomes (Table 1 and Table 2). Lactate dehydrogenase-A was also recovered in lipid rafts of prostasomes (Table 1).

5. Mesenchymal stem cell-derived exosomes, ATP and myocardial ischemia/reperfusion damage

In a study examining the complications caused by reperfusion in myocardial ischemia after surgical blood flow restoration, it could be seen that mesenchymal stem cell (MSC)-derived exosomes mediated therapeutic effects [32]. The study was undertaken in mice which underwent 30 min of ischemia, followed by reperfusion. Purified MSC-derived exosomes were administered just before reperfusion and the authors showed that the added exosomes restored ATP and NADH levels and reduced oxidative stress in the tissue. ATP deficit is one of the biochemical features of reperfusion injury. They concluded that, MSC-derived exosomes contributed with fully functional glycolytic enzymes and most likely were uptaken by endocytosis or phagocytosis and in this way restored glycolysis in reperfused myocardium. They noticed that only intact exosomes had this effect and that the therapeutic effect seemed to be a general property of all MSC-derived exosomes, regardless of their tissue source [32].

6. Human prostasomes and glycolysis

Proteomics on prostasomes and exosomes lists most of the enzymes involved in glycolysis with high scores (Table 1). In an observational study it was seen that extravesicular ATP was produced by adding fructose to purified prostasomes [33]. Hence, prostasomes operate in an environment containing fructose (semen). Furthermore, it was necessary to add an ATPase inhibitor (vanadate) in order to get a measurable ATP level since, the ATP produced by glycolysis was used-up by the surrounding prostasomal ATPases, and the net gain of ATP without the ATPase inhibitor was extremely low [33]. In the same paper, the high inherent ATPase activity was highlighted by adding concentrations of ATP directly to purified prostasomes and measuring ATP levels by a luciferin/luciferase kit after incubation at 37 °C. Addition of fructose or glucose resulted in similar production rates of ATP [33]. It could also be seen that prostasomes complemented with one of the two hexoses supplemented with glycolysis inhibitors, sodium fluoride and iodoacetate, symbiotically, lowered the amount of formed ATP, which was indicative of a glycolytic flow since iodoacetate irreversibly inhibits glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [34] and fluoride inhibits enolase [35] in the glycolytic pathway. To investigate whether the ATP production was on the cytosolic or extravesicular side of the prostasomal membrane, glyceraldehyde-3 phosphate, which is not expected to be membrane permeable, was added to purified prostasomes. Then the same glycolytic pattern was seen for glyceraldehyde-3 phosphate as for glucose and fructose, i.e. an elevated ATP count that could be lowered by adding glycolysis inhibitors, which indicated a glycolytic flow [33]. The outer surface location suggested that a possible glycolysis metabolon was situated on the surface of prostasomes. An outer surface located enzymatic chain would hardly work if not intermediates are channelled to the successive enzyme in the chain. This study concluded that human prostasomes could produce extracellular ATP by glycolysis of the fructose available in semen. The formed extracellular ATP was quickly consumed by adjacent prostasomal ATPases.

7. Prostasomes from 4 different species and glycolysis

In a following study, differences in prostasomal glycolysis between human, dog, bull, and stallion were investigated [25]. It was found that prostasomes from different species exhibited varying efficiencies in producing ATP by glycolysis and that the ATP production was matched by the ATP consumption of each specie-specific prostasome sort [25].

The ATP production was evenly balanced by ATPase activity meaning that species with a high prostasomal ATP production also had a high prostasomal ATPase activity. It is important to consider that the reproductive system differs from one species to another and that in particular dog, rely on prostate as the main accessory sex gland. Prostasomes have the same function in species, which is to promote and support sperm, even though they may differ in protein content and enzymatic activity. Prostasomes in dog showed a low glycolytic and ATPase activity while prostasomes in bull showed a high glycolytic and ATPase activity. Human and horse prostasomes displayed intermediate levels of activities between those of dog and bull [25].

8. Normal prostasomes vs prostate cancer exosomes and glycolysis

In a comparison of glycolysis and ATPase activity between prostasomes, and exosomes purified from growth media of cultivated prostate cancer cells (PC3 exosomes), it was seen that both types produced ATP by glycolysis [36]. However, the PC3 exosomes displayed a remarkably lower ATPase activity than prostasomes, thereby generating an environment with extracellular ATP in this cancerous state [36].

9. Exosomes and cancer

Heavy investments in the form of bioenergetics and biosynthetic building blocks are required by the parental cell to produce exosomes and this collides with cancer cells´ opportunistic proliferation. Surprisingly, cancer cells retain the ability to secrete exosomes. Why then would cancer cells produce exosomes? There are several reasons for cancer cells to produce exosomes including immune-regulatory functions and communication with stromal cells in the tumor microenvironment. In the following sections some advantages of secreting ATP-generating exosomes in cancer will be presented (Fig. 1).

10. Extracellular ATP and lactate building-up the tumor microenvironment

Tumor interstitial ATP levels are more than 1000 times higher than those in normal tissue of the same cell origin [37]. It has been speculated that ATP sharing in tumors is the reason for the high extracellular ATP levels in tumors [37]. In part, glycolysis by cancer exosomes could contribute to the excess of extracellular ATP found in tumor interstitium. This glycolytic ATP production would not only reduce the availability of glucose in the tumor microenvironment but also elevate the levels of lactate since lactate dehydrogenase, which converts the glycolytic end product pyruvate to lactate, is frequently identified in exosomes. A characteristic feature of the tumor environment is acidosis caused by glycolytic proton release and from released protons during degradation of glycolytically produced ATP [38]. The high levels of thereby formed lactic acid suppresses the proliferation and cytokine production of human cytotoxic T-lymphocytes [39] and promotes the development of myeloid-derived suppressor cells, which are central for tumor progression [31,40].

11. Extracellular ATP and immune regulation

The high amount of extracellular ATP in the tumor microenvironment generates elevated amounts of extracellular adenosine by the sequential activity of ectonucleoside triphosphate diphosphohydrolase-1 (CD39) and 5′-nucleotidase (CD73) [41]. CD39 and CD73 are expressed by stromal cells in the tumor microenvironment and have also been reported to associate with cancer exosomes [41]. Adenosine is a potent immuneregulator and assists in tumor immune evasion [41].

12. Extracellular ATP and seed and feed

The extracellular ATP can be uptaken into tumor cells by macropinocytosis and endocytosis and this uptake is energetically favourable [37]. The uptake of ATP in an in vitro experiment increased the intracellular ATP-levels by 30% within 45 min and more than 50% within

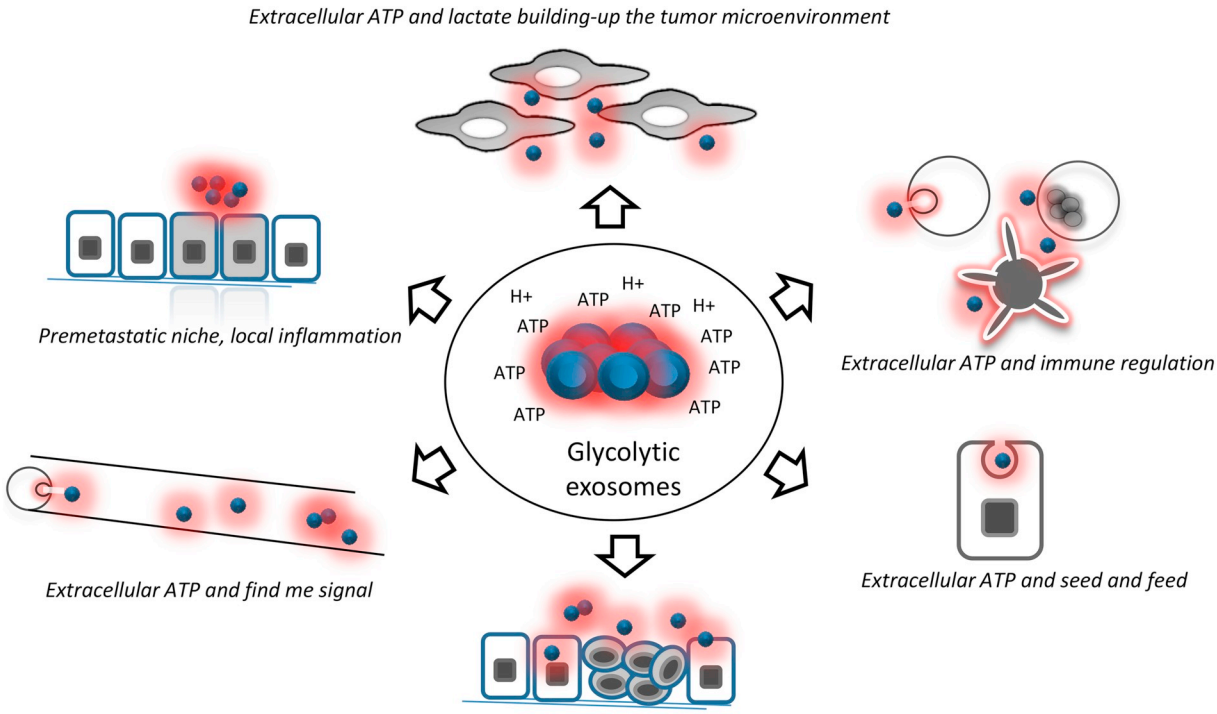

Fig. 1. Effects that tumor exosomes´ generation of ATP may have in cancer.

4 h [37]. This could indicate that the secretion of ATP-producing cancer exosomes could be part of a seed and feed system where cancer cells outsource parts of their energy production outside the cell.

13. Communication in the tumor microenvironment aided by extracellular ATP

There is evidence that exosome uptake is an energy-dependent process [36,42]. Therefore, the production of extracellular ATP may enhance exosome uptake by cells in the tumor microenvironment and facilitate cancer cells´ communication with surrounding cells.

14. Extracellular ATP and find me signal

Extracellular ATP is a potent “find me” signal used by apoptotic bodies to attract phagocytes [43]. The generation of extracellular ATP in combination with the migration of cancer-derived exosomes could build-up a long-range chemotactic gradient leading back to the tumor microenvironment.

15. Premetastatic niche, local inflammation

Metastasizing cancers often have a target tissue where primary metastases appear first [44]. This has drawn attention to exosomes to be forerunners to create a premetastatic niche for the following circulating tumor cells [45,46]. The accumulation of exosomes in such location and their production of extracellular ATP could create a local inflammatory site where the metastasizing cell could settle down.

16. Conclusion

This review brings up the possibility that exosomes like red blood cells and platelets in circulation take advantage of glycolysis to power important energy-demanding functions. This use of energy would help circulating exosomes to confront and adapt to varying conditions during their life-time in bodily fluids. However, it is important to remember that the first steps in glycolysis consume ATP and the net gain comes in later steps. This implicates that there has to be a small amount of ATP to start the glycolytic reaction. This starting ATP may be localized inside the multivesicular organelle at exocytosis or be secreted, in parallel with exosomes, from nearby organelles.

Declaration of interests

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