Exosomes as biomarker treasure chests

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ABSTRACT

Context

Although progress has been made in respect of types of markers (protein, DNA, RNA and metabolites) and the implementation of improved technologies (mass spectrometry, arrays, and deep sequencing), the discovery of novel biomarkers for prostate cancer (PCa) in complex fluids, such as serum and urine, remains a challenge. Meanwhile, recent studies have reported that many cancer-derived proteins and RNAs are secreted through small vesicles, known as exosomes.

Objective

This narrative review described recent progress in exosome research, particularly focusing on their potential role as novel biomarkers for PCa. The purpose of this review was to acquaint the clinicians and researchers in the field of urology with the potential role of exosomes as biomarker treasure chests and their clinical value.

Evidence acquisition and synthesis

Medline and Embase entries between 1966 and September 2010 were searched using the key words of exosomes, microvesicles, prostasomes, biomarkers, prostate cancer and urology. Leading publications and articles constructively contributing to exosome research were selected for this review.

Conclusions

Exosomes are small vesicles (50 to 100 nm) secreted by almost all tissues; they represent their tissue origin. Purification of prostate (cancer)-derived exosomes will allow us to profile exosomes, providing a promising source of protein and RNA biomarkers for PCa. This profiling will contribute to the discovery of novel markers for the early diagnosis and reliable prognosis of PCa. Although the initial results are promising, further investigations are required to assess the clinical value of these exosomes in PCa.

INTRODUCTION

Prostate cancer (PCa) is one of the few solid tumors with a clinically useful biomarker for both diagnostics and follow-up after treatment. This biomarker protein, prostate-specific antigen (PSA), has been considered the "gold standard" for the detection of PCa.[1] Although PSA has acceptable sensitivity, it lacks the specificity for discriminating benign prostate diseases (*e.g.,* benign prostatic hyperplasia (BPH) and infection), indolent PCa and aggressive PCa. It has also been shown that PSA-based screening leads to a decrease in the prevalence of advanced PCa and a reduction of PCa-related mortality by 20%.[2,3] However, this screening is also associated with a high risk of overdiagnosis and over-treatment based on findings on complementary diagnostic prostate biopsies. Therefore, new molecular markers for PCa are needed to more specific, to prevent unnecessary prostate biopsies and to help the urologists to decide the most optimal treatment.[4]

Searching for novel biomarkers has been the focus of many research groups, and the studies have become more extensive and sophisticated. Although exciting progress has been made in respect of novel technologies, such as mass spectrometry analysis or RNA-based arrays, discovering new biomarkers in serum and urine remains a challenge. Particularly, proteomic profiling from complex body fluids is hampered by several problems. One of these problems is that a few high-abundance proteins (albumin, immunoglobulins, transferrin, complement factors, fibrinogen, and so on) make out 97% of body fluids, whereas low-abundance proteins are generally the most promising candidates for biomarker discovery.[5] As indicated, this dynamic range of protein concentrations is very large (*e.g.,* serum contains 7.5×10^5 nmol/L albumin and 10^{-1} nmol/L PSA (3 ng/ml), meaning that for every single molecule of PSA, 7.5 million molecules of Albumin are present. Mass spectrometry has made large-scale proteomics analysis feasible; however, the high-abundance proteins reduce the detection sensitivity of this technology.[6] Most likely, promising marker proteins are probably present at the concentration of 10^{-3} to 10^{-5} nmol/L. The sensitivity of mass spectrometry has a detection limit of up to 10^2 nmol/L.[7] Due to the dynamic range issue, identification and quantification of the low-abundance proteins remains a great challenge. Therefore, even with current "state-of-the-art" technologies, discovering novel biomarkers is still like searching for a needle in a haystack.

This dynamic range problem can be partially tackled by several methodologies. For example, high-abundance proteins can be depleted by chromatography or by precipitation. Moreover, fractionating the samples into many different portions, for instance, by isoelectric focusing, mass separation or affinity chromatography, can improve the identification of low-abundance markers. Unfortunately, fractionation increases the number of measurements and, consequently, the time to process an individual sample. The detection sensitivity can increase approximately 100-fold by combining these two methods; however, it is still not enough to identify the low-abundance markers.[8]

Another option that may contribute to the better identification and detection is specific enrichment. An obvious problem with this approach is that in a discovery setting, it is not known what the protein or RNA marker of interest is. However, recent findings have revealed that small tissue-derived vesicles, the so-called exosomes, are present in serum and urine and contain a wide range of proteins and RNAs [9,10] that represent their tissue origin. These vesicles also express tissue-specific transmembrane proteins that can be used for specific isolation of the vesicles from the complex fluids. Enrichment of cancer-derived vesicles from complex body fluids may solve the dynamic range problem and allow the identification of novel biomarkers.

OBJECTIVE

Since the last decade, exosome research has been rapidly expanded, and the number of coherent publications has been gradually increasing. Therefore, it is necessary to acquaint the clinicians and researchers in the field of urology with this biological concept. The main objective of this narrative review was to describe recent progress in exosome research, especially in the field of urology, particularly focusing on their potential role as novel biomarkers for PCa.

EVIDENCE ACQUISITION AND SYNTHESIS

All entries between 1966 and September 2010 in Medline and Embase were searched to identify original studies and review articles. Leading publications and original articles constructively contributing to exosome research were included. For focusing the exosome research in the field of urology, the search was conducted using the following key words: (exosome* OR microvesicle*) AND (prostate cancer OR urology). The search was limited to the publications written in English with the full text available. Initially, we reviewed titles and abstracts for clinical relevance. A total of 25 manuscripts were reviewed, from which five were selected. Because the term of exosome has also been used in literature for a RNA-degradation complex, we manually excluded the articles describing such complex to prevent confusion.

Biogenesis and secretion of small vesicles

Exosomes (50 to 150 nm in diameter) were first described in sheep reticulocyte maturation in 1983.[11] In studies on transferrin receptor loss during reticulocyte development, it has been noticed that this plasma membrane receptor is shed through small vesicles.[12,13] The biogenesis of these vesicles starts from the internalisation of cellular membrane that,

thereby, forms an early endosome. During the formation of this endosome, cytoplasmic content is taken up by inward budding of endosomal membranes, resulting in exosome formation. When exosomes are formed, the endosome is called a multivesicular body (MVB). When the MVB fuses with the cellular membrane, the vesicles are secreted (Figure 1).[14]

The exact mechanisms involved in exosome biogenesis are not fully elucidated; however, some factors have been reported to play a role. First, specific lipids and transmembrane proteins are grouped in the cellular membrane.[15] These groups form separate microdomains, the so-called lipid rafts. These lipid rafts are enriched with glycosphingolipids and contain transmembrane cross-linked proteins.[15,16] Although the exact role of lipid rafts in exosome formation is not clear, they seem to exert an important regulatory effect. Second, for sorting and encapsulating cellular content into exosomes, protein complexes, such as "endosomal sorting complex responsible for transport (ESCRT)", and the process of protein ubiquitination are involved.[17] The function of these protein complexes is regulated by Vps4.[18] Third, exosome secretion is partially regulated by multiple Rab proteins, which control intracellular transport pathways by regulating vesicular trafficking. Especially, Rab27A, Rab27B and Rab35 have been shown to be important regulators in vesicle secretion.[19]

Although we only partially understand biogenesis of exosomes, we do know that they contain cytoplasmic content (proteins and RNAs) that is encapsulated by a cholesterol-rich phospholipid membrane consisting of a host of transmembrane proteins.[20,21] Exosomes probably represent the transmembrane and intracellular conditions of their cell origin. Furthermore, the process of the biogenesis and shedding of exosomes has been shown in many mammalian cell types, including malignant cells; it is an independent pathway, compared to the secretion of signal peptide proteins (such as PSA) that are processed through the classic consecutive route (Figure 1). Therefore, profiling the exosomes derived from specific tissues may contribute to the understanding of the pathogenesis of tissue-related diseases.

Exosomes and their functions

Exosome shedding is a process with a wide range of important regulatory functions. Their discovery in sheep reticulocyte maturation gave rise to the idea that exosomes may function as a trash bin for unnecessary and redundant proteins. Therefore, it could be an alternative pathway for lysosomal degradation.[22] Nevertheless, most attention has been paid to their role in the immune system. Functional experiments have shown that exosomes affect the immune system by expressing and processing antigens.[23] First of all, exosomes are enriched with specific antigens, compared to whole cell lysates.[24] Second, exosomes from antigen-presenting cells (APC) contain large amounts of major histocompatibility complex (MHC) class I and II molecules.[21,25] When APC-derived exo-

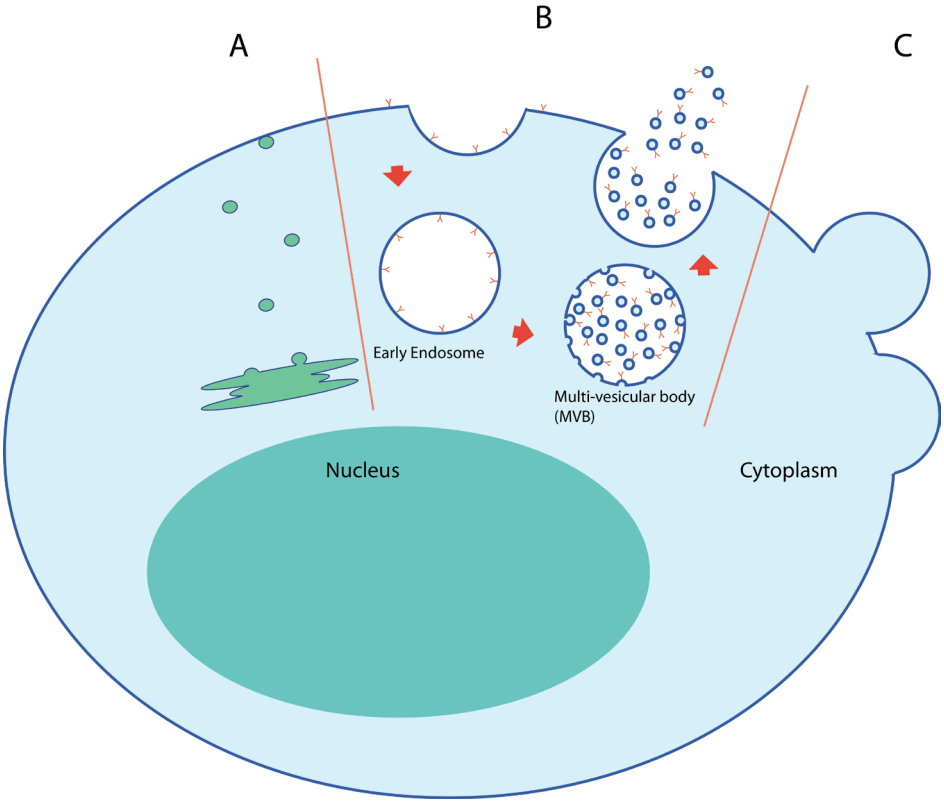

Figure 1. Schematic overview of different secretion mechanisms. (A) Secretion of signal peptide proteins through the classic consecutive route and processed through the rough endoplasmic reticulum and Golgi. (B) Merocrine secretion pathway. Cellular membrane buds inward and forms an early endosome. After the formation of such an endosome, cytoplasmic content is internalised into small vesicles (100 nm), the so-called exosomes. When exosomes are present inside the endosome, the endosome forms a multivesicular body (MVB). The MVB fuses with the cellular membrane, and the exosomes are released. (C) Apocrine secretion pathway. Proportionately larger vesicles (500 to 1000 nm), such as oncosomes, are formed by membrane shedding.

somes are incubated with donor cells, MHC could be re-expressed in these cells.[26] These results indicate that there is an exchange of membranes or membrane proteins between exosomes and cells, and that, exosomes, therefore, harbour a communicative function. Aside from the membrane transfer, exosome content, such as proteins and RNAs, can also be shuttled between cells through exosomes.[27] By transferring RNAs, exosomes are capable of transferring genetic information that can be translated into functional proteins in target cells.[28]

In the field of cancer research, there is an ongoing debate regarding their exact role as pro- or anti-tumor effectors. Experiments in mice have shown that cancer-derived exosomes can induce protective anti-tumor immune responses.[29,30] It has been demon-

strated that exosomes isolated from malignant effusions are an effective source of tumor antigens to be presented to CD8+ cytotoxic T cells.[31] *In vitro*-derived exosomes can even function as cell-free vaccines and lead to a decrease or stabilisation of tumor growth.[32] A possible explanation might be that exosomes initiate immune-mediated cell death. Nevertheless, these scenarios look promising *in vitro* under stress conditions [33] but do not seem to be well-applied to clinical settings.[24] Opposite to the anti-tumor responses are other *in vitro* studies reporting a potential role of promoting tumor cell growth.[34] Particularly, the transfer of miRNAs through glioblastoma exosomes may induce tumor growth in a benign cell line.[35] Because the effects and content of exosomes can be versatile, it is not surprising that both pro- or anti-tumor effects have been described and that the role of exosomes might change during cancer progression.

Exosomes, prostasomes and other vesicles

Many types of vesicles have been described in literature. These vesicles are heterogeneous in terms of size, content and origin; therefore, they have different names. Unfortunately, the differences in nomenclature lead to confusion. It is still unclear if all of the different vesicles are unique in biological function, or if they represent a sliding scale of one entity. Based on their biogenesis, however, vesicles could be generally divided

Table 1. Characteristics of different types of vesicles secreted by prostate or PCa cells.[33,45]

Vesicle	Size (nm)	Known protein markers	RNA marker examples	Synthesis pathway	Function	Reference
Exosomes	50 - 150	CD9, CD63, CD81, CD82, Annexins, and RAB proteins	PCA-3, TMPRSS2:ERG	Merocrine	Antigen presentation, immune regulatory, and metastatic activity	10,45-47
Prostasomes	50 - 500	CD13,CD46, CD55, CD59, Annexins, and RAB proteins	-	Merocrine and apocrine	Immunosuppressive and sperm cell motility improving	36-39
Oncosomes	50 - 500	Signal transduction proteins	DIAPH3	Apocrine	ND	48
Microvesicles	100 - 1000	Integrins, selectins, and CD40 ligand	EGFRvIII	Apocrine	Procoagulation and anticoagulation	35,49
Ectosomes (microparticles)	50 - 1000	CR1 and proteolytic enzymes	-	Apocrine	Procoagulation and anticoagulation	50,51

ND: Not Defined

into two classes: merocrine (inward budding and exocytosis, such as exosomes) and apocrine (surface shedding) synthesis (Table 1).

Studies on small vesicles in the field of urology mainly used the terms of prostasomes and exosomes. Usually, the vesicles isolated from seminal/prostatic fluids are called prostasomes. Confusion starts with vesicles isolated from prostate (cancer) cells cul-

tured *in vitro* or grafted in mice. Currently, both prostasomes and exosomes are used. Therefore, the questions are whether prostasomes are prostate-derived exosomes, and whether the prostasomes from seminal fluid are the same as the vesicles secreted by cultured cells.

Prostasomes have a pure prostatic gland origin and are present in high concentrations in seminal/prostatic fluid.[36] These vesicles are suggested to be shed through exocytosis after their formation in a MVB (merocrine), such as exosomes, and possibly also by membrane shedding (apocrine).[36] Compared to exosomes, they are enriched with cholesterol, sphingomyelin, Ca^{2+}, GDP and many transmembrane proteins (CD13, CD46, CD55 and CD59).[37-39]

Electron microscopy (EM) showed that prostasomes are round and have a mean diameter of 150 nm (50 to 500 nm). This description is highly similar to the exosomes derived from prostate epithelial cells that are also round-shaped and have a diameter of 100 nm (50 to 200 nm).[10] A striking difference is that exosomes usually contain a lipid bilayer membrane, whereas prostasomes usually contain a cholesterol-rich lipid multilayer membrane.[40] In terms of their functions, prostasomes have mainly been implicated in human reproduction by exhibiting a specific and favourable effect on the motility of spermatozoa [41] and by delaying acrosomal reaction.[42] Potential immunosuppressive activities of prostasomes have been demonstrated and are suggested to protect spermatozoa from phagocytosis by cells of the female immune system.[43] The protein content of prostasomes is comparable to that of the exosomes derived from prostate cancer cell lines. Most of the identified proteins are well-characterised intracellular proteins, including annexins, Rab proteins, heat shock proteins 70 and 90 (HSP70/HSP90) and signal transduction proteins.[43,44] The identification of biomarkers for PCa, such as PSMA (FOLH1) that is also present in exosomes, suggests that prostasomes may be a valuable source for novel biomarkers. So far, no reports have been published assessing prostasomal RNAs.

According to their marginal differences in size, morphology and content, the two types of vesicles are similar. Only their functions and potentially their lipid composition differ. Nevertheless, we hypothesised that prostasomes are exosomes derived from prostate tissue in a biological setting. Experimental comparison between exosomes and prostasomes may help differentiate their specific prostasomal properties from the more general characteristics and clarify their similarities and differences in biogenesis, content and function.

Isolation and visualisation of exosomes

Isolation

For morphological and biochemical characterisation, exosomes are usually isolated by differential ultracentrifugation. This well-developed isolation method has been shown

to be effective and can process up to 250 ml of samples. Unfortunately, this method is time-consuming (approximately 6 hours) and, therefore, is unsuitable for daily clinical practice and might affect RNA and protein quality due to degradation. Therefore, faster and simpler isolation methods, such as filtration, precipitation and immunoaffinity purification, are needed. Filtration techniques have already been established and can rapidly enrich exosomes from complex fluids.[47,52-55] Because it is particularly useful for smaller volumes, it could be easily implemented in a clinical setting.

When isolating exosomes from body fluids, it is impossible to distinguish exosomes derived from different tissues, which is a problem when searching for content in a subset of exosomes derived from a specific tissue or cell type. In this situation, immunoaffinity purification using beads or columns coated with an antibody directed against a tissue-specific transmembrane protein can be applied.[56, and unpublished work]

Visualisation

Because of the small size of exosomes, EM is the most suitable technique for morphological characterisation (Figure 2).[48,57] Using gold-labelled immune electron microscopy, it is possible to investigate whether exosomes express certain proteins on their membrane.[58] Another way of visualisation is to use confocal microscopy (CM), with the membrane of exosomes fluorescently labelled.[59] Although unlabelled exosomes are too small to be visualised by standard confocal microscopy, the lipophilic fluorescent dyes in their membranes is easily detected. Also, CM can be used to visualise exosomes isolated by

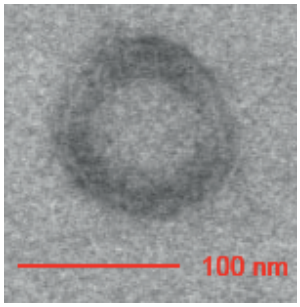

Figure 2. Electron microscopic image of an exosome. Close-up view of an exosome derived from the PC346c cell line. The diameter of the vesicle is approximately 100 nm, and the membrane is a lipid bilayer.

antibody-covered magnetic beads (Figure 3). Another advantage of this technique is the possibility of studying the functions of exosomes and their interaction with host cells.[60]

Quantification of exosomes

Counting exosomes in a sample remains a challenge. The number of exosomes is generally estimated by measuring the amount of protein.[47] The technology of fluorescence-activated cell sorting (FACS) is capable of counting exosomes; however, individually measuring each exosome (relatively tiny compared to a cell) in a flow system is difficult

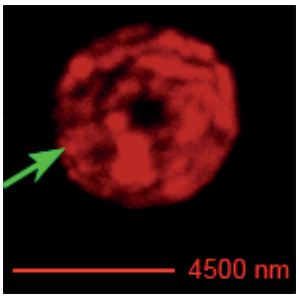

Figure 3. Exosomes attached to an immunoaffinity bead and visualised by confocal microscopy. These 4,500-nm size beads with antibodies directed against the exosomal membrane protein CD9 are incubated with fluorescently labelled exosomes derived from urine (100 nm). This close-up view of one bead shows that it contains multiple (red) exosomes. The green mark indicates a single exosome attached to the bead.

due to the resolution of the laser. These visualisation techniques (EM and CM) are not quantitative for determining the exact number of exosomes in a fraction but can be used to examine morphology and to determine transmembrane properties.

One study reported the successful use of a sandwich ELISA assay for exosome quantification.[61] In this assay, two different transmembrane proteins present on all exosomes are used. In theory, when such an assay is developed using one general transmembrane (capture) protein and one tissue- or cancer-specific transmembrane (detection) protein, the number of exosomes derived from a specific tissue can be measured.

Exosomes as biomarker treasure chests

The molecular content of exosomes is dependent on their cell origin and strongly associates with the original cellular conditions.[62] Therefore, the identification of tissue- or disease-specific exosomal proteins and RNAs will enable us to use these vesicles as a source of new biomarkers. Since the late 90s of last century, an increasing number of studies have investigated the protein content of exosomes and their potential diagnostic and prognostic values in various types of cancer, resulting in a comprehensive database consisting of 64 papers and a total of 2,400 different proteins.[63] All of these protein identifications have been obtained by mass spectrometry. In terms of RNAs, the first study on exosomes was performed in 2007.[28] Using microarray technology, they have shown that exosomes from mouse-derived bone marrow cells contain mRNAs and miRNAs. An increasingly number of papers, using microarrays as well, have described the potential role of proteins and miRNAs as diagnostic and prognostic tools.[64-66]

Until 2002, exosomes had been predominantly isolated and analysed from *in vitro* cell lines. More recent studies have showed that these vesicles can be isolated from body fluids, such as blood, urine, semen, amniotic fluid, malignant and pleural effusions, bronchoalveolar fluid, synovial fluid, saliva, and breast milk. These findings demonstrate that exosomes are present in all body fluids and can be used for determining health status.[67]

Exosomes as a diagnostic and prognostic tool for PCa

In terms of PCa, the reports on exosomes are very few. One of the first studies reported no apparent differences between exosomes of benign origin and malignant origin, regarding their synthesis, storage and release.[68] Most likely, these vesicles may differ in biochemical properties. Unfortunately, so far, no high-throughput techniques, such as mass spectrometry and microarray, have been used to evaluate the differences between exosomes of benign origin and malignant origin to identify new biomarkers. Four studies used mass spectrometry to profile exosomes derived from PCa cell lines, xenografts and metastases. To search for PCa-secreted proteins, serum from PCa-xenografted mice was analysed by mass spectrometry. All of the identified proteins were screened for human-specific sequences by extensive database searching. The proteins containing human-specific sequences were of PCa origin. Interestingly, the subcellular localisation of most of these proteins is cytoplasmic, supporting the idea that these proteins are secreted in mouse blood through exosomes. Indeed, proteomic profiling of exosomes derived from human PCa cell lines confirmed the presence of almost all of the previous identified serum proteins.[26] Two other studies analysed vesicles from prostate cell lines and vertebral prostate cancer metastases by mass spectrometry; they identified proteins related to angiogenesis, signal transduction pathways and cancer progression [48,69], including caveolin-1 (Cav-1), Akt, pyruvate kinase M2 (PKM2), programmed cell death 6 interacting protein (PDCD6IP) and poly(A)-binding protein 1 (PABPC1). Subsequent *in vitro* functional assays (such as migration and proliferation assays) demonstrated that these vesicles can influence cancer microenvironment and promote cancer progression. Although these findings are promising, further investigations are needed to fully elucidate the role of PCa exosomes in cancer development.

Aside from these biological studies, exosomes and exosomal content from patient samples have also been evaluated for their potential as potential biomarkers. Urinary exosomes from 10 organ-confined PCa patients undergoing hormonal therapy prior to radical radiotherapy were analysed.[58] Other than a considerable variation in the quantity of total exosomal proteins, no difference was observed between healthy men and PCa patients. Although these results do not specify which proteins are present in exosomes, it emphasises the technical feasibility of assessing exosomal proteins to evaluate the clinical status of PCa. However, better sample preparation, such as immunoaffinity isolation, and more robust technical approaches are needed to define significant differences with such a huge variation.

RNA expression analysis of urine-derived and PCa cell line-derived exosomes revealed that the known RNA-markers for PCa, such as the *TMPRSS2:ERG* fusion gene and *PCA3*, can be detected in exosomes by RT-PCR.[10] The *TMPRSS2:ERG* fusion transcripts were detected in urinary exosomes from two patients with high Gleason scores but not in those from two patients with low Gleason scores.[47] *PCA3* mRNA was detected in exosomes

derived from all patients. Interestingly, none of the hormone-treated patients showed detectable levels of *TMPRSS2:ERG* or *PCA3* RNAs, suggesting that the response to treatment might reduce the size of PCa tissue and, thereby, decrease the expression levels of these androgen-responsive genes.

Exosomes in other urological malignancies

Very few studies on exosomes in other urological malignancies are available. One group (Welton *et al.*) published a report on the profiling of exosomes from a bladder cancer cell line.[70] They measured exosomes derived from a single bladder cancer cell line by mass spectrometry and identified a set of protein biomarkers associating with bladder cancer, such as multiple tetraspanins and alfa-6 integrins . In respect of renal cell carcinoma, Zhang *et al.* evaluated the effects of exosomes as an immunotherapy tool by expressing GPI-IL-12 on exosomal membranes.[71] Implementation of this protein in exosomes significantly promoted T cell proliferation, contributing to an enhanced cytotoxic effect of these T cells. This effect may improve tumor rejection, therefore suggesting that exosomes may have potential application in immunotherapy.

Considerations

The studies on small vesicles in PCa describe the first step in developing new methods and identifying novel markers for the diagnosis and prognosis of PCa. Although the initial results are promising, further investigations are required to assess the exact clinical values and the biological functions of exosomes.

To investigate prostate- or PCa-derived exosomes from complex body fluids, current isolation protocols (such as ultracentrifugation) are not optimal. Procedures according to these protocols result in a heterogeneous sample of exosomes derived from several different organs. Organ-specific isolation can be achieved by immunoaffinity capture beads coated with antibodies directed against organ- or cancer-specific proteins. Experiments using latex or magnetic beads have been successfully used to achieve specific purification.[47]

For biomarker discovery from body fluids, it is important to decide which type of fluid to use. To search for markers from the prostate, serum, urine and semen are the obvious options. Collecting urine is less invasive, compared to drawing blood through venipuncture, and urinary exosomal proteins are generally more stable because the proteolytic activity in urine is lower than that in serum.[6] When urine is collected for prostasome/exosome study, the procedure is preferentially performed after prostate massage to increase the quantity of exosomes.[58]

Evaluation of exosomal content from retrospective samples with different tumor characteristics and a generally long follow-up may provide us novel diagnostic and prognostic biomarkers for PCa. To use retrospective biobank samples, the knowledge

of collection, storage, and processing conditions of urinary and plasma serum samples is essential. Storage without multiple cycles of thawing and freezing of whole urine at -80 degrees Celsius does not seem to affect exosomal content [6]. Exosomes can resist endogenous proteolytic activity in urine for at least 18 hours at 37 degrees Celsius. These findings indicate that exosomes are quite stable in complex body fluids.

CONCLUSIONS

Exosomes are small vesicles (50 to 100 nm) secreted by almost all tissues, representing their tissue origin. By isolating these exosomes, several problems of biomarker discovery from complex body fluids can be largely solved. Therefore, purification of prostate (cancer)-derived exosomes will allow us to profile the exosomes, providing a promising source of protein and RNA biomarkers for PCa. This profiling will contribute to the discovery of novel markers for the early diagnosis and reliable prognosis of PCa. Although the initial results are promising, further investigations are required to assess the clinical value of these exosomes in PCa.

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