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Junjie Xiao *Editor*

Extracellular Vesicles in Cardiovascular and Metabolic Diseases

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Extracellular Vesicles in Cardiovascular and Metabolic Diseases

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Preface

Released from diverse cells into the extracellular environment, extracellular vesicles (EVs) play essential roles in cell signaling by transporting their cargo, such as DNA, RNA, metabolites, proteins, small molecules, and lipids, to recipient cells. Those cargos in EVs can reflect their cell of origin, opening a window into altered cellular or tissue states, and their detection in biological fluids potentially offers a multicomponent diagnostic readout. Therefore, efficient exchange of cellular components through EVs can predict the diseases development. Cardiovascular and metabolic diseases commonly have complex pathogenesis and no symptoms in early period, which lead to difficulties in precise diagnosis and highly effective therapy. Since new findings and studies in the field of EV biology and metabolism are constantly being published, EVs are considered promising techniques for the diagnosis and treatment of cardiovascular and metabolic diseases.

Our group have made some efforts in studying the function of EVs and cardiovascular diseases, while other research groups worldwide have been reporting significant achievements in this field over the past decades. To advance EV research in the treatment of cardiovascular and metabolic diseases, it is necessary to publish a book summarizing the latest findings regarding the roles and mechanism of EVs in cardiovascular and metabolic diseases. In Part I, *Extraction Methods*, we summarized basic and common methodology for extracellular vesicles extraction. In Part II, *Extracellular Vesicles in Cardiovascular Diseases*, we focused on the relationship between EVs with pathophysiology of cardiovascular diseases, such as pathological cardiac hypertrophy, cardiac aging, ischemic injury, hypertension, coronary artery disease, vascular inflammation, and muscle atrophy. In Part III, *Extracellular Vesicles in Metabolic Diseases*, we emphasized the function of EVs in fatty liver, obesity, bone remodeling, and osteoporosis. In Part IV, *Therapeutic Implications*, we discussed their potential in acting as biomarkers for disease diagnosis and treatment, as well as serving as drug and gene delivery systems. In Part V, Other Aspects and Future Prospects, we discussed other aspects and future prospects about EVs. We look forward to further strengthening our findings in EVs and benefiting new approaches to cardiovascular and metabolic diseases in the near future. We'd be appreciated if our book can make any positive contribution for biologists, cardiologists, cardiovascular surgeons, endocrinologists, internists, nurses, undergraduate and graduate students in

medicine and cell biology, and others interested in cardiovascular and metabolic medicine.

We thank Dr. ZHU Yujiao for her help in preparing the book. We also acknowledge the grants from National Natural Science Foundation of China (82225005 and 82020108002), the grant from Science and Technology Commission of Shanghai Municipality (23410750100, 21XD1421300 and 20DZ2255400), and the "Dawn" Program of Shanghai Education Commission (19SG34).

Shanghai, China Junjie Xiao

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Part I

Extraction Methods

Updated Methods of Extracellular Vesicles Isolation

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Hongyun Wang, Zijiang Yang, Songwei Ai, and Junjie Xiao

Abstract

Extracellular vesicles (EVs) are considered as cargo and mediate intercellular communication. As natural biological nanoparticles, EVs can be secreted by almost all kinds of cells and exist in biofluids such as milk, urine, blood, etc. In the past decades, several methods have been utilized to isolate EVs from cell culture medium, biofluids, and tissues. Here in this chapter, we summarized conventional and novel methods and fundamental procedures of EVs extraction and purification from different biofluids (plasma, urine, milk, and saliva) and tissues (brain, intestinal tissue, muscles, and heart). The present section also discusses how to choose appropriate methods to extract EVs from tissues based on downstream analysis. This chapter will expand the horizons of EVs isolation and purification from different mediums.

Keywords

Extracellular vesicles · Isolation · Tissues · Biofluids

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1.1 Background

Extracellular vesicles (EVs) are natural membraned-enclosed nanoparticles with a diameter of 40–1000 nm [1]. Upon the size, biogenesis, and properties, the EVs are generally divided into two subgroups, including apoptotic body, microvesicles and exosomes [1]. EVs are abundant in various biofluids, cell culture medium, and tissues, which are not only potential mediators of physiological and pathological processes such as cardiovascular diseases (CVD) [2–6] but also cargo for drug delivery [7, 8]. This is because EVs have characterizations of perfect biocompatibility and they can mediate intercellular communication.

In order to investigate the effects of EVs on disease development and drug delivery,

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increasing demand of EVs isolation and purity was proposed. However, some studies demonstrated that different separation methods impact the product, purity, and biological activity of EVs. Paolini et al. extracted EVs from single or aggregated contaminants by serial centrifugation, iodixanol or sucrose density gradient and precipitation and investigated the activity of EVs. They found that using different protocols to separate EVs could strongly influence the activity of EVs [9]. Choosing an appropriate method to extract EVs should be into consideration despite there is no standard requirement now.

Here in this chapter, we overviewed the high-frequently used methods upon the source of EVs, which would give a reference when extracting EVs from different biofluids (plasma, urine, milk, and saliva) and tissues (brain, intestinal tissue, muscles, and heart). Firstly, we summarized conventional methods of EVs isolation, including ultracentrifugation, density gradient centrifugation, size-exclusion chromatography, ultrafiltration, immunoaffinity capture and precipitation (Fig. 1.1). Secondly, with the development of life science, novel methods are built to separate EVs from different mediums such as microfluidic chip and creative combination of multi methods (Fig. 1.1). Finally, not all these methods were applicable to most situation, several characterizations were still needed to be considered. Therefore, we also took an insight into the fundamental procedures of EVs extraction and purification from different biofluids (plasma, urine, milk, and saliva) and tissues (brain, intestinal tissue, muscles, and heart) (Fig. 1.2). Overall, our goal is to expand the horizons of the readers when they investigate the function and modification of EVs from different mediums and give suggestions for future clinical usages.

1.2 The Regular Methods of EVs Isolation

1.2.1 Ultracentrifugation (UC)

The ultracentrifuge was first designed by T. Svedberg in 1947 [10]. With the development of life science, UC has become one of the commonly used technologies in biochemistry and molecular biology. Especially, UC is one of the conventional techniques of EVs separation [11], which was reported by Théry C et al. in 2006 [12]. Generally, the method includes several steps, including removing cell and apoptotic debris at low speed, then eliminating larger vesicles at medium speed, and finally precipitating EVs at the high speed such as

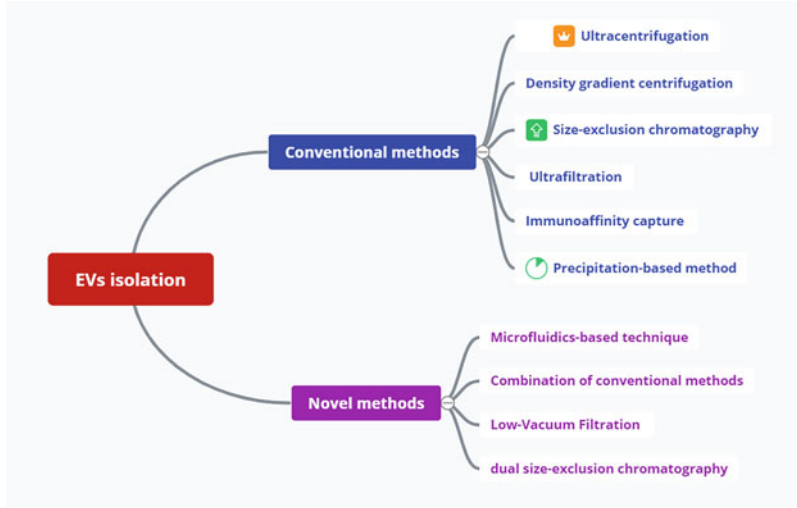

Fig. 1.1 Overview of the methods of EVs isolation

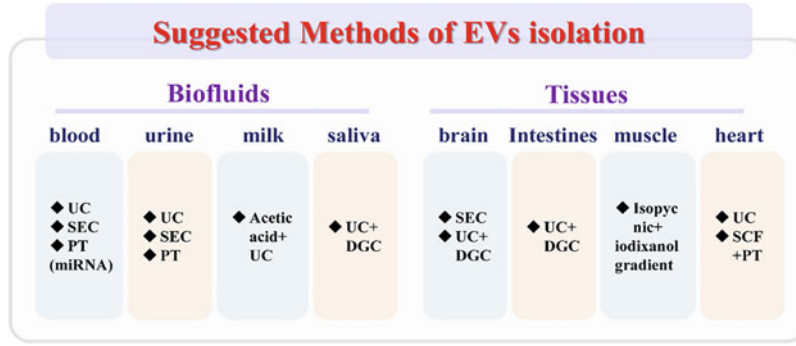

Fig. 1.2 Suggested methods for EVs isolation from different sources. *UC* ultracentrifugation, *SEC* size-exclusion chromatography, *PT* precipitation, *DGC* density gradient centrifugation, *SCF* serial centrifugation

100,000 × g. However, UC has obvious limits for clinical usage due to its low yield of isolation and time-consuming.

1.2.2 Density Gradient Centrifugation (DGC)

DGC is a technique combining UC with sucrose density gradient and can separate EVs from proteins or other lipid-like particles. It is previously used for separation of nucleic acid or ribonucleic acids [13]. Isolation of particles using DGC mainly depends on the settlement coefficient differences of particles and three kinds of mediums (cesium chloride, sucrose, and polysaccharide) are commonly used in DGC. The medium has four characterizations, including density gradient, easily adjusted pH, low osmotic pressure and non-toxic. Notably, the challenge of DGC is to extract EVs with the similar density particles and low recovery of EVs. Recently, the protocol of DGC has been improved by Li K et al. with higher recovery and purity for EVs isolation, named cushioned-density gradient ultracentrifugation (C-DGUC) [14]. The improvement mainly includes three advantages, firstly, the authors used a 60% iodixanol cushion to concentrate nanoparticles, which significantly increased the recovery and preserved the biological activity of EVs. Secondly, using density gradient ultracentrifugation to further purify EVs and remove contaminates. Finally, iodixanol itself has excellent biocompatibility and compatibility, which can be used in vivo or in vitro study with no need for its removal.

1.2.3 Size-Exclusion Chromatography (SEC)

SEC is a technique for EVs extraction upon the differences in particles' size, which depends on a column with specific diameter. The particles less than the diameter can enter through and travel along with the volume fluid [15]. SEC has been used to separate EVs from proteins and isolate EVs from different biological fluids. Interestingly, Ramesh Bokka et al. have reported that SEC can be used to isolate nanoparticles from tomato, which is an amazing study for EVs extraction from foods [16]. Similar to UC and DGC, SEC is time-consuming and not suitable for processing large samples. Despite its time-consuming, SEC shows advantages for preserving the biological structure and activity of EVs [17], and it is considered as a good candidate for EVs isolation owing to its convenient, scalable, and functional preservation [18].

1.2.4 Ultrafiltration (UF)

UF is a method that depends on the ultrafiltration membranes upon the different relative molecular weights of particles. These membranes allow the molecular particles less than its pore size to travel through and block the high molecular mass

substances, then extracting the EVs from biofluids [19]. UF is a simple and efficient technique for EVs isolation and exosomal-RNAs extraction. The disadvantage of UF is that EVs may block filtration pore, leading to a lower efficiency of EVs recovery. In addition, the existence of non-EVs particles may affect the purity of EVs [20].

1.2.5 Immunoaffinity Capture (IAC)

The IAC technique bases on the specific binding between antigen and antibody, which is mostly used in capturing tumor-derived EVs. Generally, the specific antibody is coated in microspheres or plate and directly binds to EVs. For example, immunoaffinity superparamagnetic nanoparticles (ISPN) are recently developed to bind EVs by connecting anti-CD63 antibody and then separate EVs from biofluids [21]. Notably, this method is suitable for EVs extraction from small number of samples.

1.2.6 Precipitation

The primary principle of precipitation is using a specific precipitation solution (such as polyethylene glycol, PEG) and then incubate it with samples upon its ability to entrap EVs and finally centrifugate at low speed to obtain EVs [22]. The precipitation method is a rapid approach and has been commonly used owing to its convenience, rapidness, and high efficiency in EVs recovery. However, the precipitation approach may not separate EVs from lipoproteins, which disturbs the purity and biological activity of EVs, especially plasma-derived EVs [23].

1.3 Novel Method of EVs Extraction

With the increasing attention of EVs, emerging new approaches or combination usage of classical methods have been developed for EVs isolation and purification.

1.3.1 Microfluidics-Based EVs Isolation Technique

Recently, microfluidic has been used in various particle separation, drug delivery, and diagnosis study, with advantages of high efficiency and low cost [24]. Based on microfluidics, different types of approaches have been developed to isolate EVs, such as microfluidics-based immunoaffinity capture [25] and microfluidics-based membrane filtration [26]. Here we reviewed some novel microfluidics-chip based techniques for EVs isolation. Ting-Wen Lo et al. modified the OncoBean microfluidic device and successfully improved the methods of EVs isolation by releasing EVs from the microfluidic device after capture [27]. Yeo et al. developed a centrifugal microfluidics-based label-free platform to extract EVs from small volume samples within short time and reported a high isolation efficiency (90%) and purity (85%) [28]. Differently, Kang et al. utilized a single microfluidic device, composed of four quadrants with a single inlet, successfully profiled EVs from different patient, which may promote the application of EVs in disease monitoring and diagnostics [29]. Recently, a microfluidic aqueous two-phase system was developed by Han et al. and the system was shown that it could effectively isolate EVs with a recovery of 83.4%. In addition, this system could separate the EVs with 65.4% of the proteins, increasing the purity of plasma-derived EVs [30]. To improve pre-treatment procedures of samples before EVs isolation, an integrated microfluidic system was developed and utilized in a manner of on-chip [26]. In general, microfluidics-based EVs isolation technique has made a great progress due to its advantages such as time-saving and sample-saving, while there are still some problems needed to solve.

1.3.2 Creative Combination of Different Methods

1. Combination of UC with precipitation: Ryu et al. combined UC with polymer-based precipitation to isolate EVs from cancer patients

and verified this combination was feasible for EVs extraction from human serum [31].

2. Combination of UF with SEC: Some traditional methods of EVs isolation had limitation owing to the breakage of biological activity and intact property. To solve this problem, UF and SEC were gradually used in isolating EVs owing to their advantage in preserving biophysical and functional properties of EVs. Nordin et al. creatively combined UF with size-exclusion liquid chromatography to isolate EVs with higher yields of intact and biophysically property, which was better than that of UC [32]. Lately, Guerreiro creatively used this combination to isolate the subtype of EVs (small EVs or exosome) from cell culture supernatants with low protein contamination, which provided a new strategy for exosome extraction [33].
3. Combination of UC and SEC: This combination had attracted increasing attention in blood-derived EVs isolation, which colud improve the proteomic profiling of EVs. For example, Koh et al. coupled UC with SEC to isolate EVs from blood with high yield, which enabled EVs isolation reproducible, time-efficient [34]. This combination had great potential to be used in clinical diagnosis and therapeutical intervention.
4. Combination of more than two methods: To improve the purity of EVs, three methods combination protocol had been tried by Simon et al. The researchers firstly extracted EVs from conditioned medium by UC and then processed by SEC, followed by a final concentrated procedure by an ultradevice. The UC-SEC-centrifugal filtration methods successfully obtained high yields of EVs and significantly reduced the protein contamination of EVs [35].

1.3.3 Newly Emerging Methods for EVs Isolation

Except the above techniques, several novel approaches had been verified effective for EVs extraction.

1. Low-vacuum filtration (LVF): Recently, Anna Drożdż et al. developed a low-vacuum filtration method (LVF) to isolate EVs from cultured medium [36]. This method depended on a dialysis membrane with molecular weight cut-off 1000 KDa and was applicable to high volume of samples. Briefly, the samples were filtered under low vacuum to extract EVs for the downstream analysis [36].
2. A dual size-exclusion chromatography: Jik-Han Jung et al. designed a novel dual size-exclusion chromatography column (dSEC) to isolate and purify EVs from plasma [37]. Based on this newly developed dSEC columns, the purity of EVs from plasma significantly increased when compared to conventional extraction methods of EVs.

Overall, every method has its specific advantages and disadvantages, which should be taken into consideration when using them in EVs isolation.

1.4 Recommended Methods for EVs Isolation from Biofluids

1.4.1 Plasma or Serum

Plasma and serum are widely used for EVs extraction in the past few years because EVs carry important biomarkers for disease diagnosis or drug delivery. The challenges of plasma or serum-derived EVs are purity, recovery, and biological activity. With the increasing number of attentions, several methods have been tried to isolate EVs from plasma or serum, such as UC, SEC, polymer-based precipitation, etc. Jenni Karttunen et al. evaluate the influence of precipitation-based method on EVs isolation from plasma and find that part of the EVs-specific miRNAs is lost during precipitation [38]. Precipitation is not sufficient for plasma EV-contained miRNAs extraction. To improve the purity of EVs from blood with lower protein contamination, combination of different methods has been utilized. For example, Navajas et al. utilize a method based on SEC and find that

using qEV column is fast and reliable, which can effectively separate the subpopulation of EVs upon size, etc. This small EVs enrichment approach is compatible with proteomic analysis within EVs [39].

However, a new commercial kit has been shown better than SEC for isolating EVs from plasma. Stranska et al. compare the effect of membrane affinity-based method (exoEasy kit) with SEC and find that the kit reduces the particle–protein ratio and exosome-associated proteins [40]. Actually, the downstream analysis should be taken into consideration when choosing a method to extract EVs or exosome from blood. Interestingly, some researchers have compared the protein profiling of serum-derived EVs by different methods. Askeland et al. recently performe a mass-spectrometry (MS) to compare proteomes of EVs by peptide-affinity precipitation, SEC, and UC [41]. All these three methods successfully isolate EVs from human plasma upon the nanoparticle-tracking analysis. However, the abundance, subtypes, and contamination of EVs by these three methods are different based on the label-free MS analysis. The MS results shows that the EVs isolated by UC and SEC had a high abundance than peptide-affinity precipitation. Therefore, UC and SEC derived EVs were considered as suitable methods for MS biomarker studies. Notably, the size of EVs extracted by SEC was smaller than that of UC, indicating the subtype differences between these two methods. Which method should be used to isolate EVs for MS analysis? It mainly depended on the research goals as well as the EV subtypes. In addition, Ji recently developed a new method to enrich EVs from human serum for proteomic analysis. Proteomic analysis demonstrated that the EVs extracted by this new method, hydrophobic interaction chromatography (HIC), successfully separated EVs with a high yield and reduced proteins contamination compared with conventional methods [42].

Next-generation sequencing of serum EVs demonstrated that striking method specific differences existed in EVs and EV-enclosed miRNAs. Through comparing five different methods, Buschmann et al. found two extraction methods (precipitation and membrane affinity) were suitable for miRNA biomarker discovery [43]. Precipitation-based commercial kit was gradually accepted and utilized for plasma-derived EV-miRNAs analysis [44, 45].

Specially, irregular method might be utilized to isolate EVs for special downstream analysis such as phosphor-proteomics analysis. A method based on chemical affinity purification (EVtrap) was used by researchers to isolate EVs from plasma. Through the phosphor-proteomics analysis of EVs, EVtrap showed a high recovery and fast process compared with conventional methods [46]. More investigations were still needed to find appropriate approaches to extract EVs for specific downstream analysis.

1.4.2 Urine

The concentration of EVs in urine is extremely lower than that of plasma, which is about 0.1% [47]. Therefore, isolating EVs from urine faces big challenges due to individual variation of urine, such as the pH value and osmolality. Many methods have been used in urinary EVs isolation in the past few years.

In 2015, Ramos et al. utilized SEC to isolate EVs from concentrated urine and their research showed that SEC was suitable for urinary EVs isolation with low contaminants [48]. Markowska et al. compared different commercial kits based on precipitation with UC to isolate EVs and extracellular RNAs from urine in 2017. The method invented by Ymir has shown great advantages in urinary EVs isolation compared with other precipitation methods and UC, including fast and non-toxic to cells, etc. [49]. Later, differential centrifugation method and immunoprecipitation were utilized to collect specific urinary EVs for proteomic analysis [50]. The methods mentioned above were complicated and not efficient for a high yield of urinary recovery. To overcome these difficulties, two-phase systems were created and used to isolate urinary EVs. Shin et al. combined a polyethylene glycol with aqueous two-phase systems to extract human urinary EVs with high specificity and selectively [51]. Recently, Park

et al. used three different methods (UC, precipitation-based commercial kit ExoQuick, and qEV) to isolate urinary EVs and compared the profiles of miRNAs of them. Despite these three methods have their advantages and limitations, UC, qEV, and ExoQuick were shown suitable for investing miRNAs profiling of urinary EVs [52]. Notably, methods to separate urinary EVs had multiple choices, which did not only depend on the downstream purpose but also notice the variation of urinary biophysical activity [53].

1.4.3 Milk

Human breast-milk derived EVs (mEVs) had specific effects on infants and attracted a growing attention in the past few years. Interestingly, Herwijnen et al. investigated the conservation of EV-miRNAs from human, cow, panda, and porcine milk. Several immune-related miRNAs such as let-7a and miR-148a were shared among these mammals. The conserved EV-miRNAs from milk included their sequence homology and incorporation, indicating that they were the products of evolution [54]. This discovery was helpful to form a general platform to isolate mEVs in different animals and promoted studies of mEVs. To data, multiple methods had been used to isolate EVs from milk. Despite UC was once used to isolate mEVs [55, 56], it was not suitable for EVs separation from low volume of milks. To overcome this difficult, Bickmore et al. utilized a precipitation-based method to isolate mEVs from low volume of milk [57]. However, the mEVs isolated by the above methods commonly had a low purity owing to the casein contamination. Therefore, SEC and some novels methods were utilized lately for mEVs enrichment. A method based on SEC was developed to enrich the subtype of mEVs (exosome), which had high yield compared with UC [58]. In addition, Somiya et al. developed a new method combination of acetic acid and UC and they used this method successfully separated mEVs from casein and significantly improved the purity [56].

1.4.4 Saliva

Exploring the compositions of salivary EVs (sEVs) was considered as a new direction for disease biomarkers discovery such as primary Sjögren's syndrome. For human salivary EVs isolation, methods such as SEC [59, 60], density gradient ultracentrifugation [61], precipitation-based method [62], and UC [63] have been utilized based on the different condition. Specially, microvesicles and exosomes were successfully isolated from filtered saliva samples by UC [63]. However, which method was better in isolating salivary EVs between UC and polymer-based precipitation? To answer this question, Li et al. recently analyzed the protein profiles of EVs isolated by these two methods. The proteomics data showed that the EVs isolated by UC had more specific protein markers than that isolated by precipitation, indicating that UC might prior to precipitation in isolating salivary EV-proteins [64]. Interestingly, a method combining UC with DGC was developed and used to isolate the salivary EVs from H. longicornis, which was applicable for miRNAs profiling analysis [65].

1.5 Recommended Protocols for EVs Extraction from Tissues

Recently, EVs from tissues have been attracting a wide attention owing to their roles on cell-to-cell communication, which may be helpful to discovery the mechanisms of disease. To date, some methods are tried and utilized in extracting EVs from different tissues. In this section, we expect to give some references for tissue-EVs isolation.

1.5.1 Brain

Brain tissue derived EVs (bEVs) were considered as biomarker cargos of neurodegenerative diseases. A combination usage of UC and sucrose step gradient was performed successfully to isolate EVs from human brain tissues in 2012

[66]. Later, Muraoka et al. modified this method to extract EVs from the brain tissue of human patients [67]. Similarly, the same method was also utilized to isolate the EVs from frozen human and murine neural tissues [68]. For hemibrain EVs isolation, serial UC method was mainly used after homogenization [69]. In addition, iodixanol floatation density gradient was used to purify the brain-derived EVs for downstream mass spectrometry analysis [70]. The detailed protocol for EVs isolation from whole or frozen brain tissues was described as previously [71]. However, which method was the best one for bEVs isolation was still unclear. To solve this problem, Huang et al. recently compared different methods of bEVs isolation, including SEC, DGC, and UC and evaluated the recovery and contents of EVs. The data of small RNA sequencing and proteomics analysis demonstrated that SEC may be an alternative to process large numbers of human and mouse brain tissues [72]. Notably, (1) perfusion was suggested before brain-EVs isolation, no matter which method was used; (2) The condition of tissues (such as death or frozen) and the processing may influence the subtype and composition of bEVs.

1.5.2 Intestinal Tissue

There are few researches about intestinal EVs isolation despite intestinal and gut are so hot now. For intestinal EVs isolation, Chen et al. adapted a method combination of UC with DGC [73]. Briefly, the fresh intestinal tissue should be placed on ice and then cut into small segments. Serial centrifugation was performed followed by collagenase digestion. After filtration, the EVs pallet was resuspended by PBS after UC. Finally, the intestinal EVs were further purified by DGC and diluted by PBS.

1.5.3 Muscles

Skeletal muscle is an organ responsible for glucose metabolism and is associated with insulin sensitivity. Increasing evidence show that EVs mediate intercellular communication between skeletal muscles and other tissues such as adipose tissue and liver. Therefore, exploring the role and contribution of skeletal muscle-derived EVs are crucial to understand the mechanism of metabolic diseases. However, muscle tissue are less used than muscle cells supernatant for isolating EVs among the publications, which still has some questions to answer. Specially, one recent research reportes a method based on isopycnic centrifugation/iodixanol gradient to isolate EVs from muscle tissues after trans-cardial perfusion with PBS, EVs populations separated by this method have a high purity and low contamination [74].

1.5.4 Heart

Cardiovascular diseases are the leading cause of death worldwide and multiple studies including cardiac rehabilitation and therapy, especially for exercise-induced cardioprotection, are performed [75–77]. Several types of cells such as cardiomyocyte, fibroblasts, endothelial cells exist in heart. Communications among these cells mediated by EVs play important role in maintaining cardiac function. To explore the detailed communication intra-cardiac, heart tissue derived EVs are attracting attentions. To date, there are two sources to obtain cardiac EVs, including ex vivo culture and non-culture way. Leitolis et al. dissociated the heart tissues and cultured them in ex vivo. The supernatants of cardiac dissociations culturing medium were collected and used to separate EVs by UC [78]. Differently, heart tissues had been successfully used to isolate intra-cardiac EVs. To directly extract intra-cardiac EVs from heart tissues, serial centrifugation and precipitation were used. Loyer et al. successfully extracted intra-cardiac EVs from human interventricular septum fragments by this method [79]. Besides, new method was created to isolate cardiac EVs from heart tissues. Recently, Gyun Oh utilized Langendorff-based approach followed by serial centrifugation to extract EVs successfully from heart tissue

[80]. Despite we can separate EVs from heart tissues, the recovery, the purity and the biophysical activity of cardiac EVs are still weak, which needs improvement.

1.6 Perspective

EVs are important "postmen" secreted by almost all kinds of cells and exist in biofluid. They can transfer biological information by delivering molecules such as proteins, lipids, RNAs, etc. With the development of equipment, the present methods will be modified to be applicable for EVs isolation with a higher recovery, a convenient condition, a higher purity, and biological activity. EVs isolation will have a demand of extracting specific subpopulations from multiple tissues and fluids. Preserving the biophysical activity of EVs from tissues or biofluids is very important to reveal the underlying mechanism of diseases, which will be helpful to develop drug delivery system.

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Part II

Extracellular Vesicles in Cardiovascular Disease

Extracellular Vesicles and Pathological Cardiac Hypertrophy

2

Rongrong Gao and Xinli Li

Abstract

Pathological cardiac hypertrophy is a well-recognized risk factor for cardiovascular diseases (CVDs). Although lots of efforts have been made to illustrate the underlying molecular mechanisms, many issues remain undiscovered. Recently, intercellular communication by delivering small molecules between different cell types in the progression of cardiac hypertrophy has been reported, including bioactive nucleic acids or proteins. These extracellular vesicles (EVs) may act in an autocrine or paracrine manner between cardiomyocytes and noncardiomyocytes to provoke or inhibit cardiac remodeling and hypertrophy. Besides, EVs can be used as novel diagnostic or prognostic biomarkers in cardiac hypertrophy and also may serve as potential therapeutic targets due to its biocompatible nature and low immunogenicity. In this chapter, we will first summarize the current knowledge about EVs from different cells in pathological cardiac hypertrophy. Then, we will focus on the value of EVs as therapeutic agents and biomarkers for pathological myocardial hypertrophy.

Keywords

Extracellular vesicles · Exosomes · microRNAs · Pathological cardiac hypertrophy · Therapeutic agents · Biomarkers

Abbreviations

ADSC-Exo	Exosomes from adipose-derived stem cells
CDCs	Cardiosphere-derived cell exosomes
CF-iPSCs	Cardiac fibroblast-induced pluripotent stem cells
CFs	Cardiac fibroblasts
CMs	Cardiomyocytes
CVD	Cardiovascular disease
EPCs	Endothelial progenitor cells
EVs	Extracellular vesicles
HIMF	Hypoxia-induced mitogenic factor
MAPK	Mitogen-activated protein kinase
MSCs	Mesenchymal stem cells
MVs	Microvesicles
TAC	Transverse aortic constriction
TGF-β	Transforming growth factor-β

2.1 Background

Cardiac hypertrophy is a compensatory mechanism to deal with the decreased cardiac output and a limited form of adaptation in response to an increased cardiac stress. In general, there are two

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types of hypertrophies: physiological cardiac hypertrophy and pathological cardiac hypertrophy, and they differ greatly due to the underlying molecular mechanisms, cardiac phenotype, and prognosis [1, 2]. In addition to the distinctions, physiological and pathological hypertrophy both involve cardiomyocytes enlargement, while pathological hypertrophy accompanies with ventricular remodeling. If long-term pathological factors are not removed, the heart will lead to extensive cardiac remodeling and then result in cardiac fibrosis, hypertrophy, and heart failure eventually. Briefly, pathological stimuli lead to cardiomyocytes enlargement and cause fibroblasts to proliferate and secrete extracellular matrix proteins and proinflammatory cytokines. Up to now, mounting evidence has been made to elucidate the signaling mechanisms that involve maladaptive cardiac remodeling and dysfunction: cardiomyocytes death, cardiac fibrosis, Ca^{2+}-handling proteins dysregulation, mitochondrial dysfunction, fetal gene expression reactivation, as well as sarcomere structure transformation [3, 4].

Communication between cardiomyocytes and noncardiomyocytes in the progression of cardiac hypertrophy has been reported previously. Within the cellular microenvironment, cells crosstalk through cell-to-cell contact, specific molecules, or through EVs [5, 6]. Initially, it was universally acknowledged that EVs had little impact on neighboring cells [7]. While in recent years, EVs have been well recognized to have essential roles in intercellular communication by delivering small molecules or proteins, including lipids, DNA, mRNA, miRNA, siRNA, and lncRNA into the receptor cells. These moleculars, once thought as cell debris, may act in an autocrine or paracrine manner between different cells to promote or reverse cardiac hypertrophy and remodeling. EVs, which refer to exosomes, microvesicles (MVs), and apoptotic bodies, differ with regard to biological sources, secretion methods, and contents [8]. Exosomes are produced within cell endosomes and contain about 350–400 proteins from the variety of cellular organelles or membranes [9–11]. Microvesicles are cell membrane-derived particles about 100–1000 nm in size, containing nucleic acids, lipids, or proteins. While apoptotic bodies are greater than 1000 nm and were generated from the cell membrane, which contains fragments of dying cells [12]. Exosomes, MVs, as well as apoptotic bodies all can be isolated from different biologic fluids [13, 14], which suggests that these EVs could be used as novel diagnostic or prognostic biomarkers.

In the context of pathological cardiac hypertrophy, accumulating evidence has elucidated that exosome-carried cellular proteins or noncoding RNAs were derived from different cells, including cardiomyocytes, fibroblasts, endothelial cells, and immune cells [15, 16]. Besides, EVs derived from stem cells also have been shown to be beneficial for regenerative potential of the heart [17–19]. These vesicles regulate cell-to-cell communication, promote or inhibit the activities of recipient cells, and participate in various biological processes [20]. As a new method to reverse pathological cardiac hypertrophy, EVs can break the intercellular information transmission during pathological process, which exhibits great advantages and application prospects.

In this chapter, we will discuss the current understanding of the roles of EVs in cardiac diseases, with a particular emphasis on pathological cardiac hypertrophy and perspectives for their potential value in cardiac therapies. The roles of different cell-derived EVs in pathological cardiac hypertrophy are depicted in Table 2.1 and Fig. 2.1.

2.2 Cardiomyocyte-Derived EVs on Pathological Cardiac Hypertrophy

EVs from different cell types have been shown to be involved in pathological processes. Cardiac fibroblasts, myocytes, endothelial cells, and vascular smooth muscle cells are the major cellular constituents of the heart. As shown in a previous study, the adult murine myocardium is composed of approximately 25–35% cardiomyocytes, and the rest is collectively named non-myocytes, including fibroblasts, endothelial cells, pericytes,

Table 2.1 Roles of EVs in pathological cardiac hypertrophy

Secretory cells	EVs type	Recipient cells	microRNAs or proteins	Function
CMs	Exosomes	Unknown	miR-181a	Downregulation of miR-181a resulted in the attenuation of myocardial fibrosis and hypertrophy [27]
	Exosomes	CFs	miR-217	Participate in cardiac hypertrophy and cardiac fibrosis processes through regulating PTEN [28]
	Exosomes	CFs	miR-378	Suppress p38 MAP kinase phosphorylation by targeting MKK6 in cardiac hypertrophy [29, 33]
	Exosomes	Endothelial cells and native CMs	Hsp20	Overexpression of Hsp20 significantly attenuated STZ caused cardiac dysfunction, hypertrophy, apoptosis, fibrosis, and microvascular rarefaction [34]
	Exosomes	CFs	Hsp90	Modulate collagen upregulation via biphasic activation of STAT-3 in fibroblasts during cardiac hypertrophy [35]
	Unknown	CFs	HIMF	Induce fibroblast proliferation and migration, and myofibroblast differentiation by neutralizing IL-6 [39]
	Exosomes	Endothelial and smooth muscle cells	AT1R	Modulate vascular responses to neurohormonal stimulation under condition of TAC [16]
CFs	Microvesicles	CMs	miR-146a	Suppress SUMO1 expression and induce cardiac dysfunction in maladaptive hypertrophy [50]
	Microvesicles	CMs	miR-27a	Contribute to the progression of cardiac hypertrophy through targeting PDZ and LIM domain 5 (PDLIM5) [51]
	Exosomes	CMs	miR-21-3p, miR-133a, miR-208, and miR-499	Mediate cardiomyocyte hypertrophy by silencing SORBS2 and PDLIM5 [54]
	Exosomes	CMs	Egfr and Spp1	CF exosomes upregulated RAS in cardiomyocytes via the activation of mitogen-activated protein kinases (MAPKs) and Akt [57]
Macrophage	Exosomes	CMs and CFs	miR-155	Promote cardiomyocyte pyroptosis and cardiac hypertrophy by directly targeting FoxO3a [65] Suppress fibroblast proliferation and increase fibroblast inflammation [67]
EPCs	Microvesicles	CMs	Unknown	Protect CMs from hypertrophy and apoptosis through activating the PI3K/Akt/eNOS pathway via the RNAs carried by EPC-MVs [74]
Adipocytes	Exosomes	CMs	miR-200a	Contributed to decreased TSC1 and subsequent mTOR activation in the progression of cardiomyocyte hypertrophy [75]
MSCs	Exosomes	CMs and CFs	miR-29a	Protect cardiomyocytes against pathological hypertrophy [80]
ADSCs	Exosomes	CMs	Unknown	ADSC-Exo prevents cardiac I/R injury through the miR-221/miR-222/PUMA/ETS-1 pathway [82]

(continued)

Table 2.1 (continued)

Secretory cells	EVs type	Recipient cells	microRNAs or proteins	Function
	Exosomes	CMs	IGF1	Suppress MAPKs, hypertrophy, and fibrosis Signaling as well as increases H9c2 cell viability [83]
CF-iPSCs	Exosomes	Embryoid bodies	miR22	Alter miR22 expression, which is a key regulator of cardiac hypertrophy and remodeling [85]
CPCs	Exosomes	CMs	miR-133a	Protect the hearts from pathological cardiac hypertrophy, fibrosis, and apoptosis [86]
CDCs	Exosomes	Unknown	Unknown	Decrease LV collagen content and cardiomyocyte hypertrophy while increasing vessel density [98, 99]
	Exosomes	Macrophage	EV-YF1	Attenuate cardiac hypertrophy and renal injury induced by ang II infusion in association with altered IL-10 expression [100, 101]

and telocytes [21]. Cardiomyocytes, as the most cells in the heart, play substantial roles during pathological cardiac hypertrophy, angiogenesis, cardiac fibrosis, autophagy, and apoptosis [22]. In the state of pathological cardiac hypertrophy, the release of specific exosomes by cardiomyocytes was investigated. For instance, cardiomyocyte-derived exosomes can upregulate 175 genes and downregulate 158 genes in cardiac fibroblasts [23]. Moreover, the molecular markers of cardiomyocyte-derived exosomes are the tetraspanins CD9, CD63, CD81, and the heat shock protein (Hsp)70 [24].

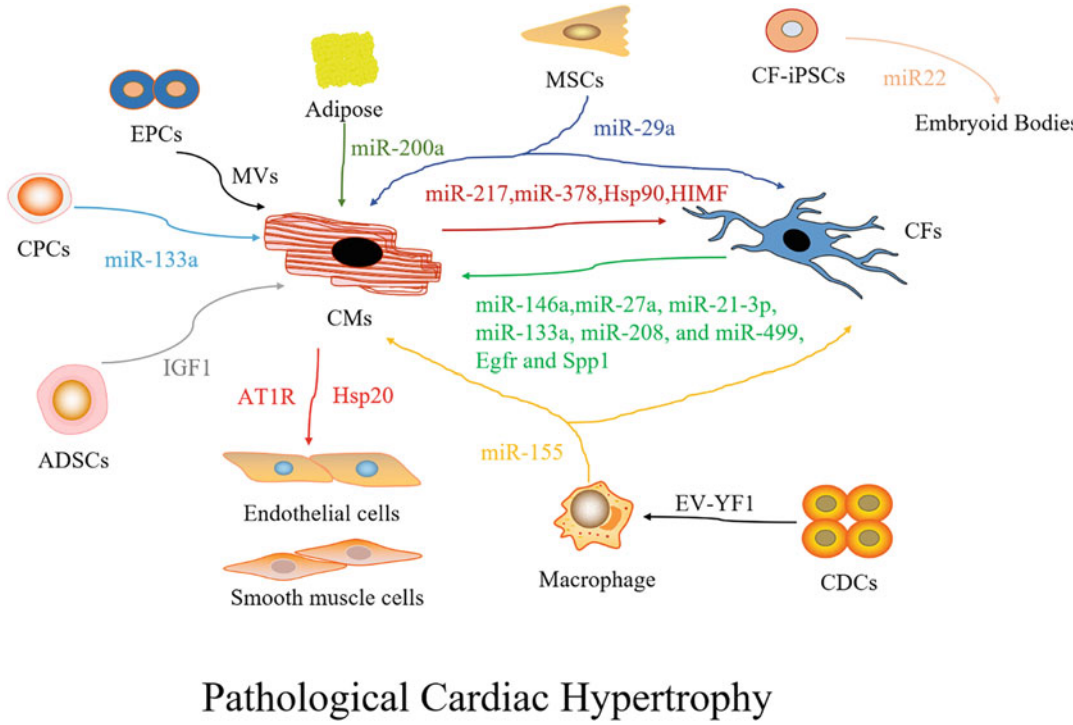

Fig. 2.1 EVs from different cells in pathological cardiac hypertrophy

Cardiomyocytes can selectively change the cargo in their exosomes and increase exosome release when suffering stresses, including glucose deprivation, hypoxia, inflammation, injury, or increased angiotensin II production [25, 26]. For example, recent studies have demonstrated that miR-181a, miR-217, miR-378 are secreted from cardiomyocytes in response to pathological hypertrophy [27–29]. miR-181a expression was decreased after Sacubitril/valsartan treatment in human iCM (human-induced pluripotent stem cell-derived cardiomyocytes), and this downregulation resulted in the alleviation of myocardial fibrosis and hypertrophy [27]. miR-181a is known as a novel regulator of cardiac remodeling, it mediates myocardial hypertrophy by regulating autophagy, p53-p21 pathway, PTEN/PI3K/AKT signaling, and the aldosterone-mineralocorticoid receptor (Aldo-MR) pathway [30–32]. Taken together, these suggested that cardiomyocyte-derived exosomes, which contain miR-181a, could protect the heart from pathological hypertrophy through multiple targets. Except miR-181a, miR-217 is another miRNA secreted from myocytes. Cardiomyocyte-derived miR-217-containing exosomes increased proliferation of fibroblasts through regulating PTEN [28]. Additionally, miR-378 is released from cardiomyocytes in response to mechanical stress and could suppress p38 MAP kinase phosphorylation by targeting MKK6 in cardiac hypertrophy through a paracrine mechanism [29]. Another study elucidated that miR-378 attenuated thoracic aortic constriction-induced cardiac hypertrophy and improved cardiac function by suppressing the MAPK signaling pathway [33].

Except these miRNAs mentioned above, cardiomyocytes also can secrete particular proteins to restore cardiac function during cardiac pathological hypertrophy. Among these proteins, two members of the heat shock proteins (Hsp20 and Hsp90) possessed important roles. One study showed that diabetic cardiomyocytes could secrete harmful exosomes, which contain lower levels of Hsp20. And Hsp20 overexpression could attenuate STZ-caused cardiac dysfunction, hypertrophy, cardiomyocyte apoptosis, cardiac fibrosis, and microvascular rarefaction [34]. As for Hsp90, Sagartirtha Sarkar found that myocyte-derived Hsp90 and myocyte-secreted IL-6 are responsible in unison for the biphasic activation of STAT-3 signaling during cardiac hypertrophy [35]. Furthermore, other studies interpreted that Hsp90 modulated cardiac ventricular hypertrophy via activation of MAPK pathway, NF-κB pathway, and stabilization of HIF-1 alpha [36–38]. Besides, HIMF (hypoxia-induced mitogenic factor) is a secreted proinflammatory cytokine and could induce cardiac fibrosis via a cardiomyocyte-to-fibroblast paracrine effect by neutralizing IL-6 [39]. Additionally, HIMF has a critical role in the development of cardiac hypertrophy via increasing the cytosolic Ca^{2+} concentration and activation of the CaN-NFAT (calcineurin-nuclear factor of activated T cell) and MAPK (mitogen-activated protein kinase) pathways [40]. Meanwhile, AT1R-enriched circulating exosomes are released from cardiomyocytes and then incorporated into endothelial and smooth muscle cells under conditions of transverse aortic constriction (TAC) [16].

Therefore, these cardiomyocyte-derived EVs can regulate myocardial remodeling through a paracrine mechanism, which provides insights into the crosstalk between different cells in this process.

2.3 Cardiac Fibroblast-Derived EVs on Pathological Cardiac Hypertrophy

Cardiac stress results in cardiomyocyte hypertrophy, whereas fibroblasts begin to proliferate, differentiate into myofibroblast, secrete extracellular matrix proteins and proinflammatory cytokines, which lead to cardiac fibrosis and remodeling eventually. Fibroblasts are known to play crucial roles in heart development and diseases as the second most cells in the heart, especially in the condition of pathological myocardial hypertrophy [41–43]. Previous study has found that subtype switching of fibroblasts was highly associated with hypertrophic responses of cardiomyocytes at initial stage of cardiac hypertrophy

[42]. Furthermore, other studies showed that adult murine cardiomyocytes develop hypertrophy when co-cultured with cardiac fibroblasts or treated with conditioned fibroblast media, which implies a fibroblast–cardiomyocyte communication during pathological processes [44, 45]. Mounting factors secreted from fibroblasts potentially affect the biological behaviors of cardiomyocytes in response to pathological insults.

Firstly, miR-146a was previously discovered as a negative regulator of innate immune responses by targeting interleukin-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6) [46], and its roles in the heart have been shown to vary by heart disease models [47–49]. In a recent study, miR-146a was reported to induce cardiac dysfunction in maladaptive hypertrophy via downregulating SUMO1 expression and modulating Ca2+ cycling, which was secreted from trans-differentiated fibroblasts and then transferred into cardiomyocytes by EV-mediated trafficking in the failing heart [50]. Besides, miR-27a is another fibroblast-derived miRNA, which is correlated with hypertrophy and contributes to the progression of cardiac hypertrophy through targeting PDZ and LIM domain 5 (PDLIM5) [51–53]. Most importantly, fibroblast exosomal-derived miR-21-3p, a star player in cardiac hypertrophy, was a potent paracrine-acting RNA molecule that could induce cardiomyocyte hypertrophy by silencing SORBS2 and PDLIM5 in Ang II-induced cardiac hypertrophy model [54–56]. Furthermore, miR-133a, miR-208, and miR-499 are all secreted by fibroblasts and involved in cardiac hypertrophy by influencing gene encoding and expressing calcium channel inositol trisphosphate 3 receptor (InsP3R) [54]. Moreover, one study demonstrated that fibroblast-derived exosomes increased the expression of renin, angiotensinogen, AT1R, and AT2R, decreased angiotensin-converting enzyme 2, and enhanced Ang II production in cultured cardiomyocytes via the activation of mitogen-activated protein kinases (MAPKs) and Akt, and this effect was blocked by both AT1R and AT2R antagonists [57]. And it revealed that specific targeting of Ang II-induced exosome release from CFs may serve as a novel therapeutic approach to treat pathological cardiac hypertrophy.

Above all, EV-mediated communication mechanism between fibroblasts and cardiomyocytes provides new understanding about pathological cardiac hypertrophy, and these fibroblast-derived EVs may serve as promising therapeutic targets in the future [58].

2.4 EVs from Other Cell Types in Pathological Cardiac Hypertrophy

Numerous studies uncovered that cardiomyocytes and fibroblasts are the dominating cell types in the process of cardiac hypertrophy and remodeling, while other cell types also have crucial roles during the progression.

The inflammatory immune microenvironment has vital roles in the development of myocardial hypertrophy. Previous study observed that macrophages were activated 5 weeks after TAC in accordance with the start of decline in cardiac function, which suggests a stage-dependent role of macrophages in the progression of pathological cardiac hypertrophy [42]. TNF-α and TGF-β are cytokines excreted by macrophages and involved in the progression of pathological cardiac remodeling, cardiac fibrosis, and hypertrophy [59–62]. Recently, interest has been gained to elucidate the role of macrophage-derived exosomes in cardiovascular diseases [63]. One previous study has shown that miR-155 was able to promote cardiac hypertrophy through Jaird2 signaling pathway in cardiomyocytes [64]. Furthermore, miR-155 was synthesized and loaded into exosomes in increased infiltration of macrophages, and these miR-155-containing exosomes could promote cardiac hypertrophy by directly targeting FoxO3a in uremic cardiomyopathy model [65]. Interestingly, exosomes derived from angiotensin II (Ang II)-induced hypertrophic cardiomyocytes could induce inflammation in macrophages via miR-155 mediated MAPK pathway [66]. Besides, macrophage-derived

miR-155, as a paracrine regulator for fibroblast proliferation and inflammation, suppressed fibroblast proliferation and increased fibroblast inflammation, leading to an impaired cardiac repair after myocardial infarction [67]. Taken together, the communication between macrophage and fibroblast or cardiomyocyte shows complex regulatory networks during pathological cardiac hypertrophy in the heart.

Except macrophages, previous evidence indicates that endothelial progenitor cells (EPCs)-derived microvesicles (MVs) can modulate endothelial cell survival and proliferation [68–70]. One clinical study showed that hypertensive patients with electrocardiographic left ventricular hypertrophy evidence have decreased circulating EPC numbers and adhesive function compared to those without left ventricular hypertrophy [71]. These findings may explain the pathogenetic processes that link left ventricular hypertrophy and endothelial injury in cardiovascular disease [72, 73]. Moreover, to elucidate the underlying mechanism, Yanfang Chen et al. found that EPC-derived MVs could protect cardiomyocytes from Ang II-induced hypertrophy and apoptosis accompanied with the upregulation of Akt/p-Akt and its downstream eNOS/p-eNOS [74].

In addition, myocardial hypertrophy can be modulated by endocrine organs and adipose tissue is a vital endocrine organ that maintains metabolic homeostasis in the heart. Nanping Wang et al. demonstrated that activation of PPARγ signaling in adipocytes increased miR-200a expression and secretion through utilizing cocultures of adipocytes and cardiomyocytes. And delivery of miR-200a in adipocyte-derived exosomes to cardiomyocytes contributes to decreased TSC1 and subsequent mTOR activation in the progression of cardiomyocyte hypertrophy [75]. The results provide insights into understanding the crosstalk between adipocytes and cardiomyocytes in regulating cardiac hypertrophy and remodeling.

Therefore, targeting the functional properties of these EVs could open a novel therapeutic approach for pathological cardiac hypertrophy.

2.5 Stem Cell-Derived Exosomes on Pathological Cardiac Hypertrophy

Increasing evidence validates the value of stem cell-derived exosomes on clinical and preclinical myocardial injury models [76]. Besides, stem cell-derived exosomes are less immunogenic than parental cells, because of a lower content of membrane-bound proteins.

Mesenchymal stem cells (MSCs) are self-renewing multipotent stromal cells, which can be isolated from various tissues, and are capable of differentiating into diverse cell types. MSCs have been verified in ischemic and reperfusion diseases through regulating myocardial energy metabolism and inhibiting cell death pathway [77–79]. With regard to myocardial hypertrophy, one study showed that MSC-derived exosomes significantly protected myocardium against pathological remodeling, preserved heart function when pressure overload induced by transverse aortic constriction (TAC) [80]. Besides, adipose-derived stem cells (ADSCs) play vital roles in wound repair [81]. Exosomes from adipose-derived stem cells (ADSC-Exo) can be absorbed and internalized by cardiomyocytes to inhibit apoptosis and hypertrophy by reducing the expression of PUMA and ETS-1 [82]. Furthermore, Pg-LPS damaged H9c2 cells co-cultured with ADSCs increase cardiomyocyte viability through suppressing hypertrophy, apoptosis, fibrosis, and MAPK markers [83]. And ADSC-encapsulated hydrogetics could regulate anti-inflammatory factors in cardiomyocytes, which are important instruments for the potential treatment of cellular vesicles and myocardial infarctions in hypertrophy [84]. Taken together, these data provide strong evidence that ADSC-Exo has broad prospects for clinical application in pathological cardiac hypertrophy. Furthermore, exosomes secreted from cardiac fibroblast-induced pluripotent stem cells (CF-iPSCs) may alter several miRNAs expression, especially miR-22, which is a key regulator of cardiac hypertrophy and remodeling [85].

A study by Izarra et al. revealed that the existence of a group of endogenous stem cells in the adult heart, named cardiac progenitor cells (CPCs), contributes to the progression of cardiac hypertrophy. CPC-derived exosomal miR-133a could protect the hearts from pathological cardiac hypertrophy, fibrosis, and apoptosis [86], while the authors failed to identify downstream partners.

Above all, mesenchymal stem cells are more suitable for the treatment of cardiac hypertrophy because they involve fewer ethical issues, higher self-renewal ability, and lower immunogenicity [87].

2.6 Exosomes from Cardiosphere-Derived Cells on Pathological Cardiac Hypertrophy

For decades, the adult mammalian heart was thought to be a post-mitotic organ. While recent studies changed this view with the identification of endogenous cardiac stem cells residing within the adult heart—cardiac progenitor cells (CPCs) [88, 89]. Cardiosphere-derived cells (CDCs) are a kind of CPCs that form spherical aggregates as cultured suspension. CDCs are a mixed cell population that have shown great potential in stimulating endogenous mechanisms of cardiac repair and attenuating adverse remodeling [90, 91]. CDCs can be extracted and isolated from the patient's myocardium and then administered by intramyocardial injection or intracoronary infusion [92]. After previous success in the animal experiments, several clinical trials have elucidated the safety and efficacy of autologous CDC therapy in humans [92–96]. Accumulating studies have shown that the benefits of CDCs are mediated by the secretion of extracellular vesicles [97]. A randomized placebo-controlled study demonstrated that cardiosphere-derived cell exosomes can decrease acute ischemia-reperfusion injury and halt chronic post-MI adverse remodeling in pigs [98]. Besides, another study also confirmed that CDC-derived exosomes could attenuate pressure overload-induced right ventricular dysfunction in juvenile Yorkshire pigs [99]. While future studies are needed to include qualitative and quantitative evaluation of growth factor and miRNAs concentration in these two studies. Meanwhile, Eduardo Marbán and Geoffrey de Couto demonstrated that infusions of CDC-derived exosomes or the Y RNA fragment EV-YF1, a CDCexo-derived noncoding RNA, can attenuate cardiac hypertrophy induced by Ang II infusion, without affecting blood pressure, in association with changes in the expression of the anti-inflammatory cytokine IL-10 [100, 101].

2.7 Translational Perspective

The heart undergoes hypertrophy in response to pressure overload to improve contractility and reduce ventricular wall stress in the beginning, but this adaptive hypertrophy leads to heart failure through pathological remodeling eventually. EVs derived from different cells have been widely recognized for their therapeutic potential in cardiovascular diseases [102, 103]. The cell sources of EVs used in experimental studies were different types of stem and progenitor cells, including MSCs, ESCs, CPCs, and others [103–105]. Compared to stem cell therapy, cell-free products containing different bioactive substances such as mRNAs, miRNAs, or proteins have more advantages [106]. Mounting evidence has validated the positive impact of EVs on clinical and preclinical experiments [76, 98, 99]. For example, MSC-derived exosomes exert a pleiotropic protective effect on pulmonary hypertension and right ventricular hypertrophy [107].

Moreover, EVs can be measured in most body fluids, while studies of EV-based biomarkers for CVDs are still in its infancy [10, 108]. EV-containing miRNAs are widely investigated, which may serve as potential diagnostic or prognostic biomarkers in diverse cardiovascular pathologies. For instance, one study showed that circulating exosomal miR-133a and miR-1 were higher in patients with acute coronary

syndromes than normal control [109]. Additionally, high levels of endothelial cell-derived miRNAs, including miR-126 and miR-199a, were in accordance with a lower major adverse cardiovascular event rate in patients with stable coronary artery disease [110]. Besides, particular proteins enriched in EVs also were investigated as potential biomarkers. As for pathological cardiac hypertrophy, AT1R-enriched circulating exosomes are released from cardiomyocytes under conditions of transverse aortic constriction (TAC) [16]. Despite EVs have obvious advantages, they have not been tested in realistic large-animal disease models. Besides, the exact mechanisms of cargo selection and packaging as well as the communication between EVs and target cells also remain unexplored. To further validate EVs as biomarkers in CVDs, particularly in pathological cardiac hypertrophy, large randomized clinical trials are needed [111].

2.8 Conclusion

Exosomes, the most effective active molecules, play vital roles in intercellular communication and have promising potential for regulating pathological cardiac hypertrophy. Stem cell therapy has its drawbacks when considering clinical trials. While cell products, such as secreted EVs, may be considered as a clinical tool. Furthermore, to establish EVs as diagnostic or prognostic biomarkers in pathological cardiac hypertrophy, more efforts are needed to conduct large randomized clinical trials.

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Extracellular Vesicles and Cardiac Aging

3

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Abstract

Global population aging is a major challenge to health and socioeconomic policies. The prevalence of diseases progressively increases with aging, with cardiovascular disease being the major cause of mortality among elderly people. The allostatic overload imposed by the accumulation of cardiac senescent cells has been suggested to play a pivotal role in the aging-related deterioration of cardiovascular function. Senescent cells exhibit intrinsic disorders and release a senescence-associated secretory phenotype (SASP). Most of these SASP compounds and damaged molecules are released from senescent cells by extracellular vesicles (EVs). Once secreted, these EVs can be readily incorporated by recipient neighboring cells and elicit cellular damage or otherwise can promote extracellular matrix remodeling. This has been associated with the development of cardiac dysfunction, fibrosis, and vascular calcification, among others. The molecular signature of these EVs is highly variable and might provide important information for the development of aging-related biomarkers. Conversely, EVs released by the stem and progenitor cells can exert a rejuvenating effect, raising the possibility of future anti-aging therapies.

Keywords

Aging · Senescence · Extracellular vesicles · Cardiac aging

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3.1 Background

So you run and you run to catch up with the sun but it's sinking
Racing around to come up behind you again.

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The sun is the same in a relative way but you're older,
Shorter of breath and one day closer to death.
—"Time," by Waters, Gilmour, Wright, Mason [1].
The Dark Side of the Moon, Pink Floyd,
Harvest Records (UK) and Capitol Records (USA), 1973.

Aging is a ubiquitous biological phenomenon. Despite the natural acquisition of wisdom that follows aging, as early highlighted in *De Senectute* by the Greek philosopher Cicero, it has provided many challenges to all generations. The interest of scientific and governmental authorities in the biology of aging has grown significantly since health records showed that the global population is progressively aging. This demographic shift has been driven by an increase in life expectancy and a decrease in birth rate initiated after the post-World War II baby boomer generation. While people aged ≥65 years accounted for 8.0% of the total population in 2012, this proportion will increase to 35% by 2050, totaling 2.1 billion people [2].

However, the increase in longevity has not been followed by a proportional increase in health span. Although the prevalence of infectious and parasitic diseases is decreasing, the global population is experiencing a rise in chronic and degenerative diseases, particularly cardiovascular diseases (CVDs) [3]. Currently, CVDs are the main causes of morbidity and mortality in the population aged ≥60 years, particularly ischemic heart and cerebrovascular diseases, congestive heart failure, and cardiac arrhythmias [3]. It has been estimated that with the rising prevalence of the elderly population, the percentage of deaths from CVDs will increase to 40%, while the cost attributed to this group of diseases will triplicate [4].

Aging is indeed a dominant risk factor for cardiovascular dysfunction, irrespective of other risk factors. Healthy elderly people frequently exhibit a progressive stiffening of the myocardium, mainly due to interstitial fibrosis. The resulting rise of ventricular diastolic pressure prolongs the isovolumic relaxation time and impairs early diastolic filling, which is highly dependent on passive blood flow [5]. This leads to a shift toward late diastolic filling, dependent on atrial contraction, as evidenced by a decrease of the *E/A* ratio in echo-Doppler cardiogram recordings. Atrioventricular valve prolapse and blood regurgitation also arise as a consequence of the ventricular diastolic dysfunction, leading to atrial enlargement [5]. Conversely, the basal systolic function is preserved, as the ejection fraction remains at approximately 65%. Even so, the inotropic reserve is often reduced, as evidenced in ergometric tests and after injection of β-adrenergic agonists [6]. Therefore, despite the increase in the maximal end-diastolic volume, there is no proportional reduction in the stroke volume, consistent with uncoupling of the Frank-Starling mechanism. Altogether, these changes increase the risk of heart failure with preserved ejection fraction (HFpEF), the most prevalent heart failure type among elderly people. Vascular abnormalities include an increase in collagen deposition, calcification, and stiffening. The consequent increase in the afterload and systolic pressure is followed by a decrease in the diastolic pressure, resulting in the characteristic pulse pressure widening observed in elderly people [7, 8].

The mechanisms whereby cardiovascular homeostasis deteriorates during aging remain largely unclear. The accumulation of senescent cells and impairment of healing and regenerative mechanisms are thought to exert pivotal roles. Also, extracellular vesicles (EVs) have arisen as important players in the senescence and aging processes. Both the concentration of EVs and their cargo change significantly with aging. EVs are now thought to be part of the *senescence-associated secretory phenotype* (SASP), and it is not surprising that these EVs contribute to the progression of the senescent wave and aging. Conversely, EVs secreted by the stem and progenitor cells from healthy and young donors promote anti-aging and might provide salutary effects in aging-related diseases. Here we will discuss the involvement of EVs in the process of cardiac aging and the development of aging-related cardiac dysfunction, as well as potential therapeutic approaches.

3.2 Accumulation of Cellular Injury and Cell Senescence

Aging is a complex biological process that involves the accumulation of cellular damage throughout life. This is constantly influenced by endogenous (genetic predisposition and exposure to chronic diseases) and environmental factors (pollution and ultraviolet radiation) that cause cellular dysfunctions and increase the levels of cell stress. Over time, the rate of cell death by necrosis and apoptosis increases, whereas a considerable part of the reminiscent cellular pool shifts toward a senescence state. Senescence is an allostatic state characterized by cell cycle arrest, resistance to cell death, abnormal cellular phenotype, and changes in the transcriptome [9]. Accordingly, Hayflick and Moorhead reported that human fibroblasts have a limited number of divisions before entering a state of permanent cell cycle arrest, introducing a pivotal concept in the field of cellular biology, later coined as the *Hayflick limit* by the Nobel laureate Macfarlane Burnett [10, 11]. This study also paved the way for posterior studies on *replicative senescence* and *aging*.

Due to the arrangement of DNA strands (e.g., 3′-5′ and 5′-3′) and the capacity of DNA polymerase to replicate the DNA strands only in the 5′ to 3′ sense, small base pair sequences in the chromosomal ends would be lost in each cell division. To avoid strand breaks in gene-coding sequences, the ends of each DNA strand have a sequence of base pair repeats (e.g., TTAGGG) sheltered by a protein complex, namely telomeres [12]. These structures prevent the free chromosomal ends to be recognized as strand breaks, which would otherwise trigger a DNA damage response (DDR), the cornerstone in the process of senescence. However, telomeres per se can become critically short, leading to DDR activation and senescence [12]. This view has been challenged by findings showing short telomere sequences in non-senescent cells, while this process can also be attenuated by the expression of telomerase [12]. Alternatively, telomeres are highly prone to accumulate oxidative damage, particularly in the loop region, due to poor access of repair proteins [13]. Therefore, even post-mitotic cells can undergo senescence when exposed to stress conditions that lead to DNA breaks, telomere-associated damage, or inhibition of DNA polymerase complex, such as ionizing irradiation and anthracycline treatment. The DDR response is thought to give time for the cell to repair the DNA breaks by non-homologous end-joining or homologous recombination and to prevent mutation spread and cancer formation.

The DDR pathway involves the activation of ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad3 (ATR) protein kinases [14]. Both bind to damaged regions in the DNA and catalyze the phosphorylation of serine and threonine substrates of several proteins, particularly checkpoint kinases (Chk) 1 and 2, a pivotal step to activate the protein p53. Under normal conditions, p53 is very unstable, being rapidly degraded in proteasomes, a process dependent on the ubiquitin ligase Mdm2 [14]. Upon DNA damage, the Chk-induced phosphorylation stabilizes p53 and prevents its proteolytic degradation, enabling it to stimulate the expression of cyclin-dependent protein kinase (CDKs) inhibitors (CDKIs), such as p21, p27, and $p16^{INK4a}$ [15]. CDKIs inhibit the complexes formed by CDKs in the G1 and S phases of the cell cycle, resulting in cell cycle arrest [14, 15]. Senescent cells also exhibit higher volume and flattened phenotype, besides positive staining for senescence-associated β-galactosidase (SA-βgal), and markers of DNA damage (e.g., γH2AX) [16].

Despite the physiological importance of senescence, the allostatic overload imposed by the accumulation of senescent cells is determinant for the deterioration of physiological regulation of the *milieu intérieur*. This is partially due to the plethora of cellular dysfunctions elicited by senescence [17]. Particularly, mitochondrial dysfunction has been regarded as a pivotal dysfunction that can be either a trigger or a consequence of senescence and aging. With aging, mitochondria undergo changes in the morphology, abundance, and activity of oxidative

phosphorylation complexes [18]. Dysfunctional mitochondria have been observed in several aged organs, including skeletal muscle, heart, and adipose tissue [19]. Mitochondrial function in aged tissues is impaired at several levels, including mitochondrial content and morphology, the activity of electron transport chain (ETC) complexes, the opening of the mitochondrial permeability transition pore (MPTP), the formation of ROS, and mitochondrial dynamics and less ATP production [20]. Additionally, there is an increasing accumulation of mitochondrial DNA mutations in mammals as they age, leading to a dysfunction of oxidative phosphorylation complexes, which, nonetheless, can affect only subsets of cells in a tissue [21]. This can be driven either by mitochondrial DNA or extracellular signals, such as hormones, which makes it difficult to distinguish between the origins of mitochondrial dysfunction in aging. Noteworthy, senescence-associated mitochondrial dysfunction, characterized by a distinct phenotype, exhibits a unique non-autonomous cellular program that is likely responsible for the altered metabolism and aberrant cell differentiation observed in aged animals [22].

The senescent wave is propagated to neighboring cells through the secretion of autocrine and paracrine factors composing the *senescence-associated secretory phenotype* (SASP) [23]. SASP is driven by the transcription factors nuclear factor-κB (NFκB) and CCAAT/enhancer-binding protein-β (CEBPβ) and includes soluble factors (interleukins and chemokines), secreted proteases (metalloproteinases), and insoluble proteins [24]. The SASP exerts an important regulatory role in the immune response to stimulate the clearance of senescent cells [24]. However, it also promotes several adverse effects, including remodeling of the extracellular matrix, sterile inflammation, dysfunction, apoptosis, and senescence of surrounding cells [24]. As a result, the accumulation of senescent cells and long-term exposure to the SASP-induced effects contribute to the deterioration of physiological homeostasis.

3.2.1 EVs, Aging, and Cell Senescence

EVs are surrounded by a lipid bilayer and participate in the homeostatic regulation of all cellular phenotypes, from the elimination of metabolic residues to paracrine communication and modulation of components of the extracellular milieu. EVs are classified according to their size in exosomes (≤120 nM) and microvesicles (100–500 nM) [25]. Exosomes originate from endosomes and are produced in multivesicular bodies that fuse with the plasma membrane. Microvesicles, however, are formed by direct budding from the plasma membrane. Their content varies according to physiological conditions and cell phenotype and may include micro-RNA (miR), messenger RNA (mRNAs), proteins, lipids, and even organelles, such as mitochondria [26, 27]. Once secreted into the extracellular milieu, EVs can be incorporated by recipient cells and thereby regulate cellular homeostasis.

Aging is characterized by profound changes in the concentration of circulating EVs and their cargo. A higher concentration of circulating EVs have been observed in elderly individuals, in keeping with findings in aged mice [28–30]. Although conflicting evidence suggested that the levels of circulating EVs might otherwise decrease with aging, this is most likely due to a more rapid internalization by B lymphocytes [31]. Among all EVs fractions, only the prevalence of small exosomes (<50 nm) increases with aging, consistent with an increase in exosome biogenesis and release [29]. In addition, aging is followed by a shift in the cargo of EVs. However, the high variability of EVs cargo has hampered the process of characterizing a precise molecular signature so far. Experimental findings demonstrated lower levels of antioxidant enzymes and galectin-3, while higher levels of reactive oxygen species and transmembrane protein 33 were observed in the EVs obtained from aged rodents compared to their young counterparts [29, 32–35]. Particularly, the miR profile carried by circulating EVs is also altered,

as EVs obtained from aged mice can exhibit higher expression of miR-146a, -21, -22, -223, -145, and let-7a compared to EVs from young mice [29]. MicroRNAs (miRs) are non-coding RNAs that can regulate gene expression by binding to target RNAs and thereby modulate protein expression [29, 34, 36]. The ontological analysis revealed that this miR cluster is involved in the regulation of gene transcription, signal transduction, and nucleotide-binding [29]. An immunomodulatory effect was also observed, as the exposure of lipopolysaccharide-stimulated macrophages of young mice to EVs isolated from old mice elicited augmented phagocytic capacity and expression of arginase 1, mannose receptor C-type 1, transforming growth factor β-1 (TGFβ), while reducing interleukin (IL)-6 and inducible nitric oxide synthase (NOS) compared to vehicle and EVs obtained from young mice [29]. This effect might be associated with the development of a systemic proinflammatory status, although in vivo evidence will be necessary to ratify this hypothesis.

Even though the mechanisms underpinning the aging-related changes in EVs homeostasis remain unclear, growing evidence has suggested a prominent role of senescence. The release of EVs increases under biological conditions widely known for eliciting cell senescence, such as redox imbalance, ionizing irradiation, and telomere attrition, an effect likely dependent on p53 and its downstream gene, tumor suppression-activated pathway 6 (TSAP6) [37]. Stabilization of p53 with nutlin-3, an Mdm2 antagonist, elicited premature senescence and an increase in exosome biosynthesis. Furthermore, EVs release is also increased during doxorubicin administration, an experimental model of senescence [38]. TSAP6, a glycosylated protein highly expressed in the Golgi apparatus, endosomal compartments, and plasma membrane, is known for exerting an important role in exosome biosynthesis, as evidenced in TSAP-deficient mice [39].

The specific cargo profile of senescent EVs is variable and dependent on the cellular origin [40–43]. Overall, however, it has been reported that senescent EVs carry abnormal concentrations of lipids, nucleic acids, and proteins compared to EVs released by non-senescent cells. Proteomic analyses revealed an upregulation in the prevalence of proteins associated with extracellular matrix remodeling, hemostasis, cell adhesion, and hydroelectrolytic balance [42, 44, 45]. Interestingly, a hallmark of senescent EVs is the accumulation of γH2AX-positive chromatin fragments, consistent with damaged DNA [46]. The release of damaged DNA is crucial to maintain cell viability since inhibition of this process increases the apoptotic rate of senescent cells [46]. However, the senescent EVs can also elicit DNA damage and propagate senescence to neighboring cells, as demonstrated by the increase in SA-βGal staining, $p16^{INK4a}$ expression, and γH2AX in cells incubated with EVs released by $H\text{-}RAS^{G12V}$senescent fibroblasts [41]. When incorporated by recipient cells, the damaged DNA fragments of senescent EVs can activate cyclic cGMP-cAMP synthases, thereby increasing cytoplasmic concentrations of cyclic nucleotides. Ultimately, this leads to the activation of NFκB, a master regulator of several SASP components [47]. In vitro evidence indicates that inhibition of both exosome biogenesis or neutralization of the RNA and protein fractions within senescent EVs can prevent this effect [41, 48]. The miR signature is another important player in this field, although as in the case of aging, it is highly variable in senescent EVs as well [29]. Accordingly, several miRs can regulate the expression of gene-encoding proteins associated with the progression of cell cycle and SASP components, including let7, miR-146a, -25-5p, -217, -133b, -106a, -106b, -19b, -20a, -34a, -181a, and -221 [36, 49, 50].

Analogous to EVs, mitochondria-derived vesicles (MDVs) are also involved in many physiological aspects of cell senescence. The production of MDVs arises during mitochondria germination, regardless of mechanisms linked to mitochondrial fission [51]. The insertion of the translocase protein of outer mitochondrial membrane 20 in the membrane of these MDVs facilitates their incorporation into multivesicular bodies (precursors of EVs). Once inserted in the multivesicular bodies, components of the endolysosomal system are translocated to the

intracellular surface of the plasma membrane, forming small intraluminal vesicles (ILVs) [52]. Depending on the characteristics of the ILV protein cargo, they can fuse with the plasma membrane and release mitochondria-derived components into the extracellular matrix [53]. MDVs are also considered pivotal to the process of mitochondrial quality control and to deal with damaged mitochondria [53, 54]. Although there is an age-related decline in the mitochondrial load within circulating EVs, most are damaged or poorly functional. Thus, it has been proposed that intercellular transfer of autologous mitochondria might be a potential SASP component. Impaired mitochondria from EVs can increase the production of reactive oxygen species and elicit mitochondrial damage in the recipient cells, leading to activation of the DDR and subsequent cell senescence [55]. As previously demonstrated, mitochondria carried by monocyte-derived EVs elicit proinflammatory activity in endothelial cells, thereby being intercellular mediators for cardiovascular diseases and other proinflammatory conditions associated with the activity of interferon-1 and tumor necrosis factor [56].

3.3 Cardiac Aging and Cell Senescence

As observed in virtually all organs, there is a natural loss of viable cardiac cells with aging, whereas the prevalence of $p16^{INK4a}$-positive senescent cells and expression of SASP components increase [57, 58]. The accumulation of cardiac senescent cells is thought to play a pivotal role in the aging-related deterioration of cardiovascular homeostasis, as suggested by findings with experimental models of doxorubicin treatment [59–61]. Furthermore, the cardiotoxicity elicited by radiotherapy is further aggravated when the radiation is focused in the left precordial area, although the role of senescence in this condition remains to be elucidated [62]. More recently, several reports demonstrated that there is an accumulation of senescent cells in the heart throughout aging-related cardiometabolic diseases, such as ischemic heart disease and diabetes [63–68]. Conversely, either genetic or pharmacological clearance of senescent cells can provide important cardiovascular salutary effects in aged rats and mice, such as improvements of vascular reactivity, ejection fraction, aerobic capacity [69–72].

3.3.1 Cardiomyocytes

Cardiomyocytes, the cardiac functional unit, account for 30.1% and 49.2% of cardiac cells in atria and ventricles, respectively [73]. Although evidence of cardiomyocyte mitosis has been observed in adult hearts, most of these cells lose the proliferative capacity early in post-uterine life, which makes them unlikely to undergo replicative senescence [74, 75]. Alternatively, cardiomyocytes can become senescent when exposed to telomere-associated damage, mainly due to oxidative damage and accumulation of advanced glycation-end products [76, 77]. In contrast to genomic DNA damage, telomeric damage is irreparable and thereby can trigger a persistent DDR signaling and senescence in these cells, which are characterized by an increase in the expression of SA-βgal and CDKIs [59, 76, 78–80].

Structural abnormalities exhibited by senescent cardiomyocytes include an increase in the expression of cytoskeletal proteins, while nucleic proteins laminin A and C are frequently downregulated [81, 82]. Sarcolemmal fluidity and elasticity decrease, partially contributing to the increased stiffness of aged hearts [83]. Consistent with the flattened phenotype of other senescent cells, hypertrophy is a common feature observed in senescent cardiomyocytes [84]. In keeping with this, aged mice frequently exhibit an increase in the LV mass compared to their young counterparts, while this is partially improved by pharmacological clearance of senescent cells [72]. Even though cardiomyocyte hypertrophy compensates for the natural decline in the prevalence of viable cells observed in aged hearts, it might be followed by restrictive cardiomyopathy. Accordingly, persistent telomere attrition predisposes to ventricular dilation, impairment of the hemodynamic performance,

and ultimately heart failure, as evidenced in telomerase knockout mice [85]. Furthermore, the lower expression and abnormal distribution of connexin 43 throughout the sarcolemma of senescent cardiomyocytes are associated with an electrical uncoupling, slower conduction velocity, and higher susceptibility to arrhythmic episodes [86, 87].

Another important hallmark of cardiomyocyte senescence is mitochondrial dysfunction [59, 88]. Mitochondria of senescent cardiomyocytes exhibit an increase in both proton leak and reactive oxygen species (ROS) production, which is further aggravated by the downregulation of antioxidant enzymes [76, 88–90]. The resulting redox imbalance is especially important in the context of senescence since telomeres are highly susceptible to irreparable oxidative damage and persistent DDR in cardiomyocytes [76]. Particularly, interfibrillar mitochondria of aged hearts can develop calcium overload due to calcium leak from the sarcoplasmic reticulum [77]. Together, both mitochondrial calcium overload and redox imbalance contribute to the stabilization of the mitochondrial permeability transition pore, the release of cytochrome c, and the recruitment of pro-apoptotic caspases [91]. As expected, the metabolic efficiency of mitochondria of senescent cardiomyocytes is impaired, especially fatty acid and glucose oxidation capacity [92–95]. In such a condition, a shift toward oxidation of ketone bodies is thought to improve the redox balance and excitation-contraction coupling, although it is unlikely that this metabolic process is enough to keep cellular ATP levels within the physiological levels necessary for cellular demands [96–98]. Therefore, since mitochondria are the powerhouse of eukaryotic cells, the reduced mitochondrial respiratory capacity is a major determinant of the decline in the myocardial aerobic capacity observed in elderly individuals [77].

The accumulation of senescent cardiomyocytes is thought to underpin most of the systolic dysfunction exhibited by aged hearts. This is most likely associated with a decrease in the calcium current density, although conflicting evidence demonstrated an increase in calcium transient and current density in aged sheep hearts [99–102]. Alternatively, it has been proposed that baseline contractile performance and calcium transient remain preserved, whereas the contractile reserve is nonetheless impaired, as demonstrated upon noradrenaline stimulation and pacing frequencies >4 Hz in senescent cardiomyocytes of aged rat hearts [99, 103]. This agrees with findings in healthy elderly individuals, whose baseline ejection fraction is reported to be within physiological levels, while the inotropic reserve is frequently decreased [6]. Furthermore, the endurance performance in treadmill tests can be improved by the clearance of senescent cells in aged mice, suggesting that the aging-related decline in cardiopulmonary performance is at least partially associated with the accumulation of senescent cells [71].

In addition to the intrinsic dysfunction, senescent cardiomyocytes also contribute to the ongoing spread of senescence to neighboring cells through their SASP. Due to the relative novelty of this field and the difficulties in studying senescence in cardiomyocytes, most of the knowledge about the signature of their SASP is only in its first steps. Most findings have been collected using experimental models of cardiovascular diseases and senescence, such as ionizing irradiation and doxorubicin. As such, it was recently reported that senescent cardiomyocytes release a soluble non-inflammatory SASP composed of endothelin 3, TGFβ, growth/differentiation factor 15 (GDF15), and CCN family member 1 (CCN1) [76, 79]. In vitro, these factors attenuated fibroblast proliferation and propagated senescence, an effect evidenced by SA-βGal staining [76]. While this is important to regulate scar formation after myocardial infarction, the physiological repercussion in aged hearts remains unclear [79]. It is reasonable to hypothesize, however, that the accumulation of senescent cardiomyocytes and the chronic exposure to their SASP contribute to the remodeling of the extracellular matrix observed in aged hearts.

These cardiomyocyte-derived SASP components can also be released within EVs, although this field remains largely unexplored in

aged hearts so far. During doxorubicin treatment, there is an early increase in the levels of circulating EVs enriched with the glycogen phosphorylase isoenzyme BB, mostly derived from cardiomyocytes [59, 104, 105]. Interestingly, these senescent EVs exhibit a high concentration of 4-hydroxynonenal (4HNE), a toxic product of lipid peroxidation [105]. This metabolite is highly reactive and can interact with several cellular macromolecules, particularly proteins enriched in amino acids containing nucleophilic groups. Furthermore, 4HNE released by foam cells was reported to induce endothelial cell senescence by activation of the peroxisome proliferator-activated receptor δ, raising the question of whether 4HNE-enriched EVs secreted by senescent cardiomyocytes could also contribute to the spread of senescence [106]. At least in vitro, senescent cardiomyocyte-derived EVs can shift macrophages toward a proinflammatory phenotype [104]. This effect is associated with a downregulation of cytoplasmic thioredoxin levels and consequent recruitment of the NFκB pathway [104]. Conversely, doxorubicin-induced cardiomyocyte senescence was abrogated by serum EVs isolated from healthy humans, a mechanism associated with the downregulation of miR-34a [107]. Taken together, these findings suggest that senescent cardiomyocytes secrete EVs with a likely pro-senescent cargo and thereby might contribute to the aging-related deterioration of cardiovascular function.

3.3.2 Fibroblasts

In contrast to cardiomyocytes, evidence on cell senescence in fibroblasts is vast and diverse, although mostly focused on fibroblasts from other origins than the heart itself. According to the landmark study by Hayflick and Moorhead, cultured human fibroblasts exhibit cell cycle arrest after a finite number of cell divisions [11]. Telomere shortening was first proposed to underpin the replicative senescence of fibroblasts since it was delayed by ectopic expression of the catalytic subunit of telomerase [108]. However, non-senescent fibroblasts can exhibit short telomeric repeats as well, challenging this concept. Instead, telomere oxidative damage was later coined as the likely mechanism of replicative senescence in fibroblasts, especially in the telomeric loop. Indeed, consistent findings demonstrate that fibroblast growth is inversely correlated with the oxygen partial pressure, even at physiological ranges [13, 109]. This is associated with impaired access of repair proteins to damaged telomeric repeats, thereby triggering a persistent DDR, cell cycle arrest, and ultimately senescence [109, 110].

In aged mice hearts, there is an increased prevalence of fibroblasts expressing X-Gal crystals, a senescence biomarker [111]. An upregulation of miR-22 and the non-coding circular RNA circ-Foxo3 is likely involved, as evidenced in aged heart samples and in vitro findings with cardiac fibroblasts [112, 113]. Accordingly, ectopic expression of miR-22 and circ-Foxo3 elicited an increase in the expression of SA-βGal in mouse cardiac and embryonic fibroblasts, while it was partially blunted by circ-Foxo3 silencing [112, 113]. It was proposed that by interacting with anti-senescence proteins E2F1 and Id1, circ-Foxo3 increases the susceptibility of cardiac fibroblasts to senescence, particularly under conditions of redox imbalance [113]. Also, recent findings suggest that cardiac fibroblast senescence is also modulated by osteopontin (OPN). OPN is a protein originally associated with left ventricular remodeling and fibrosis in cardiac pathological conditions and obesity [114, 115]. Consistent with an OPN-induced pro-senescence effect, cardiac fibroblasts of aged $OPN^{-/-}$ mice exhibited a lower SA-βGal expression and higher proliferating rate when compared to the wild-type group.

The fibroblast is the cardiac cell phenotype with the most altered transcriptional profile in aged mouse hearts. Senescent fibroblasts exhibit significant alteration in the expression of genes associated with inflammation, extracellular matrix turnover, and angiogenesis [84, 112, 116, 117]. Since fibroblasts are a highly prevalent cellular phenotype in the heart and the main source of extracellular matrix components and modifying enzymes, it is not surprising that

fibroblast senescence imposes a profound effect on the myocardial composition. In keeping with this, cardiac fibrosis is associated with an increase in the prevalence of $CD45^+$ myeloid-derived fibroblasts in aged C57BL/6 mice [118]. The hearts of individuals >65 years of age show a 50% increase in the collagen content, while the diameter of each fiber may increase by 200% when compared to young individuals, especially type I collagen fibers [119, 120]. In rodents, the cardiac collagen content also increases with aging [121–123].

However, the mechanisms whereby the myocardial extracellular matrix is remodeled by senescent fibroblasts remain controversial, since collagen synthesis by these cells is reduced compared to non-senescent fibroblasts [78, 79, 124]. This is further corroborated by evidence showing that fibroblasts obtained from aged rat hearts exhibit a reduced net collagen synthesis in response to angiotensin II, a hormone that is widely known for its pro-fibrotic effect [125]. Instead, it has been proposed that this is most likely associated with an altered expression of hydroxylysylpyridinoline and metalloproteinases (MMPs) [121–123, 126]. Hydroxylysylpyridinoline, a pyridinoline-derivative, crosslinks collagen fibers and higher expression of this molecule is associated with an increase in collagen tension, which is further aggravated upon glycation [127, 128]. Senescent fibroblasts of aged hearts can also acquire an osteogenic profile, which might further contribute to the process of myocardial stiffening and cardiac valves calcification [129, 130]. Conversely, conflicting findings that either clearance of senescent cells or p53 silencing elicit an increase in fibrosis after myocardial infarction and transverse aortic constriction challenged the hypothesis of a potential pro-fibrotic role of senescent fibroblasts, although clearer evidence on cardiac aging per se is lacking [131, 132].

The identity of the SASP components released by senescent fibroblasts has been investigated with transgenic and pharmacological models of cell senescence. Recent reports demonstrated a significant increase in the release of the overall proteins by senescent fibroblasts in vitro. The core soluble SASP pathways of both irradiation- and Ras-induced fibroblast senescence are composed of proteins mainly associated with extracellular matrix organization, growth, and metabolism, including GDF15, insulin-like growth factor-binding protein, C-X-C motif ligands (CXCL), MMPs, stanniocalcin 1, cystatin-S, and C-C motif chemokine 3 [42, 43, 133]. Noteworthy, the CXCL family is known for reinforcing cell senescence [133]. Only 1–6% of all secreted proteins are downregulated after fibroblasts shift to a senescent state [42]. Proteins associated with hemostasis and both platelet activation and degranulation are upregulated as well, including Serpines 1, 2, B1, and B6, thrombospondin 1, filamin A-alpha, integrin B1, and plasminogen activator/urokinase (PLAU) [43]. This is consistent with the finding that the increase in the coagulability state observed during doxorubicin treatment was reduced by the clearance of senescent cells using the transgenic mouse model p16-3MR [43]. Although more robust evidence on aging per se is necessary, most soluble SASP components released by senescent fibroblast have also been regarded as aging biomarkers, including Serpins, GDF15, MMPs, and stanniocalcin-1 [42].

Senescent fibroblasts also exhibit an increase in the production of EVs compared to quiescent cells, while the size of these vesicles is approximately 2% lower compared to those released by non-senescent fibroblasts [42]. Interestingly, a recent report indicated that incubation of fibroblasts with serum EVs isolated from old rats resulted in the increased shift into myofibroblasts, an effect associated with reduced HSP70 levels contained in these organelles [30]. The high fibronectin expression in the surface of the EVs released by senescent fibroblasts is thought to facilitate their incorporation by recipient cells since it was blunted by the blockade of fibronectin receptor α5β1 integrin [134]. The interaction of fibronectin with α5β1 integrin leads to the recruitment of focal adhesion kinase and Src kinases, ultimately increasing the invasive and migratory capacity of previously quiescent fibroblasts [134]. EVs released by

senescent fibroblasts also exhibit abnormal lipid content, including an increase in the expression of hydroxylated sphingomyelin and phospholipids, although the physiological repercussion remains unclear [45].

Overall protein content carried by these EVs also increases, particularly those associated with inflammation (e.g., complement system and prostaglandin turnover), membrane organization (e.g., cell–cell adhesion, vesicle-mediated transport, and cell junction), cellular signaling (RAS signaling and G-protein signaling), ribosomal proteins, and epithelial cell differentiation pathways [42]. Binding-proteins and proteins associated with endocytosis are highly abundant. Furthermore, nicotinamide phosphoribosyltransferase (NAMPT), CXCL ligands 1, 2, and 3, PLAU, and calcium/calmodulin-dependent protein kinase type II (CAMKII) subunit β are important aging-related proteins among the top 20 proteins upregulated in the EVs of senescent fibroblasts [42]. Interestingly, circulating levels of NAMPT decline with aging in humans and mice, while aged mice transplanted with EVs enriched with NAMPT from young donors exhibited extended lifespan and improved aerobic performance in treadmill tests [135]. Since NAMPT is the rate-limiting enzyme in NAD^+ biosynthesis, a metabolic process widely reported to be downregulated in elderly people with heart failure, the higher release of NAMPT-enriched EVs by senescent fibroblasts might play an important but still unclear role in cardiac aging [136]. EV-containing CXCL ligands and PLAU, also expressed in the soluble SASP, can reinforce senescence in recipient cells and elicit a hypercoagulability state, respectively. Finally, the aging-related increase in the expression of cardiac CAMKII is thought to underpin the higher susceptibility to atrial fibrillation in aged mouse cardiomyocytes, since the CAMKII inhibitor KN-93 was able to decrease the diastolic calcium peak and the frequency of spontaneous calcium transients [137]. It remains to be elucidated whether EV-derived CAMKII contributes to this process as well.

Another change worth noting is the miR signature. EVs released by senescent fibroblasts exhibit an increase of approximately 80% in the expression of miRs, particularly miR-23a-5p and -137, whereas the expression of miR-625-3p, -766-3p, 17-3p, 199b-3p, 381-3p is downregulated [138]. Enrichment analysis of the interactions between miRs and targeted-genes revealed that the top 20 upregulated miRs contained within these EVs can inhibit genes encoding pro-apoptotic transcription factors. As such, these EVs can exert an antiapoptotic effect in recipient fibroblasts exposed to high concentrations of H_2O_2, although it remains unclear whether this effect was indeed driven by the alluded miRs [138]. In addition to the paracrine signaling per se, the authors raised the hypothesis that the packaging of a selective miR cluster might be associated with the disposal of potential tumorigenic signals, which might further reinforce senescence in the secreting cell. Future studies will be necessary to elucidate whether these cargo alterations also occur in aged cardiac fibroblasts as well.

3.3.3 Endothelial Cells

Endothelial cells (ECs) are widely present in the cardiovascular system, particularly in the endothelial layer of all blood vessels, from the heart to small capillaries. These cells regulate the vasomotor tonus, blood pressure, blood coagulation, angiogenesis, and the cellular and molecular interchange between vascular and extra-vascular compartments. Importantly, the prevalence of senescent ECs increases with aging, as demonstrated by the expression of p53, p21, and p16 in ECs obtained from the antecubital vein of sedentary elderly individuals (60 ± 1 years) [139]. This was also observed in the senescence-accelerated mice strain model and was associated with a higher susceptibility to HFpEF elicited by a high-fat diet [63]. Conversely, downregulation of p53 in ECs provided salutary effects in experimental models of heart failure induced by pressure overload and diabetes [140–142].

Similar to fibroblasts and cardiomyocytes, telomere-associated damage is a major trigger of senescence in ECs [143]. Telomere length of ECs

decreases as a function of populational doubling and is also dependent on the blood vessel origin, being shorter in blood vessels exposed to higher hemodynamic stress [144, 145]. Furthermore, senescent ECs exhibit a high prevalence of DNA and telomeric damage compared to non-senescent ECs. This is associated with a reduced expression of TRF1, an important component of the protein sheltering complex that protects the telomeric repeat ends [146]. TRF1 is also recruited by ATM protein to damaged DNA foci, where it facilitates DNA repair by homologous recombination, while TRF1 downregulation or lower activation is associated with a higher sensitization to senescent stimuli [147]. Mitochondria are the main source of ROS in this case. The burst on mitochondria-derived ROS leads to translocation of telomerase from the nucleus into the cytoplasm and activation of Src-kinase, altogether increasing the susceptibility of ECs to replicative senescence [148]. Consistent with this, incubation of ECs with the antioxidant compound N-acetylcysteine attenuated the development of premature replicative senescence [148].

Senescent ECs exhibit a flattened and slightly enlarged phenotype, most frequently accompanied by polymorphic nuclei [144]. As expected, there is an accumulation of post-translated, modified proteins, mainly due to oxidation, nitrosylation, and carbonylation [149]. The expression of CX43 declines as a function of populational doubling, while the otherwise expected increase in its expression elicited by epithelial growth factor is abrogated, suggesting that the integrity of the endothelial barrier deteriorates as EC shifts to senescence [150]. Together with an increase in the expression of CD44, vascular cell adhesion molecule 1 (VCAM1), and intercellular adhesion molecule 1 (ICAM1), these changes likely contribute to the development of cardiac sterile inflammation, a hallmark of aging and aging-related diseases [141, 151]. The changes in the transcriptomic profile involve mostly proteins associated with metabolic pathways, histone-modifying enzymes, heat-shock proteins, and integrins, as evidenced in irradiated ECs [149]. Since senescent ECs exhibit an increase in the nuclear translocation of NFκB, it is not surprising that their SASP is composed of inflammatory molecules and extracellular matrix-remodeling proteins as well, such as monocyte-chemoattractant protein [140, 152]. In keeping with this, p53 deletion from endothelial cells was followed by a decrease in the infiltration of inflammatory cells and the expression of genes encoding TNFα and monocyte-chemoattractant molecule in an experimental model of heart failure induced by pressure overload [141]. This is further enhanced by a shift in the expression of proinflammatory miRs, including miR-125a and -126 [140].

Particularly, senescent ECs exhibit an impaired expression of vasomotor molecules. The ECs expression of vasoconstrictor molecules increases with aging, including endothelin-1, as evidenced in ECs isolated from the brachial artery of elderly and young individuals [153]. Conversely, the activity of eNOS and NO production is downregulated [142, 154–157]. This leads to impaired endothelial-dependent vasodilation, a dysfunction correlated with the accumulation of senescent EC in elderly individuals [139, 142, 153]. Notwithstanding, in vitro evidence demonstrated that incubation of human umbilical vein ECs with the NO donor S-nitrosopenicillamine elicited an increase in telomerase activity and reduced SA-βGAL staining after 30 populational doublings, while the opposite was promoted by inhibition of NOS with N^{G}-mono-methyl-$_{L}$-arginine [158]. These findings suggest that the reduced production of NO contributes to the reinforcement of senescence in vascular cells. In addition, ECs also exhibit a p53-dependent decrease in sprouting and vasculogenic capacity, while the reendothelization process declines with the accumulation of senescent endothelial progenitors, which together impair the vascular adaptation to ischemic diseases and endothelial damage [140, 142, 159].

Part of the SASP of senescent ECs is released within EVs. Recent evidence indicated an increase in the biosynthesis and release of EVs expressing $CD63^{+}$ $CD9^{+}$ $CD81^{+}$ when ECs undergo replicative senescence [160]. This is consistent with findings that elderly individuals

exhibit higher circulating levels of annexin 5^+ $CD31^+$ $CD42^-$ EVs, an endothelial-associated EVs phenotype [28]. As expected, the cargo shift of these senescent EVs imposes profound physiological effects. Accordingly, the high expression of miR-21-5p and -217 is thought to elicit senescence in the recipient cells, since their predicted targets include the DNMT1 and Sirtuin1 [50]. Indeed, incubation of ECs with these senescent EVs elicited a decrease in the expression of cyclins D1 and A, higher expression of p16 and SASP components IL6 and IL8, and a reduced proliferative capacity evidenced by Ki67 staining [50]. Both expression and distribution of vascular endothelial cadherin and β-cadherin can be significantly impaired in recipient cells as well [160, 161]. These findings suggest that these senescent EVs contribute to the further deterioration of the endothelial barrier, as aforementioned, which facilitates the infiltration of circulating inflammatory cells and molecules. However, future studies will be necessary to confirm this in vivo, particularly in highly sealed vessels such as in the blood–brain barrier.

Recent findings indicate that EVs secreted by senescent ECs can promote calcification of human aortic smooth muscle cells, as evidenced by the alizarin red and phenolsulfonphthalein staining [28]. Importantly, the report demonstrated that this effect was also elicited by EVs isolated from elderly individuals and was associated with an augmented prevalence of EVs presenting high concentration of calcium and pro-calcification proteins (e.g., Bone morphogenic protein 2), respectively, determined by the microanalytic comparison of atomic composition and western blot assay. Vascular calcification is a major aging-related disorder, especially in the aorta and atherosclerotic plaque. Although the mechanisms remain unclear so far, these findings suggest that the accumulation of senescent ECs and the cargo shift exhibited by their EVs might play an important role. These findings also raise the hypothesis on whether this cargo shift contributes to the development of valve calcification, another important disorder reported in elderly individuals.

3.3.4 Stem and Progenitor Cells

The accumulation of cellular damage is normally balanced by healing and regenerative mechanisms. This is mainly dependent on the proliferation and differentiation of stem and progenitor cells. The bone marrow is an important source of circulating hematopoietic stem cells (HSCs), endothelial progenitor cells (EPCs), and mesenchymal stem cells (MSCs). These cells can engraft in remote tissues and differentiate into several cellular phenotypes, although with a limited capacity [162–167]. Most tissues have a pool of resident progenitor cells as well. This cellular pool also releases several molecules that promote cytoprotection and thereby contribute to the preservation of cellular integrity during exposure to inhospitable conditions, mostly through the release of EVs [168]. Through these mechanisms, they exert immunomodulatory, anti-inflammatory, proliferative, angiogenic, anti-fibrotic, and antiapoptotic effects.

For many years the adult human heart was regarded as an organ devoid of regenerative capacity. Several conflicting findings were raised until the ground-breaking report by Bergmann and co-workers demonstrated that it can indeed regenerate itself, although with an extremely limited rate estimated at 1% per year [74]. In conditions of cardiac injury, the rate of cardiomyocyte turnover can reach approximately 15% nonetheless, as reported in experimental models of myocardial infarction in transgenic Cre-lox mice [169]. In addition to a limited cardiomyocyte proliferation, stem cell differentiation has been hypothesized to play an important role in this process. However, the existence and characterization of a resident cardiac progenitor cell (CPC) remain controversial. Several candidates have arisen so far, with c-kit^+ cells, Sca-1^+ cells, and cardiosphere-derived cells (CDC) being the most investigated, either as endogenous sources of cardiomyocyte turnover or therapeutic approaches in regenerative medicine [170–174]. Conversely, in a recent report on the cellular composition of human heart samples using *state-of-the-art analyses of large-scale*

single-cell and single-nucleus transcriptomes, the presence of these potential cardiac progenitor cells was not detected [73].

With aging, however, cardiomyocyte turnover further decreases to an estimated rate of 0.45% per year [74]. Together with the progressive accumulation of senescent cardiac cells, the impaired regenerative capacity increases the risk of cardiac dysfunction in elderly individuals. An important limiting factor is the SASP released by the resident senescent cells. These compounds can turn the recipient tissues into inhospitable microenvironments for chemoattraction, engraftment, survival, and differentiation of stem and progenitor cells. This paradigm has been widely supported by findings with stem cell transplantation and parabiosis. As such, the capacity of homing and grafting of bone marrow-derived stem cells obtained from adult mice was impaired when transplanted into aged versus adult mice recipients [175]. Circulating systemic factors in young mice recipients likely increase the potential for endogenous HSC grafting in comparison to aged recipients, as demonstrated in mice subjected to heterochronic parabiosis [176, 177]. Furthermore, the proliferative capacity of liver progenitor cells obtained from young mice was significantly greater when transplanted into aged recipients surgically joined by heterochronic parabiosis, when compared to those submitted to the isochronic model [178].

Stem cells per se are also highly prone to undergo both replicative- and stress-induced senescence. The expression of senescence and oxidative damage biomarkers increases, while genes encoding proteins that regulate DNA replication and repair are downregulated with aging [179–182]. Despite all the controversies concerning the CPC phenotype, recent findings suggested an accumulation of senescent c-kit^{+} CD31^{-} CD34^{-}CD45^{-} CD90^{+} CD166^{+} CD105^{+} CD140α^{+} cells in aged human hearts, as demonstrated by the high levels of SA-βGal, p16^{INK4a}, γH2AX, and impaired proliferative and clonogenic capacity [183]. Furthermore, their paracrine SASP was able to elicit senescence in neighboring cells. Using the CAST mice strain with short telomeres, Matsumoto et al. demonstrated that telomere shortening can elicit an accumulation of cardiac c-kit^{+} cells expressing p53, p16^{INK4a}, p27, and SA-βGal [184]. Similar findings were observed in human and rat hearts after doxorubicin treatment [59, 185]. In isolated human cardiac c-kit^{+} cells, exposure to doxorubicin was followed by a decrease in the expression of cyclin D1, Cdnk4, and phosphorylated Rb^{Ser798}, ultimately reducing cell proliferation, estimated by BrdU and Ki67 labeling. Conversely, biomarkers of cell senescence were increased, including γH2AX, SA-βGal, p16^{INK4a}, and phosphorylated-p53^{Ser15} and -21^{Clip1} [185].

As expected, the shift toward senescence is followed by a profound change in the cargo of their EVs, resulting in an impaired cytoprotective capacity and propagation of senescence [186, 187]. This is supported by experimental findings demonstrating that treatment with EVs obtained from young donors can improve both the lifespan and health span of aged recipients [188]. As such, infusion of NAMPT-enriched EVs obtained from young mice not only increased the longevity of old mice but also mitigated the decline in aerobic performance during treadmill tests [135]. At least in part, this rejuvenating effect is associated with a senolytic potential [189–192]. So far, however, few reports have investigated the cardiac rejuvenating potential of stem cells and their derivatives. The salutary effects of treatments with stem cell and derivatives in the cardiotoxicity induced by doxorubicin have been widely demonstrated, although evidence linking the physiological improvements with senolysis remain scarce [193]. A recent report by Zhuang et al. demonstrated that treatment with MSC-derived exosomes elicited a senolytic effect in hearts of doxorubicin-treated mice, an effect evidenced by lower levels of cardiac p27, p16, and p21 mRNA [194]. This was partially attributed to the incorporation of exosome-contained long non-coding RNA NEAT1 by recipient cardiac cells and activation of Sirt2, ultimately resulting in an improvement of systolic function. Recently, emerging findings suggested that aging-related cardiac dysfunction can be attenuated by CDC transplantation. Using

an experimental model of heterochronic transplantation, Grigorian-Shamagian et al. demonstrated that aged healthy rats treated with 1×10^6 CDCs obtained from young rats exhibited a slight improvement in the diastolic function compared to the placebo-treated group [195]. Furthermore, both echocardiography and histological findings suggested that aging-related cardiac hypertrophy and fibrosis were attenuated as well. The transcriptomic analysis suggested that these salutary effects were associated with a CDC-induced senolysis, which was further corroborated by the increased prevalence of cardiac cells within the highest quartile of telomere length. Interestingly, CDC-derived exosomes also increased the prevalence of cardiac cells with longer telomeres, suggesting a prominent role for CDC-derived EVs [195].

3.4 Promising Approaches: Mitochondria-Enriched Vesicles

Another promising approach is the use of mitochondria-enriched EVs as a rejuvenating therapy, although it has been poorly explored so far [196, 197]. EVs show advantages since they serve as reservoirs for mitochondrial components and protect them from degrading enzymes [198, 199]. In such conditions, the mitochondria material and information are retained as complete as possible, meeting the requirement to reprogram recipient cells to accomplish complex adjustments. For example, in the treatment of acute respiratory distress syndrome, which worsens with aging, the mitochondrial constituent present in MSC-derived EVs not only inhibits the release of proinflammatory cytokines but also improves the capacity for bacterial elimination of alveolar macrophages [27, 200]. Interestingly, recent evidence suggests that transplantation of a primary allogenous mitochondrial mixture reduces cell aging caused by ultraviolet radiation [201]. Additionally, it is possible to achieve oocyte rejuvenation by mitochondrial supplementation, achieving variable success rates after approaches that include heterologous or autologous mitochondrial replacement [202, 203]. However, despite the current data pointing to a promising future of anti-aging therapies based on mitochondrial-enriched EVs, future studies will be necessary to investigate whether they can indeed improve the functions, morphology, and mitochondrial genetic content, in addition to overcoming the population of resident impaired mitochondria [204, 205].

3.5 Perspectives

Cell senescence is a major determinant of aging-related deterioration of cardiovascular homeostasis. In the heart, all cardiac cells undergo senescence, either replicative or stress-induced. In addition to the intrinsic cellular dysfunction, the SASP compounds released by senescent cells not only propagate the senescent wave to neighboring cells but also impose drastic changes to the extracellular matrix. Most of these compounds are released within EVs that can be readily incorporated by recipient neighboring cells. In senescent cardiomyocytes, the role played by these EVs remains largely unclear, particularly in aged hearts. Conversely, EVs released by senescent fibroblasts, ECs, and CPCs candidates exhibit significant changes and contribute to the process of cardiac dysfunction in either prematurely senescent or aged hearts. Further analyses will be necessary to better characterize the molecular signatures of these EVs for their potential use as aging-related biomarkers. In contrast, EVs secreted by the stem and progenitor cells obtained from young and healthy donors promote senolytic and rejuvenating effects in aged and senescent hearts, raising the possibility of alternative anti-aging therapy.

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Extracellular Vesicles and Ischemic Cardiovascular Diseases

4

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Abstract

Characterized by coronary artery obstruction or stenosis, ischemic cardiovascular diseases as advanced stages of coronary heart diseases commonly lead to left ventricular aneurysm, ventricular septal defect, and mitral insufficiency. Extracellular vesicles (EVs) secreted by diverse cells in the body exert roles in cell–cell interactions and intrinsic cellular regulations. With a lipid double-layer membrane and biological components such as DNA, protein, mRNA, microRNAs (miRNA), and siRNA inside, the EVs function as paracrine signaling for the pathophysiology of ischemic cardiovascular diseases and maintenance of the cardiac homeostasis. Unlike stem cell transplantation with the potential tumorigenicity and immunogenicity, the EV-based therapeutic strategy is proposed to satisfy the demand for cardiac repair and regeneration while the circulating EVs detected by a noninvasive approach can act as precious biomarkers. In this chapter, we extensively summarize the cardioprotective functions of native EVs and bioengineered EVs released from stem cells, cardiomyocytes, cardiac progenitor cells (CPCs), endothelial cells, fibroblast, smooth muscle cells, and immune cells. In addition, the potential of EVs as robust molecule biomarkers is discussed for clinical diagnosis of ischemic cardiovascular disease, attributed to the same pathology of EVs as that of their origin. Finally, we highlight EV-based therapy as a biocompatible alternative to direct cell-based therapy for ischemic cardiovascular diseases.

Keywords

Ischemic cardiovascular diseases · Native extracellular vesicles · Bioengineered extracellular vesicles · Biomarkers

4.1 Background

Ischemic cardiovascular diseases including myocardial infarction (MI) and heart failure are the leading causes of morbidity and mortality worldwide [1, 2]. The cardiovascular system supplies blood to all tissues in the body during which oxygen and nutrients are simultaneously carried to cells. Decrease or block of local blood perfusion leads to ischemia which also deprives cells of oxygen and nutrients such as glucose and growth factors [3]. Once myocardial ischemia

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occurs, cardiomyocytes would undergo cell death (apoptosis, pyroptosis, and necrosis) within 20 min, followed by complete necrosis at 2–4 h after persistent coronary arterial occlusion [4, 5]. Clinical therapy of ischemic cardiovascular diseases is timely re-establishing blood flow in the ischemic cardiac tissue. However, reperfusion increases oxidative stress and causes paradoxical cardiomyocyte impairment, which is named ischemia–reperfusion (I/R) and determines the final infarct size after successful revascularization following MI [6, 7].

Without sufficient blood supply to the heart, myocardial ischemia could hinder the supply of glucose and oxygen, as well as delay the clearance of metabolic by-products such as lactic acid and CO_2 [8]. Although hypoxia is a key determinant to induce cellular damage in ischemic conditions, other abnormal events involving acidosis, increases in oxidative stress, disturbed calcium homeostasis, decreased levels of adenosine triphosphate (ATP), and mitochondrial and DNA damage exert significant effects on inflammation, neovascularization, and collateral formation [9]. Anaerobic glycolysis consuming glycogen storage allows short periods of ischemia. Glucose transporters (Glut1 and Glut4) are carried to the muscle fiber membrane to facilitate additional glucose uptake [10]. Ischemia could lead to acidosis and decrease the cardiac pH from approximately 7.2 to 6.5, which is necessary to alter the activities of various enzymes including phosphofructokinase and phospholipid burning enzymes. Besides, acidosis can be further enhanced by inhibition of mitochondrial NADH oxidation and vacuolar proton ATPase, increased CO_2 levels in tissues, excessive glycogen conversion, anaerobic glycolysis, and ATP hydrolysis [11–14]. As a result, intracellular acidosis triggers apoptosis of cardiomyocytes via cavitation proton ATPase [15].

Cell-to-cell communication is essential for maintaining tissue homeostasis and disease progression. Two classic pathways are direct cell-to-cell contact with short-range cell crosstalk and long-distance communication of cytokines or hormones [16]. Another intercellular communication mechanism emerges as the intercellular transfer of extracellular vesicles (EVs), which could deliver various biological signaling to receptor cells at an exceeded level than that of soluble factor signaling, attributed to a large number of bioactive molecules, surface receptors, and genetic information in EVs [17]. EVs are extracellular structures surrounded by lipid bilayers and secreted by almost all known cell types. According to their sizes, EVs have been classified into three categories involving exosomes (sizes of 30–150 nm), apoptotic bodies (sizes of 50 nm–10 μm), and microparticles or microvesicles (sizes of 100–1000 nm) [18, 19]. Exosomes are intracavinal vesicles that are formed through the membrane invagination of multivesicular endosomes and released into the extracellular space followed by the fusion of multivesicular endosomes with cell membranes [20]. Microvesicles (50–1000 nm) are heterogeneous EVs characterized by their origin and secretion to the exobud through the plasma membrane. Apoptotic bodies are released by dying cells after apoptosis. Responsible for intercellular communication, EVs carry molecules such as DNA, proteins, lipids, RNA, and/or microRNAs (miRNAs) [21]. These functional components vary from cell origin and specific pathophysiological conditions at the time of EV packaging and secretion. Extensive evidence suggests that EVs get involved in diverse cardiovascular physiological and pathological processes including regulation of angiogenesis and blood pressure, cardiomyocyte hypertrophy, apoptosis/survival, and cardiac fibrosis. Also, due to their wide distribution in human plasma, bronchoalveolar fluid, serum, saliva, urine, semen, bile, cerebrospinal fluid, amniotic fluid, tumor effusion, ascites, and milk, EVs have been employed as potential biomarkers for cardiovascular disease [22–28]. Cell-to-cell communication transferred by EVs between cardiomyocytes and vessel cells has an impact on cardiovascular pathology, diagnosis, and therapy [29–32].

In this chapter, we would summarize a variety of natural/engineered EVs derived from different types of cells, with an emphasis on their biogenesis and cargo formation, and performances as biomarkers of ischemic cardiovascular disease

for cardiac repair and regeneration. Finally, we would discuss the potential and challenges of EVs in clinical therapy of ischemic cardiovascular disease.

4.2 Different Sources of Extracellular Vesicles for Therapy of Ischemic Cardiovascular Diseases

Current treatments for ischemic cardiovascular diseases mainly focus on slowing the progression of diseases, rather than repairing and regenerating damaged heart muscle [33]. Although cell transplantation is considered one of the most promising ways to promote the proliferation of cardiomyocytes, it suffers from immunogenicity, risk of post-transplant arrhythmia and tumorigenesis, and uncertain differentiation and retention rate of cells [34]. A series of cytokines and growth factors, such as VEGF, HGF, Ang-1, SDF-1A, IGF-1, SFRP-2, TGF-β, and eNOS/iNOS, are involved in the paracrine effect of cell-based therapy [35]. These factors are beneficial to protect cardiomyocytes from apoptosis and necrosis, promote angiogenesis in infarcted myocardium, delay interstitial remodeling, and increase the recruitment of circulating progenitor cells [36]. With the same bioactive factors as their source cells, EVs can function as an alternative to cell transplantation. EVs are isolated from all major cell types found in the heart ranging from primary adult cardiomyocytes, primary cardiac endothelial cells, primary cardiac fibroblasts, and vascular smooth muscle cells to cardiac progenitor cells (CPCs) [37]. The majority of EVs in healthy people's plasma is derived from platelets and red blood cells, but plasma EVs are also released from white blood cells, endothelial cells, monocytes, neutrophils, and lymphocytes [38].

1. Cardiomyocyte-derived EVs: Cardiomyocytes may be an important type of parent cells to secrete EVs, especially under stress conditions such as myocardial ischemia and failure. Cardiomyocytes can release EVs containing heat shock proteins HSP70 and HSP90 and HSP60 in response to hypoxia and reoxygenation injury in vitro [39]. EVs containing tumor necrosis factor (TNF)-α can also be released by cardiomyocytes for inflammatory response. Glucose deprivation induces the loading of functional glucose transporters and glycolytic enzymes into EVs that are derived from neonatal rat cardiomyocytes [40]. Thus, cardiomyocytes can specifically regulate the function of neighboring cells by releasing specific EVs to respond to environmental stress.
2. Cardiac progenitor cell-derived EVs: a kind of cells with the ability to proliferate and differentiate into cardiomyocytes are called CPCs [41]. There were 857 unique gene products and 150 miRNAs in CPC-derived EVs, compared to CPCs. The miR-22 in CPC-derived EVs could inhibit methylCpG binding protein 2 and reduce apoptosis of the ischemic cardiomyocytes [42]. Hypoxia stimulates CPCs to release EVs which upregulate the expression of proangiogenic genes, antifibrosis genes, and a cluster of miRs (miR-210, miR-132, and miR-146a-3p), as well as increase their capacity to improve cardiac function after I/R injury in rats. After being inoculated into the ischemic/reperfusion heart, the CPC-derived EVs could elevate ATP and NADH levels in vivo [43]. With matrix metalloproteinases (MMPs) and extracellular matrix metalloproteinases (ECMPs) inside, EVs derived from CPCs could mediate proangiogenic efficiency. Therefore, CPC-derived EVs may play key roles in the EV-based therapy of ischemic cardiovascular disease [44].
3. Endothelial cell-derived EVs: The endothelial cells can release EVs containing miR-146a to stimulate angiogenesis [45]. Hypoxia changes the composition of mRNA and protein in EVs released from cultured endothelial cells in vitro. Exosomal intercellular adhesion protein expression was increased after TNF-α treatment of endothelial cells. These findings exemplify the protective function of endothelium-derived EVs against ischemic

cardiovascular disease, which may also make them biomarkers for cardiac stress and diseases [46].

4. Fibroblast and smooth muscle cell-derived-EVs: Cardiac fibroblasts secrete miRNA-27a*-enriched EVs into the extracellular space in response to stimulation of Angiotensin II, which inhibits PDLIM5 translation, thereby leading to the expression of hypertrophic gene in cardiomyocytes [47]. EVs released by cardiac fibroblasts contain high levels of miR-21-3p/miR-21 which can induce cardiomyocyte hypertrophy [48]. EVs released by smooth muscle cells are associated with vascular calcification and atherosclerosis. Different conditions such as ischemia, stress, and volume overload are able to induce fibroblasts, cardiomyocytes, endothelial cells, and inflammatory cells to regulate mast cell responses through EVs-mediated intercellular communication.
5. Mesenchymal stem cell-derived EVs: Mesenchymal stem cells (MSCs) are present in almost all tissues and play a major role in tissue repair and regeneration. Many signaling molecules from mesenchymal stem cells are associated with self-renewal and differentiation, which also have been found in the EVs derived from MSCs. Thus, these MSC-derived EVs could influence cell cycle, proliferation, cell adhesion, cell migration, and cell morphogenesis. Similarly, miRNAs shuttling through MSC-derived EVs mainly in their precursor form drive downstream signaling pathways. In addition, MSC-derived EVs carry anti-inflammatory cytokines such as interleukin-10 and tumor growth factor (TGF)-β to affect the lymphocyte proliferation [49].
6. Immune cell-derived EV: B cells and dendritic cells in immune cells mediate secretion of EVs with major histocompatibility complex (MHC)-dependent immune responses. These EVs express specific adhesion molecules to target specific receptor cells. NK cells-derived EVs surrounding perforin and granulase B could mediate antitumor activity in vitro and in vivo. Taking into consideration that macrophages can release IL-1β during inflammasome activation, the EVs secreted by macrophages exert roles in pro-inflammatory activity and initiating immune response [50].
7. Platelet-derived EVs: Studies have shown that increased cell adhesion factors, thrombopoietic factors, and inflammatory factors in EVs that are released by platelet in vascular plaques, thrombosis, and atherosclerosis can promote the delivery of platelet EVs to endothelial cells and macrophages in the vascular lesion sites. Platelet-derived EVs also stimulate angiogenesis, and intramuscular injection may improve vascular remodeling after ischemia [51].

4.3 Native Extracellular Vesicles for Ischemic Cardiovascular Therapeutics

The first study on EVs as a potential therapeutic intervention for cardiovascular disease was published in 2010. Since then, different types of cell transplantation were found to repair the infarcted heart. Besides, exploration of the underlying mechanism revealed that the protective effect of cell transplantation is mainly through the paracrine mechanism, especially EVs secreted by surviving cells, instead of directly mediation by cells [52]. EVs are naturally suitable for the transport of proteins and nucleic acids as well as cell-to-cell crosstalk, which makes them particularly attractive as drug delivery agents. In addition, due to their biophysical properties, EVs are easy to isolate while their contents such as RNA and protein can be easily manipulated [53]. Although EVs must contend with low cardiac osmotic and endocytosis rates, they can overcome poor transplantation by being directly internalized by recipient cells when compared to cells [54]. The limitations of EVs in cardiovascular therapy are the lack of effective target for the damaged myocardium. Optimizing the storage, isolation, and purification procedures for EVs is

challenging to move EV-based therapy from the laboratory bench to the clinic [55].

The therapeutic effect of EVs in recipient cells is mainly attributed to the delivery of proteins and/or non-coding RNAs, especially miRNAs. For example, miRNA-19, miRNA-21, miRNA-24, and miRNA-210 have been reported to get involved in cardiovascular repair, while several novel miRNAs (miRNA-22, miRNA-29a, miRNA-143, miRNA-146, miRNA-181b, miRNA-222, miRNA-294-3p, and miR-126) favor cardiovascular protective effects of exosomes [56]. Another important component of EVs, which is also related to their bioactivity, is proteins such as platelet-derived growth factor D and pregnancy-associated plasma protein A. Highly expressed in exosomes, pregnancy-associated plasma protein A has been shown to mediate cardiac protection and angiogenesis [57]. EVs could regulate autophagy, activate pro-survival signaling pathways, and reduce oxidative stress, thereby improving the survival rate of cardiomyocytes and endothelial cells. In addition, it can regulate the inflammatory response and cytokine secretion, as well as increase the activation of CD4 positive T cells by affecting the polarization of immune cells [58].

It has been confirmed that EVs can be secreted by cultured heart and vascular cells, stem cells. EVs have been shown to mediate communication between endothelial cells and smooth muscle cells, endothelial cells (ECs) and pericytes, cardiomyocytes and ECs, and fibroblasts and cardiomyocytes [59]. Smooth muscle cells play important roles in the formation of atherosclerotic plaque which can lead to MI. Studies have indicated that ECs release microvesicles rich in miR-143/145, which are absorbed by smooth muscle cells and regulate gene expression in receptor cells. Injection of EVs containing miR-143/145 into a mouse model of atherosclerosis reduced the formation of atherosclerotic lesions [60]. Cardiac fibroblasts have been demonstrated to secrete miR-21-rich EVs as key paracrine signaling mediators for cardiac hypertrophy. MiR-21 is shuttled to cardiomyocytes and affects the expression of its miR-21 target genes, thereby leading to cell hypertrophy [61]. EVs in the rat heart after ischemic preconditioning were responsible for the transmission of remote conditioning signals to protect the heart. The proangiogenic activity of pericytes is partially dependent on the released miR-132, especially in response to hypoxia. Pericyte-derived miR-132 was absorbed by ECs, thereby resulting in a higher proangiogenic capacity [62]. A recent report suggested that both ischemic and healthy human and mouse cardiomyocytes might release exosomal-like vesicles in vivo [63]. In mice with acute MI, circulating miRNA-1 was released into the bloodstream via EVs to inhibit the expression of the SDF-1 receptor CXCR-4 in bone marrow mononuclear cells [64]. These studies reveal that EV-mediated communication mechanisms can effectively favor cardiac repair and regeneration.

4.4 Bioengineered Extracellular Vesicles for Ischemic Cardiovascular Therapeutics

Although native EVs function as a delivery modality with their unique characteristics, they have inherent limitations of unclear heterogeneity and lack of targeting. The bioengineering operation can endow the EVs with improved target as therapeutic tools for the treatment of cardiovascular disease [65]. In detail, engineered EVs overcome their limitations by addressing the bioactivity, stability, internalization, and targeting of EVs. Exosome modifications are classified from a technical point of view depending on whether they are performed before EVs are secreted by donor cells or after the purification of EVs from culture medium or liquid. From a biological point of view, these modifications occur on the membrane or in the cavity of EVs [66]. Regulation of EVs-secreting cells has two different procedures: culture under stress conditions (hunger, hypoxia, inflammation) and transfection of exogenous compounds such as miRNAs, plasmid DNA, and small molecules to enhance their bioactivity. Some cell platforms have been customized to enrich EVs with specific proteins and RNAs [67]. Additionally, few studies have used cellular mechanisms to design EVs

with specific epitopes to the heart. Although the biophysical properties of EVs can be kept relatively intact, overexpression may produce unforeseen biological consequences that ultimately interfere with the biogenesis of EVs [68]. Tracking EVs and their biological distribution in vivo are important for the full evaluation of their therapeutic potential for cardiovascular disease. Most EVs have been isolated and labeled with fluoro groups, luminescent reporters, or radioactive tracers. In a few cases, reporter genes are expressed by transgenic treatment of EVs-secreting cells. EVs can be monitored in vitro or in vivo by luminescence or fluorescence [69]. To enhance the targeting of EVs, EVs have been modified with exogenous peptides (such as integrin αVβ3 high-affinity cyclic RGD peptides, ischemic targeting peptides, and cardiomyocyte-specific peptides), proteins (such as streptavidin), or lipids [70]. To enhance the internalization and endosomal escape of EVs, vesicles were modified with cationic lipids, pH-sensitive peptides, and cell-penetrating peptides. Taken together, these studies demonstrate the possibility of improving the bioactivity, tracer, targeting, and internalization of engineered EVs, compared with naked EVs [71].

4.5 EVs as Potential Biomarkers of Ischemic Cardiovascular Diseases

EVs originate from different subcellular compartments and are released in the extracellular space. By transferring their cargos to targeted cells and tissues, they act as new regulators of cell-to-cell communication between adjacent and distal cells. Since their vesicle composition, biological content, and protein markers are the individual characteristics of cell activation and damage, most EVs detected in serum and saliva can be concentrated as diagnostic and prognostic biomarkers which could suggest the occurrence of ischemic cardiovascular diseases [72–74]. Their isolation within the membrane also protects proteins, RNA, and DNA from degradation [75]. The features of circulating EVs or non-vesicular binding nucleic acids are valuable tools for the diagnosis and monitoring of cardiovascular disease, recently referred to as liquid biopsy. In epidemiological investigations, circulating EVs provide a noninvasive and nearly continuous flow of information about disease status [76–78]. Finally, the cell-specific application of genetic engineering and EVs may provide a new therapeutic approach for the treatment of ischemic cardiovascular diseases, offering hope for the application of EVs in ischemic cardiovascular disease [79].

With a variety of proteins, lipids, mRNAs, non-transcriptional RNAs, miRNAs, and small RNAs that represent their cellular origin and reflect the pathology of their source cells, EVs have potential as biomarkers for clinical diagnosis of ischemic cardiovascular disease [80]. The possibility of isolating and characterizing EVs from body fluids makes them very attractive as diagnostic markers [81]. CPC-derived EVs possess several cardioprotective and angiogenic microRNAs, such as miR-132, miR-210, and miR-146. Compared with CPC itself, CPC-derived EVs contained a portion of specific miRNAs that were specifically enriched in miR-146a, suggesting that miRNA enrichment into EVs may have occurred through a specific mechanism rather than random selection [82]. Recently, the elevated serum miR-192 levels (specifically EVs) were significantly associated with patients who developed heart failure within 1 year after MI, in comparison with a matched control group that did not develop a cardiovascular event after discharge [83]. In addition, miR-133 and miR-328 in plasma were elevated in patients with MI, both of which are considered novel biomarkers for acute MI [84]. A study of EVs in patients with acute coronary syndrome showed that miRNA-208a expression was significantly upregulated in serum EVs from patients with acute coronary syndrome [85]. In addition, survival was reduced in patients with high miRNA-208A expression, suggesting that exosomal miRNA-208A can be employed for early diagnosis and prognosis of acute coronary syndromes. Another study revealed an increase in the number of EVs binding cardiac miRNAs after

coronary artery bypass surgery [86]. In the future, diverse EVs can function as new biomarkers of persistent myocardial ischemia, vascular injury without cell death, non-infarct or asymptomatic myocardial ischemia, and different types of angina and microvascular angina. Besides, a biomarker of myocardial ischemia with low persistence and no cell death would help identify the disease at an early stage [87]. Similarly, the diagnosis of acute coronary syndromes needs to be improved, especially at the early time point after MI, microvascular angina, and non-ST-segment elevation of acute coronary syndrome (ACS) [88]. Given the wide range of cardiac cell types that are able to secrete EVs, circulating EVs which originate from coronary and peripheral arteries could provide a potentially significant identifying biomarker to support diagnosis and reflect the formation of coronary thrombotic occlusion in patients of MI [89]. However, the practical application of exosomal-derived proteins or miRNAs as biomarkers has not been implemented in clinical practice despite the presence of a large number of exosomes in biological fluids, mainly due to the lack of a rapid and effective method to process large numbers of biological samples. Several commercial companies have begun to develop EV-based cancer diagnostics, while EVs as biomarkers of cardiovascular diseases are still an unexplored world that we are committed to pioneering [90].

4.6 Conclusion and Future Perspectives

Circulating EVs can be detected in the plasma of patients with cardiovascular diseases, thus EVs expression patterns can be used as diagnostic and prognostic biomarkers for a variety of cardiovascular diseases. The multiple functional efficiencies of EVs on the progression of ischemic cardiovascular disease vary from the origin of cells, the functional status of source cells, and the transport capacity of functional bioactive molecules in the vesicle [91].

Since EVs get involved in the physiological and pathological processes of ischemic cardiovascular disease, research about their biogenesis, contents, and functional effects on target cells can provide new diagnostic and prognostic information for patients with cardiovascular diseases [92, 93]. Firstly, EVs are known to exert therapeutic effects on ischemic cardiovascular disease in preclinical MI models, which lights the future of EV-based therapy for cardiovascular diseases. Secondly, with advantages of modifiability, high viability, and inherency, EVs has similar therapeutic effect when compared to stem cell/progenitor cells. Thirdly, the therapeutic effect of EVs can be enhanced by increasing the stability and targeting of EVs, enriching their therapeutic content, improving their internalization and intracellular transport, and controlling their spatial and temporal release in biomaterials [94]. The implementation of standard separation and characterization of EVs are urgently required to explore EVs as a potential approach for ischemic cardiovascular disease [95–97]. Autologous EVs have the advantage of immune compatibility, but they cannot be collected on demand, and are difficult to be standardized as clinical products based on individual factors such as the donor's disease and age. EVs provided by exogenous sources have the advantage of being easier to standardize and bulk storage [98, 99]. In addition, loading exogenous molecules into EVs and controlling their delivery in vivo provides many opportunities to enhance the bioactivity of EVs [100]. Therefore, targeted technologies that increase the accumulation of EVs in the cardiovascular system to reduce the necessary injected dose, as well as strategies that enrich specific biomolecules in EVs, may be the key approaches to unlocking its clinical application. In the future, EVs can become clinical biomarkers for ischemic cardiovascular disease due to the specificity of their inclusivity. Native and engineered EVs represent a promising cell-free, safe, and customizable therapeutic approach to improving the therapeutic efficiency of cardiovascular diseases.

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Extracellular Vesicles and Hypertension

5

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Abstract

Hypertension implicates multiple organs and systems, accounting for the majority of cardiovascular diseases and cardiac death worldwide. Extracellular vesicles derived from various types of cells could transfer a variety of substances such as proteins, lipids, and nucleic acids from cells to cells, playing essential roles in both physiological and pathological processes. Extracellular vesicles are demonstrated to be closely associated with the development of essential hypertension by mediating the renin–angiotensin–aldosterone system and crosstalk between multiple vascular cells. Extracellular vesicles also participate in various kinds of pathogenesis of secondary hypertensions including acute kidney injury, renal parenchymal diseases, kidney transplantation, secretory diseases (primary aldosteronism, pheochromocytoma and paraganglioma, Cushing's syndrome), and obstructive sleep apnea. Extracellular vesicles have been proved to have the potential to be served as new biomarkers in the diagnosis, treatment, and prognosis assessment of hypertension. In the future, large multicenter cohorts are highly in demand for further verifying the sensitivity and specificity of extracellular vesicles as biomarkers.

Keywords

Extracellular vesicles · Hypertension · Vascular cells · Renin–angiotensin–aldosterone system · Secondary hypertension

5.1 Background

Hypertension is a multifactorial disease with complex pathogenesis that implicates a variety of systems and organs. Previous studies suggest that the etiology of hypertension is mainly due to the interaction between genetic and environmental factors leading to changes in pathological pathways [1, 2]. Hypertension has become the leading cause for cardiovascular disease (CVD) and cardiac death [3–5], affecting 1.39 billion people worldwide [6], thus a wide concern around the world [7].

Extracellular vesicles (EVs) are 30–100 nm vesicles which can be released by various types of cells. EVs contain a variety of substances such as proteins, lipids, and nucleic acids, mediating substances transferring and signal transduction between cells, thus regulating the dynamic homeostasis of human body [8]. EVs have been

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proved to be extensively involved in the development of various cardiovascular diseases, such as hypertension, coronary artery diseases, cardiomyopathy, valvular heart disease, and heart failure [9, 10]. This chapter summarizes the research progresses of roles of EVs in essential and secondary hypertension.

5.2 Extracellular Vesicles and Essential Hypertension

Hypertension can be divided by etiologies into two main subtypes, essential hypertension and secondary hypertension, with essential hypertension to be the majority [11]. The pathogenesis of essential hypertension is extremely complex, partly including activation of renin–angiotensin–aldosterone system (RAAS), endothelial dysfunction, and vascular remodeling [12], of which molecular mechanism remains to be fully understood.

5.2.1 Roles of Extracellular Vesicles in Renin–Angiotensin–Aldosterone System

RAAS is known to have a fundamental role in the mechanism of hypertension [13]. RAAS is composed of renin, angiotensinogen, angiotensin-converting enzyme (ACE), angiotensin II (Ang II), angiotensin II type 1 receptor (AT_1R), and angiotensin II type 2 receptor (AT_2R). Under pathological circumstances, excessive Ang II binds to AT_1R and causes pathological consequences such as vascular injury and remodeling by initiating inflammatory responses [14]. In hypertensive mice model induced by transverse aortic constriction (TAC), EVs rich in AT_1R were secreted and released from cardiomyocytes and thus circulating AT_1R-riched EVs significantly increased. Interestingly, transferring of these AT_1R-riched EVs into AT_1R gene knockout (-KO) non-hypertensive mice could reduce the hypertension caused by excessive Ang II [15]. This study confirmed that EVs could transport functional cell receptors like AT_1R from cells to cells, mediating key physiological and pathological processes. Notably, in the condition of already formed circulating hypertension, circulating AT_1R-riched EVs derived from cardiomyocytes may further exacerbate blood pressure. Therefore, despite using drugs such as ACE inhibitors (ACEIs) or angiotensin receptor blockers (ARBs), strategies which prevent the release of harmful EVs derived from cardiomyocytes to circulation may be necessary to attenuate the elevated blood pressure enhanced by positive feedback.

5.2.2 Roles of Extracellular Vesicles in Vascular Endothelial Cells

Blood pressure is mainly modulated by vascular endothelium, with Ang II and thromboxane A2 to be vasoconstrictors and nitric oxide (NO) to be a vasodilator. Both the vasoconstrictors and vasodilators can be transported by EVs. The level of circulating EVs increased in patients with cardiovascular diseases. Meanwhile, endothelial NO synthase (eNOS) expression was downregulated significantly, which led to endothelial dysfunction in these patients [16]. Endothelium-derived EVs inhibited endothelium-mediated vasodilation and eNOS-mediated NO generation [17]. Interestingly, EVs derived from other cells also had anti-vasodilatory function. T cells-derived EVs could decrease eNOS expression and increase caveolin-1 expression, thus inducing endothelial dysfunction in both conductance and resistance arteries [18]. Meanwhile, platelet-derived EVs could induce thromboxane A2-dependent vasoconstriction [19].

Inflammatory-induced dysfunction of vascular endothelial cells (ECs) promotes the development of hypertension [15]. EVs derived from dendritic cells exaggerated inflammation of vascular ECs by activating the endothelial nuclear factor kappa B (NF-κB) signaling, increasing the expression of pro-inflammatory factors including vascular cell adhesion molecule-1, intercellular adhesion molecule-1 (ICAM-1), and selectin E [20]. It was confirmed that the inflammatory injury of

ECs in hypertension was also caused by EVs produced by macrophages. The level of microRNA-17 (miR-17), a negative regulator of ICAM1 expression, was significantly dropped in EVs isolated from serum of hypertensive rat compared with that of control rats, and transferring of EVs with decreased miR-17 level isolated from hypertensive rats could lead to overexpression of ICAM-1 and thus induce inflammatory injury of ECs [21].

5.2.3 Roles of Extracellular Vesicles in Vascular Smooth Muscle Cells

The abnormal crosstalk between ECs and vascular smooth muscle cell (VSMCs) accounts for various kinds of cardiovascular diseases including hypertension [22–25]. Growth arrest-specific 5 (GAS5), which was a long non-coding RNA (lncRNA) expressed both in VSMCs and ECs, was involved in vascular remodeling and thus inducing hypertension, with its expression significantly downregulated. Knockdown of GAS5 affected endothelial activation, endothelial proliferation, VSMCs phenotypic conversion, and ECs-VSMCs communication through β-catenin signaling [26]. It was also reported that endothelial-derived EVs enhanced expression of vascular cell adhesion molecule-1 (VCAM-1), upregulated high-mobility group box (HMGB) 1 and HMGB2, and thus accelerated leukocyte adhesion in VSMCs, promoting inflammatory [27]. MicroRNA-126 (miR-126) were confirmed to be transmitted directly from ECs to VSMCs. Endothelial miR-126 was demonstrated to modulate turnover of VSMCs. Co-culture of VSMCs with ECs or media of ECs increased level of miR-126 in VSMCs and thus induced turnover of VSMCs; meanwhile, inhibition of miR-126 could reverse these effects [28]. Uptake of endothelial-derived EVs by VSMCs could diminish VSMCs proliferation and migration. The underlying mechanisms were that transferring of microRNA-125 (miR-125) by endothelial-derived EVs into VSMCs downregulated expression of low-density lipoprotein receptor-related protein 6 (LRP6) [29]. Similarly, it was identified that microRNA-195 (miR-195) was packaged in EVs and transferred from ECs to VSMCs, downregulating 5-hydroxytryptamine transporter (5-HTT) expression in VSMCs and thus inhibiting VSMCs proliferation [30].

On the other hand, VSMCs-derived EVs also affect physiological functions of ECs. Overexpression of Krüppel-like factor 5 (KLF5) in VSMCs significantly reinforced the expression and secretion of microRNA-155 (miR-155). Then VSMCs-derived EVs transferred KLF5-induced miR-155 from VSMCs to ECs, and destroyed endothelial barriers, increasing endothelial permeability and thus promoting atherosclerosis [31]. Transferring of cZFP609 from VSMCs in Sirtuin1 (SITR1)- transgenic (−Tg) mice to ECs by EVs was proved to reduce endothelial angiogenic activity. cZFP609 interacted with hypoxia-inducible factor 1α (HIF1α), downregulating vascular endothelial growth factor (VEGFA) expression and thus inhibiting endothelial angiogenic functions [32].

5.2.4 Roles of Extracellular Vesicles in Vascular Fibroblasts

Migration of VSMCs participates in the vascular remodeling, and the crosstalk between vascular fibroblasts and VSMCs plays a crucial role in the regulation of migration of VSMCs. Fibroblasts-derived EVs from spontaneously hypertensive rats (SHR) could significantly promote the migration of VSMCs compared to Wistar-Kyoto (WKY) rats, which could be blocked by the EVs inhibitor GW4869, the ARB losartan, and the ACEI captopril. Although no significant differences were observed in Ang II and AT_1R levels between fibroblasts-derived EVs from SHR and WKY rats, fibroblasts-derived EVs from SHR could increase levels of Ang II and ACE, and enhance ACE activity in VSMCs of WKY rats. Therefore, it was ACE which transferred by fibroblasts-derived EVs that increasing Ang II level and promoting migration of VSMCs [33]. It was further disclosed that transferring of fibroblast-derived EVs from WKY rats into SHR could attenuate VSMCs migration through

delivering miR-155-5p into VSMCs and downregulating the expression of ACE, which may become a future therapeutic target [34]. Similarly, overexpression of miR-155-5p could also inhibit the VSMCs migration [35]. In conclusion, fibroblast-derived EVs could mediate the migration of VSMCs during the progress of hypertension.

5.3 Extracellular Vesicles and Secondary Hypertension

Secondary hypertension refers to hypertension due to identifiable causes, which account for approximately 5% of hypertensive patients [11]. EVs are proved to participate in various causes of secondary hypertension, including acute kidney injury, renal parenchymal diseases, kidney transplantation, secretory diseases (primary aldosteronism, pheochromocytoma and paraganglioma, Cushing's syndrome), and obstructive sleep apnea.

5.3.1 Extracellular Vesicles and Acute Kidney Injury

Acute kidney injury (AKI) refers to a sudden decline in renal function, caused by a broad spectrum of intrinsic and extrinsic factors such as shock, sepsis, urinary tract obstruction, and drug abuse [36, 37], and leads to severe clinical problems. Urinary EVs, which were isolated and purified from urine by Pisitkun, etc. for the first time, can be secreted from all nephron segments [38], providing evidence for early detection of different types of AKI. Tubular epithelial cells-secreted microRNA-19-3p (miR-19-3p) was reported to increase in lipopolysaccharide-induced AKI model, suppressing cytokine signaling-1, leading to M1 macrophage activation and interstitial inflammation [39]. Aquaporin-1 protein in urinary EVs was proved to decrease in renal ischemia/reperfusion (I/R) -induced AKI model [40]. Fetuin-A in urinary EVs significantly increased in renal I/R-induced AKI model, cisplatin-induced AKI model, and AKI patients, and the elevation of fetuin-A in cisplatin-induced AKI model before the elevation of serum creatinine and morphological injury [41]. The level of messenger RNAs (mRNAs) encoding transcriptional repressor activating transcription factor 3 (ATF3) in urinary EVs was elevated in renal I/R-induced AKI model [42], and levels of ATF3 protein in urinary EVs were also elevated in both cisplatin-induced AKI model and AKI patients earlier than serum creatinine [43]. Additionally, urinary ATF3 was shown to indicate sepsis-induced AKI [44].

5.3.2 Extracellular Vesicles and Renal Parenchymal Diseases

Immunoglobulin-A nephropathy (IgAN) is the main cause of glomerulonephritis, and urinary EVs may help diagnose early IgAN. Proteomic analysis of proteins in EVs in IgAN and thin basement membrane nephropathy (TBMN) showed that levels of α-1-antitrypsin and ceruloplasmin were higher in IgAN patients [45]. Additionally, the chemokine ligand-2 (CCL2) level in urinary EVs was upregulated in IgAN patients, which positively related to histologic injury and decline in renal function [46].

For other major pathological types, Wilms' tumor-1 (WT-1) in urinary EVs was observed to be significantly elevated in focal segmental glomerulosclerosis (FSGS) patients than in steroid-sensitive nephrotic syndrome (SSNS) patients or healthy controls, and dropped after recovery. In addition, the level of WT-1 in urinary EVs may become a predictor of podocyte injury, which shown to be earlier than albuminuria in both mice model and patients [47].

Diabetic kidney disease is the most common etiology for chronic kidney disease; however, microalbuminuria cannot perfectly predict diabetic kidney disease in all cases [48]. Expression of WT-1 was reported to increase in urinary EVs isolated from patients with diabetes, which was connected with declined renal function, suggesting WT-1 in urinary EVs as an indicator of podocyte injury, which is the main pathological change during the progress of diabetic kidney

disease [49]. Additionally, urinary EVs rich in microRNA-15-5p (miR-15-5p) isolated from type 2 diabetic patients could promote apoptosis of mesangial cells by targeting B cell lymphoma 2 (Bcl2) under high glucose [50].

Systemic lupus erythematosus (SLE) is a systemic disease which could lead to various clinical manifestations, of which lupus nephritis is the most serious one, accounting for the majority of deaths [51]. It was recently reported that levels of microRNA-146a (miR-146a) increased in urinary EVs in both mouse model and patients with lupus nephritis [52, 53]. In addition, microRNA-221 (miR-221) and microRNA-222 (miR-222) expressions in urinary sediment was proved to be associated with lupus nephritis disease activity [54].

For tubular damage, kidney solute and water transporters in urinary EVs were potential biomarkers [38, 55]. Na^+-K^+-Cl^- cotransporter in urinary EVs was found absent in Bartter syndrome [55], while Na^+-Cl^- cotransporter was found absent in Gittelman's syndrome [56].

Polycystic kidney disease (PKD), which is characterized by multiple cysts in both kidney and other organs, commonly accompanied by hypertension. PKD is caused by a mutation in genes encoding for proteins polycystin-1, polycystin-2, and fibrocystin, which played chief roles in maintaining the function of primary cilia. It was demonstrated that smoothened, cystin, and adenosine diphosphate (ADP)-ribosylation factor-like 6 (ARF6) were involved in PKD [57]. Quantitative proteomics on urinary EVs isolated from advanced stages of PKD patients also confirmed higher abundances of periplakin, envoplakin, and villin-1 [58].

5.3.3 Extracellular Vesicles and Kidney Transplantation

Kidney transplantation is to some degree the only option for end-stage kidney diseases. Since transplanted kidneys are vulnerable to be injured, monitoring of the status of transplanted kidneys after kidney transplantation is of great importance [59], and EVs may be of help in this situation. Urinary $CD133^+$ EVs were found absent in end-stage kidney diseases. Urinary $CD133^+$ EVs usually increased 7 days after kidney transplantation, but were not shown to increase in patients with severe renal dysfunction before transplantation, suggesting that urinary $CD133^+$ EVs could reflect the regenerative potential of kidney [60]. It was found in urinary EVs isolated from transplanted patients that the level of neutrophil gelatinase-associated lipocalin (NGAL) was elevated in patients with delayed graft function (DGF), which could be served as a biomarker of DGF after kidney transplantation [61]. High level of urinary $CD3^+$ EVs was reported in patients with organ rejection after kidney transplantations with high detection accuracy and may become a noninvasive method to detect organ rejection [62]. In addition, mRNA transcripts of antibody-mediated rejection-associated genes glycoprotein 130 (gp130), SH2 domain containing 1B (SH2D1B), tumor necrosis factor-α (TNF-α), and chemokine ligand-4 (CCL4) in circulating EVs were extremely higher in antibody-mediated rejection than cell-mediated rejection, which may be served as noninvasive biomarkers for antibody-medicated rejection after kidney transplantation [63].

5.3.4 Extracellular Vesicles and Secretory Hypertension

Primary aldosteronism (PA), characterized by an inappropriately high level of aldosterone in circulation, results in hypertension, hypokalemia, and metabolic alkalosis [64, 65]. Concentration of circulating EVs isolated from patients with PA increased [66] due to the fact that aldosterone could activate endothelial exocytosis [67]. Concentration of urinary EVs also increased in patients with PA. Differentially expressed proteins in PA, such as alpha-1-acid glycoprotein (AGP1) [68], NGAL, and lipocalin-2 were reported as biomarkers for diagnosis and prognosis assessment of PA [69]. Some microRNAs (miRNAs) presented in EVs were proved to be related to aldosterone metabolism. For example, microRNA-4516 (miR-4516) in urinary EVs was

shown to participate in the regulation of renal sodium transporters associated with PA pathophysiology [70–72].

Pheochromocytoma and paraganglioma (PPGLs) are chromaffin cell tumors and account for 0.2%–0.6% of hypertension [73–76], associated with significant cardiovascular morbidity [77–80]. Since causes of majority of PPGLs patients only involve somatic mutations, it is more suitable to monitor somatic mutations [81]. Double-stranded DNA (dsDNA) fragments in the circulating EVs of patients with PPGLs were found to share the same mutations with that of tumor cells. Moreover, dsDNA fragments in circulating EVs was highly consistent with the genomes of tumor cells, thus becoming a genetic marker for diagnosing PPGLs [82].

Cushing's syndrome (CS), characterized by chronic hypercortisolism, is related to increased mortality mainly due to cardiovascular disease [83, 84]. Hypertension is one of the common clinical features of CS, and occurs in about 75% of all cases [85, 86]. Increased transporters for renal sodium reabsorption in urinary EVs such as Na^+-K^+-Cl^- cotransporter and Na^+-Cl^- cotransporter were proved to be contributed to hypertension in CS [87].

5.3.5 Extracellular Vesicles and Obstructive Sleep Apnea

Obstructive sleep apnea (OSA), the most common form of sleep disordered breathing, is the main cause of secondary hypertension. OSA is characterized by intermittent hypoxia, sleep fragmentation, and episodic hypercapnia, and could result in sudden death [88].

It was proved that miRNAs in EVs were closely correlated with OSA [89–91]. Expression of microRNA-630 (miR-630) in EVs was extremely lower in children with endothelial dysfunction which was associated with OSA, which had 416 gene targets in ECs, and corresponded to 10 major functional pathways [92, 93]. Additionally, circulating $CD31^+$ $CD41^+$ and $CD41^+$ AV^+ platelet-derived EVs significantly increased in OSA patients, and were positively related to OSA severity [94–96]. Similarly, $CD11b^+$ and $CD45^+$ leukocyte-derived EVs levels increased in children with moderate-to-severe OSA and in symptomatic adults with OSA, and $CD11b^+$ leukocyte-derived EVs were positively correlated with OSA severity [94, 95]. And for endothelium-derived EVs, $CD31^+$ $CD42b^-$ endothelium-derived EVs levels were extremely higher in patients with OSA, and were positively corresponded to endothelial dysfunction. Continuous positive airway pressure (CPAP) therapy tended to downregulate $CD31^+$ $CD42b^-$ endothelium-derived EVs levels [97]. $CD31^+$ $CD42b^-$ and $CD62E^+$ endothelium-derived EVs levels were extremely higher in OSA, with $CD31^+$ $CD41^-$ EMP levels positively related to apnea–hypopnea index and intima media thickness and $CD62E^+$ endothelium-derived EVs levels negatively correlated with remission of OSA by CPAP treatment [98, 99].

5.4 Perspective

Growing evidence has shown that EVs are closely involved in both essential hypertension (Fig. 5.1) and many subtypes of secondary hypertension (Table 5.1) and has the potential to become new biomarkers in the diagnosis, treatment, and prognosis assessment of hypertension. Compared with tradition diagnostic methods, diagnosing by EVs has the advantages of being noninvasive, more convenient, and less costly. Multiple biological information carried by EVs derived from different systems and organs can help diagnose different subtypes of hypertension and thus allowing patients to undergo more appropriate treatment, help identify changes in disease procedures and thus ensuring doctors to adjust the treatment in time, and help assess the prognosis, thus improving patients' quality of lives. However, large multicenter cohorts are desired to further confirm the specificity and sensitivity of EVs as biomarkers.

This chapter summarized the roles of EVs in essential and secondary hypertension, hoping to provide new ideas for future applications of EVs.

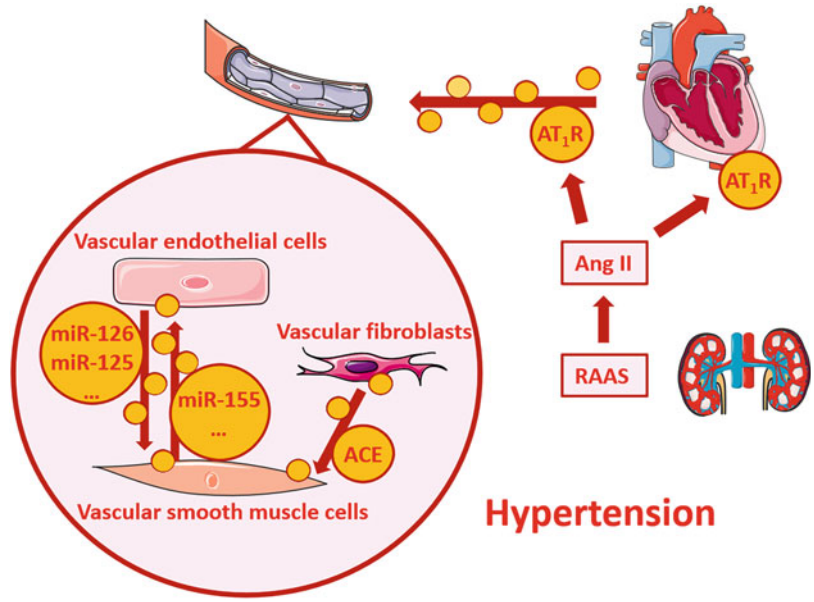

Fig. 5.1 Roles of extracellular vesicles in essential hypertension

Table 5.1 Roles of extracellular vesicles in secondary hypertension

Diseases	EVs and EVs contents	Function	Reference
Acute kidney injury	MiR-19b-3p	Increase in acute kidney injury	[39]
	Aquaporin-1	Decrease in acute kidney injury	[40]
	Fetuin-A	Increase in acute kidney injury earlier than serum creatinine and morphological injury	[41]
	ATF3	Increase in acute kidney injury earlier than serum creatinine	[42–44]
Renal parenchymal diseases	α-1-antitrypsin, ceruloplasmin	Increase in immunoglobulin-A nephropathy	[45]
	CCL2	Increase in immunoglobulin-A nephropathy, correlated with histologic injury and decline in renal function	[46]
	WT-1	Increase in focal segmental glomerulosclerosis earlier than albuminuria	[47]
	WT-1	Increase in diabetic kidney disease	[49]
	MiR-15-5p	Increase in diabetic kidney disease and enhance mesangial cells apoptosis	[50]
	MiR-146a	Increase in lupus nephritis	[52, 53]
	MiR-221, miR-222	Correlated with lupus nephritis disease activity	[54]
	Na^+-K^+-Cl^- cotransporter	Absent in urinary EVs in Bartter syndrome	[55]
	Na^+-Cl^- cotransporter	Absent in urinary EVs in Gittelman's syndrome	[56]
	Smoothened, cystin, ARF6	Involved in polycystic kidney disease	[57]
	Periplakin, envoplakin, villin-1	Increase in polycystic kidney disease	[58]
Kidney transplantation	Urinary $CD133^+$ EVs	Absent in patients with end-stage kidney diseases, reflect regenerative potential of kidney	[60]
	NGAL	Biomarker of delayed graft function	[61]
	Urinary $CD3^+$ EVs	Reflect rejection	[62]
	Gp130, SH2D1B, TNFα, CCL4	Reflect antibody-mediated rejection	[63]

(continued)

Table 5.1 (continued)

Secretory hypertension	AGP1, NGAL, lipocalin-2	Biomarkers for diagnosis and prognosis assessment of primary aldosteronism	[68, 69]
	MiR-4516	Regulate renal sodium transporters involved in primary aldosteronism pathophysiology	[70–72]
	DsDNA fragments	Genetic marker for diagnosis of pheochromocytoma and paraganglioma	[82]
	Na^+-K^+-Cl^- cotransporter, Na^+-Cl^- cotransporter	Contribute to hypertension in Cushing's syndrome	[87]
Obstructive sleep apnea	MiR-630	Decrease in obstructive sleep apnea	[92, 93]
	$CD31^+$ $CD41^+$, $CD41^+$ AV^+ platelet-derived EVs	Increase in obstructive sleep apnea	[94–96]
	$CD11b^+$, $CD45^+$ leukocyte-derived EVs	Increase in obstructive sleep apnea	[94, 95]
	$CD31^+$ $CD42b^-$ endothelium-derived EVs	Increase in obstructive sleep apnea	[97]
	$CD31^+$ $CD42b^-CD62E^+$ endothelium-derived EVs	Increase in obstructive sleep apnea	[98, 99]

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Extracellular Vesicles in Coronary Artery Disease

6

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Abstract

Coronary artery disease (CAD) is the leading cause of death and disability worldwide. Despite recent progress in the diagnosis and treatment of CAD, evidence gaps remain, including pathogenesis, the most efficient diagnostic strategy, prognosis of individual patients, monitoring of therapy, and novel therapeutic strategies. These gaps could all be filled by developing novel, minimally invasive, blood-based biomarkers. Potentially, extracellular vesicles (EVs) could fill such gaps. EVs are lipid membrane particles released from cells into blood and other body fluids. Because the concentration, composition, and functions of EVs change during disease, and because all cell types involved in the development and progression of CAD release EVs, currently available guidelines potentially enable reliable and reproducible measurements of EVs in clinical trials, offering a wide range of opportunities. In this chapter, we provide an overview of the associations reported between EVs and CAD, including (1) the role of EVs in CAD pathogenesis, (2) EVs as biomarkers to diagnose CAD, predict prognosis, and monitor therapy in individual patients, and (3) EVs as new therapeutic targets and/or drug delivery vehicles. In addition, we summarize the challenges encountered in EV isolation and detection, and the lack of standardization, which has hampered real clinical applications of EVs. Since most conclusions are based on animal models and single-center studies, the knowledge and insights into the roles and opportunities of EVs as biomarkers in CAD are still changing, and therefore, the content of this chapter should be seen as a snapshot in time rather than a final and complete compendium of knowledge on EVs in CAD.

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Keywords

Extracellular vesicles · Coronary artery disease · Biomarkers · Pathogenesis · Diagnosis · Prognosis · Treatment

6.1 Background

Coronary artery disease (CAD) is the leading cause of death and disability worldwide, responsible for about one-third of deaths in people above 35 years of age, and a major economic burden [1].

CAD is characterized by atherosclerotic plaque accumulation in epicardial arteries, thereby narrowing their lumen and limiting myocardial oxygen supply [2]. An imbalance between oxygen consumption and supply causes myocardial ischemia, which clinically manifests in angina. Angina occurs during physical exercise and is relieved by rest or nitrates [3]. CAD has long, stable periods (chronic coronary syndrome, CCS), but can also become unstable, typically due to atherosclerotic plaque rupture or erosion, which results in complete vessel occlusion (acute coronary syndromes, ACS). Despite recent progress in the diagnosis and treatment of CAD, evidence gaps remain, including (1) CAD pathogenesis, (2) the most efficient diagnostic strategy, (3) prognosis of individual patients with CAD, and (4) monitoring of CAD therapy. These gaps could be filled by the development of novel minimally invasive blood-based biomarkers.

Extracellular vesicles (EVs) are lipid membrane particles released from cells into body fluids, including blood [4]. The first evidence of EVs dates back to 1967 when Peter Wolf identified EVs from platelets as "platelet dust," i.e., subcellular particles promoting plasma clotting [5]. Since then, over 200 articles have been published on EVs and CAD (PubMed search 10.03.2021, query: ("extracellular vesicles" OR "exosomes" OR "EV") AND ("coronary artery disease" or "CAD")). Because (1) blood is a complex body fluid and EVs are a small fraction of both serum and plasma, (2) the association between EVs and CAD has been studied mostly in single-center studies, and (3) experimental models and conditions are often incompletely described; the results published thus far on EVs in CAD are often inconsistent, incomparable, and controversial. These results, together with possible explanations for discrepancies and proposed solutions, are summarized in Table 6.1.

Methodological improvements during the last decade enabled critical evaluation of the previous concepts on EVs [6] and have resulted in guidelines and standard operating procedures to measure EVs [7, 8]. There is potentially a wide range of opportunities for EV applications in patients with CAD, because all cell types participating in the development and progression of CAD, including platelets, macrophages, vascular endothelial cells, and vascular smooth muscle cells, release EVs [9], and because the currently available guidelines support reliable and reproducible EV analysis in multicenter studies.

This chapter provides an overview about our current knowledge regarding the role of EVs in CAD pathogenesis, EVs as biomarkers to diagnose, predict prognosis, and monitor therapy in CAD patients, and EVs as new therapeutic targets and/or drug delivery vehicles in CAD. The overall structure of the chapter is presented in Fig. 6.1.

6.2 Methodological Challenges

EVs in blood are more difficult to study than cells for three reasons. Firstly, EVs are substantially smaller than platelets, which are the smallest cells present in blood [10, 11]. Most EVs have a diameter < 200 nm, whereas platelets are typically 2–3 μm in diameter [12, 13]. Secondly, EVs are outnumbered by lipoproteins which overlap in size and density [14]. The reported plasma concentration of EVs is typically below 10^{11} mL^{-1}, whereas the concentration of lipoproteins exceeds 10^{16} mL^{-1} [15–17]. Thirdly, collection and handling of blood results in the generation of additional EVs, which makes it difficult to establish the relationship between ex vivo measurements of EVs and in vivo observations [7]. This section discusses the challenges accompanying the collection and handling of

Table 6.1 Controversial results regarding extracellular vesicles (EVs) in coronary artery disease (CAD) as discussed in this chapter, potential reasons for discrepancies, and proposed solutions. *ACS* acute coronary syndrome, *ADMA* asymmetric dimethylarginine, *CCS* chronic coronary syndrome, *DAPT* double antiplatelet therapy, *EVs* extracellular vesicles, *HDL* high-density lipoproteins, *NO* nitric oxide, *NSTEMI* non-ST-elevation myocardial infarction, *PS* phosphatidylserine, *STEMI* ST-elevation myocardial infarction

Process/disease	Statement	Counterstatement	Reason for discrepancy	Solution
CAD pathogenesis (paragraph 3)				
Endothelial dysfunction	EVs induce endothelial dysfunction [26, 27]	Co-isolated contaminants (NO synthase inhibitor) induce endothelial dysfunction [28]	Impossible to separate EVs and contaminants using centrifugation or precipitation [7]	Determine the presence and biological activity of co-isolation contaminants [7]
Vascular wall inflammation	EVs activate endothelial cells and leukocytes [29]	Co-isolated contaminants (cytokines, growth factors) induce inflammation [30]		
Plaque rupture	Plaque-derived EVs are procoagulant due to PS exposure [31–33]	Plaque-derived EVs are a mixture of EVs originate from issue dissociation		Isolate EVs without plaque dissociation (e.g., in culture medium) [34]
		PS exposure is sensitive to freeze-thawing [35, 36]. In fresh plasma, 50% EVs no PS [12]		Minimize manipulations during blood collection, centrifugation, freeze-thawing [37]
CAD diagnosis (paragraph 4)				
ACS vs. CCS vs. healthy volunteers	Elevated concentration of platelet EVs in ACS compared to CCS and healthy controls [38]	Comparable concentrations [39, 40]	Comorbidities and drugs affect EV concentration and composition [41, 42] Variations in pre-analytics and instruments are larger than variations between different stages of CAD [21] Lack of robust isolation and detection protocols hamper reproducibility	Consider the effect of comorbidities and administered drugs on EVs Apply instrument calibration [10] Perform measurements according to recent guidelines [7, 8, 25]
STEMI vs. NSTEMI	Elevated concentration of platelet EVs in STEMI compared to NSTEMI [43, 44]	Lower concentration [45]		
CCS vs. healthy volunteers	Elevated concentrations of platelet EVs in CCS compared to healthy controls [46]	Comparable concentrations [47]		
CAD prognosis (paragraph 5)				
Plaque rupture prediction	EV-derived miRNAs contribute to atherosclerotic plaque rupture [48]	Co-isolated contaminants as argonaut proteins and HDL carry miRNA [49, 50] Only a small	Impossible to separate EVs and contaminants using centrifugation or precipitation [7]	Consider EV contamination with protein and HDL-associated miRNA [52]

(continued)

Table 6.1 (continued)

Process/disease	Statement	Counterstatement	Reason for discrepancy	Solution
		fraction of EVs carry miRNAs which do not affect cell function [51]		
Antiplatelet therapy monitoring (paragraph 6)				
Lack of response to DAPT	Elevated platelet EV concentration in patients non-responsive to DAPT [53] Correlation between platelet EV concentration and platelet reactivity [54]	No correlation between platelet EV concentration and platelet reactivity during DAPT [42]	Platelet reactivity tests may not reflect platelet functional status in vivo [55, 56]	Standardize the time between blood collection and platelet function analysis [37] Consider flow-based methods to assess platelet function in a more physiological way [57]

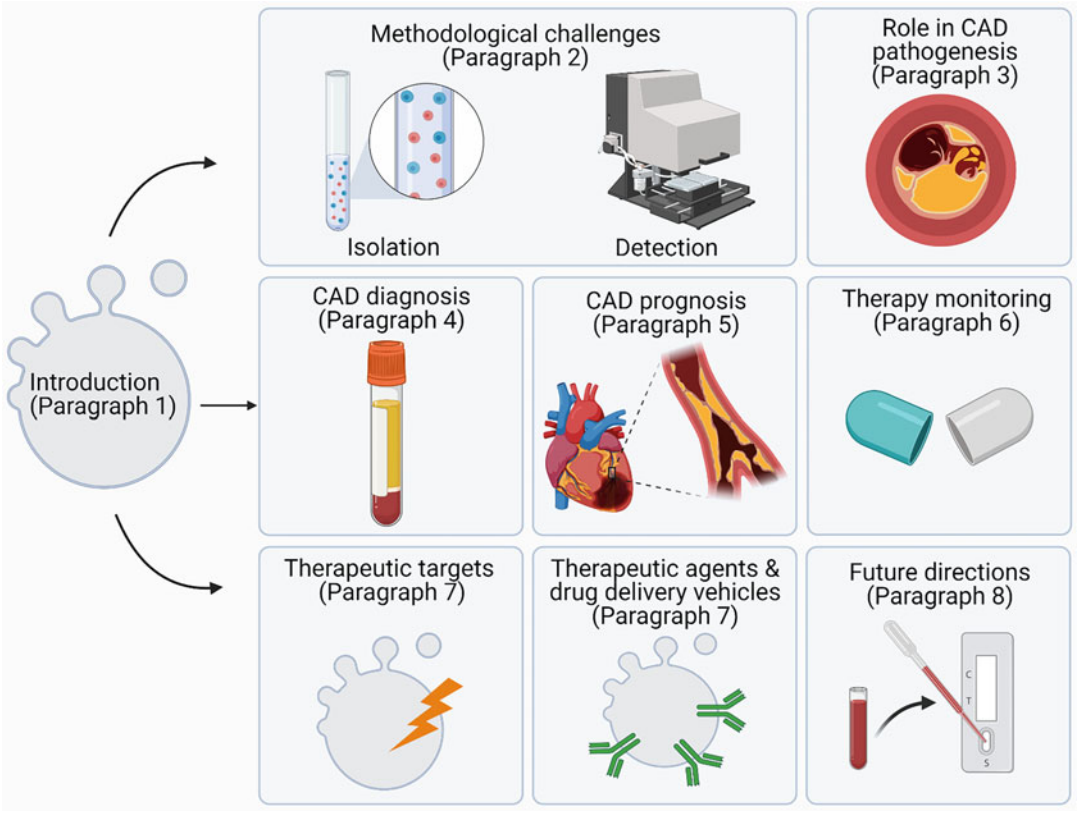

Fig. 6.1 Structure of the chapter. Extracellular vesicles (EVs) are released by cells known to participate in the development and progression of coronary artery disease (CAD), and therefore, EVs from such cells are potential biomarkers for diagnosis, prognosis, and monitoring of therapy, as well as therapeutic targets and/or drug delivery vehicles. Guidelines for EV isolation and detection promote reliable and reproducible EV analysis in clinical trials, offering opportunities for EV applications in CAD

blood, the storage of blood plasma, and the techniques to isolate and characterize EVs. Special attention will be paid to flow cytometry, which offers the possibility to detect single EVs directly in blood plasma, and thus does not require isolation.

EVs are present both in plasma and serum, but the EV concentrations in plasma best reflect the in vivo situation. Serum contains additional EVs that were generated during clot formation in vitro, which mostly originate from platelets [18]. Thus, blood collection for EV research aims to collect blood and prepare plasma samples in such a way that the EVs present resemble the in vivo situation as close as possible. Collection and handling of blood are aimed at preventing generation of additional EVs while preserving the EVs originally present within the blood.

The main reason to prepare plasma is to remove cells, which is important for three reasons. Firstly, many techniques cannot detect both cells and EVs simultaneously. Secondly, removal of cells makes the release of additional EVs impossible. Thirdly, in clinical studies the collected plasma samples are often stored by freezing. Freezing ruptures cells, thereby releasing fragments and particles that overlap in size with EVs. Specific considerations and recommendations for blood collection, plasma preparation, and storage for EV research can be found in the methodological guidelines of the American Heart Association [7]. Also, to standardize reporting of EV studies, a public online platform EV-TRACK was launched (www.evtrack.org) [19].

Techniques to measure EVs in plasma can be divided into bulk detection and single-particle detection techniques. Bulk detection techniques are applied after isolation of EVs, are widely available, and are often scalable to clinical exploitability. A disadvantage, however, is that the origin of the generated signals often remains unclear. This is particularly a problem for plasma because plasma EVs are derived from multiple cell types and outnumbered by non-EV particles [12].

Single-particle detection techniques detect EVs one by one. Examples are flow cytometry, microscopy methods, resistive pulse sensing, and single-particle tracking. From all single-particle detection techniques, flow cytometry is routinely used in clinical laboratories, and only flow cytometry is capable of (1) measuring EVs directly in plasma, (2) differentiating EVs from non-EV particles, (3) establishing the cellular origin of EVs, and (4) measuring at such a high throughput that statistically significant results can be obtained within a clinically meaningful time frame, e.g., less than 1 min [20]. Therefore, this section provides extra background about flow cytometry.

Flow cytometry was developed to detect single cells in suspension, but not subcellular particles. Thus, most flow cytometers lack sensitivity to detect (all) EVs. Out of 46 tested clinical flow cytometers in 2018, 14 flow cytometers were too insensitive to detect EVs [21]. Considering differences in sensitivity between instruments and the fact that relevant data of flow cytometers are expressed in arbitrary units, which make data comparison impossible, at present a strategy is being developed to calibrate flow cytometers to enable comparable concentration measurements of EVs [21–24]. To improve reproducibility, the EV flow cytometry working group (http://evflowcytometry.org/) developed a framework for reporting EV flow cytometry methods and results, called MIFlowCyt-EV [8, 25]. To disseminate the recent knowledge involving standardization and reporting of EV flow cytometry experiments, this working group, with members from ISEV (International Society of Extracellular Vesicles), ISAC (International Society for Advancement of Cytometry, and ISTH (International Society on Thrombosis and Haemostasis), currently prepares an educational compendium to provide the background knowledge that is required to understand the biological, chemical, and physical principles underlying flow cytometry experiments of subcellular particles.

Regardless of the applied technology, there are a few considerations that apply to all EV measurements in plasma. First, all details should be reported to improve reproducibility [4, 8, 19]. Second, assay controls should be included to verify that signals indeed originate from EVs.

Third, only use techniques that are suitable to measure EV in complex samples such as plasma. For example, dynamic light scattering is often used to measure the size distribution of EVs in plasma or serum, but this method is unsuitable to measure the size distribution of polydisperse EV-containing samples [11]. Fourth, the detection range of the instrument and how the detection range may affect statistics should be taken into account. Here, detection range refers to the minimum and maximum signal that can be detected, such as the smallest and largest diameter or the lowest or highest concentration of EVs. For example, single-particle tracking is often used to determine the "mean diameter of EVs," which is misleading because the lower limit of detection, typically about 70–90 nm, makes it impossible to measure smaller EVs, which may be more abundantly present [10]. Therefore, it is best practice to report sample statistics with the quantified detection limit of the used instrument.

6.3 Pathogenesis

During the past decade, our understanding of the pathophysiology of CAD has undergone a remarkable evolution. Previously considered a cholesterol storage disease, we currently view atherosclerosis as a chronic inflammation of the vessel wall [58].

Arterial endothelial cell dysfunction is considered the earliest event in atherosclerosis [59]. Healthy endothelium exerts a number of vasoprotective effects, largely mediated by nitric oxide (NO), the most potent endogenous vasodilator [60]. Traditional cardiovascular risk factors such as hypercholesterolemia, hypertension, smoking, and diabetes all lead to endothelial cell dysfunction and decreased NO production. As a result, arterial endothelial cells express adhesion molecules that bind to leukocytes, thereby enabling leukocyte migration from the blood into the intima, where leukocytes promote a local inflammatory response [61, 62]. For example, macrophages express scavenger receptors binding oxidized low-density lipoproteins (LDLs), which, when consumed by the macrophages, promote their transformation into foam cells [63]. As inflammation continues, activated leukocytes and endothelial cells release matrix metalloproteinases that promote the replication of arterial smooth muscle cells, which ultimately leads to the formation of the advanced atherosclerosis lesion [64].

Since cells involved in the development and progression of atherosclerotic lesions release EVs, EVs may play a role in the development of atherosclerosis, including (1) endothelial dysfunction, (2) vascular wall inflammation, and (3) atherosclerotic plaque rupture [65, 66]. Involvement of EVs in the development of atherosclerosis is shown in Fig. 6.2.

When reading this chapter, the reader is advised to remain critical when interpreting the results published thus far. First, the concentration and/or composition of EVs depends on experimental conditions and limitations of analytical methods [10]. At present, variations in published EV concentrations measured by different instruments is larger than the variations between the reported EV concentrations in health and disease, or in different stages of CAD (Table 6.1) [21]. Regarding the biochemical composition of EVs, the techniques most often applied for isolation of EVs are centrifugation- and precipitation-based methods, which result in co-isolation of non-EV-associated soluble proteins such as cytokines and growth factors. Although for biomarker applications it is irrelevant to know whether a potential biomarker is truly associated with EVs, imperfect isolation of EVs hampers studies aimed to unravel their true contribution to the pathogenesis of CAD.

Endothelial cell dysfunction is the earliest event in atherosclerosis. Activated endothelial cells express adhesion receptors that can bind leukocytes. Adhesion of leukocytes enables leukocyte migration from the blood into the intima, where the leucocytes become activated and promote local inflammation. As inflammation continues, activated leukocytes and endothelial cells release matrix metalloproteinases that promote the proliferation of arterial smooth muscle cells, leading to the formation of advanced atherosclerotic plaques. In turn, plaque rupture

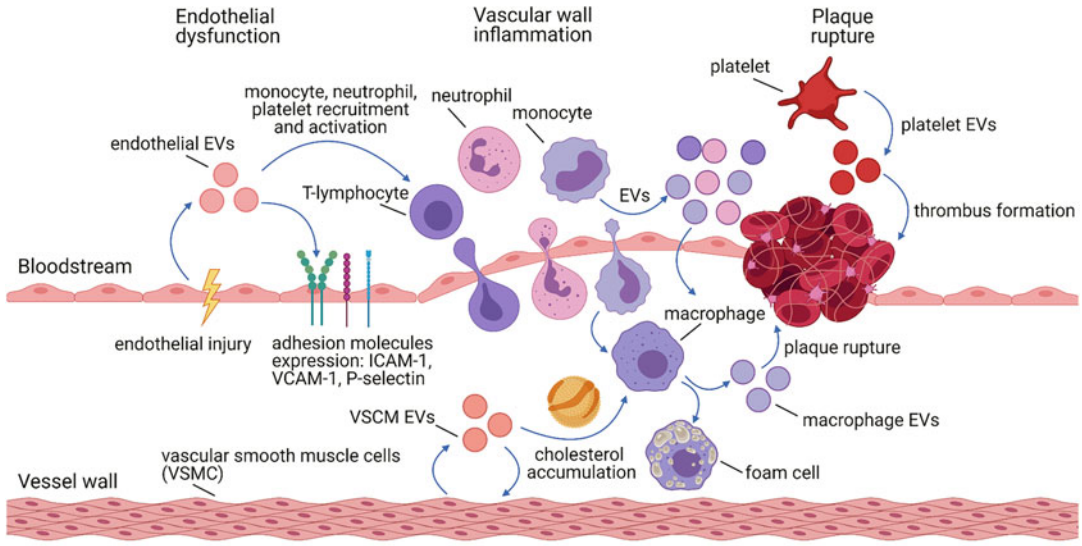

Fig. 6.2 Involvement of extracellular vesicles (EVs) in atherosclerosis

triggers platelet activation and aggregation, leading to blood vessel occlusion. Since endothelial cells, leukocytes, platelets, and vascular smooth muscle cells involved in the development and progression of atherosclerotic lesions all release EVs, EVs may participate in all stages of atherosclerosis, including endothelial cell activation, vascular wall inflammation, and atherosclerotic plaque rupture. ICAM-1: intercellular adhesion molecules; VCAM-1: vascular cell adhesion molecule-1.

6.3.1 Endothelial Dysfunction

Endothelial dysfunction results from the imbalance between the production and release of vasoprotective and vasoconstricting endothelium-derived mediators [29]. Endothelial EVs are reported to contribute to endothelial dysfunction by impairing vasorelaxation and increasing local oxidative stress [26, 27], although molecules with similar effects such as asymmetric dimethylarginine may co-isolate (Table 6.1) [28]. Thus, whether the observed effects of EVs on endothelial dysfunction are really attributable to (endothelial) EVs still remains questionable.

Underlying vascular diseases may affect plasma concentration and function of blood-borne EVs. For example, the plasma concentration of endothelial EVs was reported to increase with the severity of CAD. The lowest concentration was found in healthy controls, and increasing concentrations were found in patients with chronic coronary syndromes (CCS), followed by ACS, and recurrent ACS [67]. Regarding the function of blood-borne EVs, EVs isolated from the blood of patients with atherosclerosis induced endothelial dysfunction of rat aortic rings, whereas EVs from healthy subjects were did not induce this [68–70]. Whether such effects are due to differences in composition and/or concentration of EVs, or confounders such as asymmetric dimethylarginine, is unclear. For example, concentrations of 10^3–10^4 EVs from human umbilical vein endothelial cells (HUVECs) per mL have proangiogenic properties by regulating matrix metalloproteinase production in vitro [71], whereas concentrations of 10^5–10^6 per mL have anti-angiogenic properties, stimulate the production of reactive oxygen species (ROS), and decrease the formation of capillary-like structures [72]. These effects may depend on the presence of confounders in isolated EVs, but also on the type

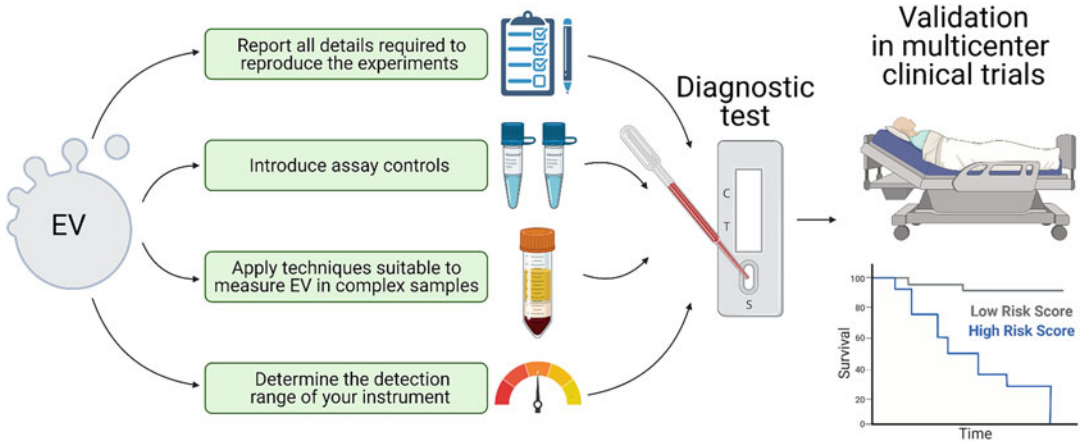

Fig. 6.3 Simplified roadmap to develop extracellular vesicle (EV)-based clinically relevant diagnostic tests. Following development of a test, a validation in multicenter clinical studies will be required

of endothelial cells studied, since venous and arterial endothelial cells differ in structure, composition, and function. Atherosclerosis develops following the injury of *arterial* endothelial cells, whereas the most frequent models used to study endothelial EVs are *venous* endothelial cells (HUVECs). Since atherosclerosis does not develop in the veins, the use of HUVECs as model to study the contribution of EVs to arterial endothelial dysfunction might be questioned.

6.3.2 Vascular Wall Inflammation

Vascular wall inflammation is initiated by infiltration of leukocytes, following endothelial injury and apoptosis (Fig. 6.3). EVs mediate the interactions between cells involved in vascular wall inflammation in vitro and in animal models. On the molecular level, EVs may contribute to inflammation by transporting bioactive molecules, including proteins, lipids, RNA, and DNA, to recipient cells [73]. For example, EVs from apoptotic endothelial cells, platelets, and activated T-cells were reported to (1) induce the release of the pro-inflammatory cytokines from leukocytes, (2) facilitate the adhesion of leukocytes to endothelium, (3) induce cholesterol accumulation in macrophages, (4) induce a phenotypic change of macrophages from an anti-inflammatory to a pro-inflammatory phenotype, and (5) induce a pro-inflammatory smooth muscle cell phenotype [29, 74–77]. Altogether, EVs are thought to participate in all key events that contribute to atherosclerotic plaque formation [78–80].

6.3.3 Plaque Rupture

The direct cause of coronary thrombosis and subsequent myocardial infarction is the rupture of the atherosclerotic plaque, which leads to vascular occlusion [81]. EVs were identified in human atherosclerotic plaques in 1998 [82]. Atherosclerotic plaques were separated from the underlying arterial wall, minced, enzymatically digested, and then centrifuged to obtain cell-free but EV-containing plaque homogenate [31–33, 83]. The concentration of EVs in the plaque homogenate was about 200-fold higher than in plasma, suggesting that EVs accumulate within atherosclerotic plaques [29, 32]. Atherosclerotic plaque-derived EVs originated from endothelial cells, erythrocytes, leukocytes, and smooth muscle cells (Fig. 6.3), and are thought to contribute to the procoagulant activity of atherosclerotic plaques [31–33]. As the plaque ruptures, released EVs promote coagulation by exposing phosphatidylserine (PS) and tissue factor (TF).

PS provides the surface for the assembly of coagulation factors, thus linking platelet activation within the ruptured atherosclerotic plaque to coagulation [84]. TF, in turn, triggers coagulation and thus the formation of thrombin, which results in fibrin formation and crosslinking of fibrin by activated factor XIII, thereby stabilizing the platelet plug so that a real thrombus is formed [81]. TF-exposing EVs were reported to be present in atherosclerotic plaque homogenates [32, 85]. EVs have also been implicated in atherosclerotic plaque rupture, as both circulating and plaque-derived EVs promote neovascularization, thereby stimulating intraplaque hemorrhage and plaque instability in vivo [86–88].

Although the results of previous studies suggest that EVs contribute to atherosclerotic plaque formation and rupture, isolation of tissue- and plaque-derived EVs is not straightforward. Plaque-derived EVs are isolated following mechanical [31–33] and/or enzymatic dissociation of the tissue [83]. As a result, the obtained EVs are likely a mixture of real tissue-derived EVs, EVs released from the cells in vivo, EVs present in the interstitial fluid of tissues, and EVs from intracellular origin released during tissue and cell dissociation in vitro. Moreover, because LDL particles also accumulate in atherosclerotic plaques, and the density of these particles increases during oxidation [89], LDL particles from atherosclerotic plaques overlap in density with EVs. Since contamination of EV samples with LDL particles was not measured, it cannot be excluded that at least a fraction of "EVs" that were isolated from atherosclerotic plaques are in fact oxidized LDLs. Thus, whether the measured "EVs" are truly derived from atherosclerotic plaques, and whether the measured "EVs" are indeed real EVs, requires confirmation.

It has been suggested that to measure EVs derived from atherosclerotic plaques, it is better to culture plaques and analyze the EVs released into the supernatant, instead of isolating EVs following mechanical or enzymatic plaque dissociation [7]. However, EVs released from cells cultured in vitro likely differ from those released in vivo. Whereas the development of atherosclerosis is a lifelong process and involves multiple cell types, cell cultures have life spans of days or weeks and are less complex. Moreover, the effects of culture-dependent pre-analytical variables, such as the type of culture medium and the number of cell passages, have not been established [37]. Recently, new protocols have been published to isolate EVs from tissues [90], and application of such protocols may shed new light on the presence of EVs within atherosclerotic plaques.

6.4 Diagnosis

The dynamic nature of CAD results in various clinical presentations, which can be categorized as either CCS or ACS (please see: Introduction) [3]. Biomarkers that are currently used to diagnose ACS, such as cardiac troponin, suffer from several limitations. For example, an elevated concentration of cardiac troponin in blood may result from conditions other than ACS, and cardiac troponin is also measured in the blood of healthy individuals when high-sensitivity assays are being used [91]. Moreover, cardiac troponin is released *after* cardiomyocyte necrosis, and hence such troponin does not reflect the preceding, or early stage of ACS [91]. Thus, novel biomarkers are needed to improve the sensitivity and specificity of CAD diagnosis at an early stage.

Since activated platelets initiate and propagate coronary thrombosis in the course of ACS, biomarkers of platelet activation such as platelet EVs have the potential to become biomarkers to diagnose ACS. For example, in a meta-analysis of 11 clinical studies including over 700 patients, the concentrations of platelet EVs were twofold higher in patients with ACS, compared to healthy, age-and gender-matched controls [38], indicating that concentrations of platelet EVs can potentially be used as biomarker to diagnose ACS. In addition, in ACS patients the concentrations of EVs exposing TF, EVs derived from granulocytes ($CD66b^+$), and activated endothelial cells ($CD62E^+$) were higher in the culprit coronary artery than in peripheral blood, and inversely

correlated with the time of coronary occlusion, i.e., onset of pain-to-percutaneous coronary intervention (PCI) time, suggesting that these EVs may indicate the stage of thrombus formation [92]. However, when concentrations of EVs were measured more recently with novel, more sensitive flow cytometers, no differences in concentration of platelet EVs were observed between patients with ACS, CCS, and controls (Table 6.1) [39, 40]. Hence, more studies are required to elucidate the diagnostic utility of both platelet and non-platelet EVs in ACS.

There are controversies regarding the reported concentrations of EVs between different ACS subtypes. ACS is classified into (1) ST-elevation myocardial infarction (STEMI), which is a transmural infarction with electrocardiographic (ECG) changes indicating acute coronary occlusion, (2) non-STEMI (NSTEMI), which is a subendocardial infarction with ECG changes indicating ischemia, but no acute occlusion, and (3) unstable angina pectoris, which is a pre-infarction state characterized by typical angina, but without biochemical or ECG signs of myocardial necrosis [93]. Especially the last-mentioned patients pose a diagnostic challenge, because it is difficult to differentiate between a cardiac and non-cardiac cause of chest pain in the absence of any objective proof of ischemia. Since EVs are released from platelets probably during and following thrombus formation, measuring platelet EV concentrations may facilitate the diagnostics of developing coronary thrombosis [94]. However, at present patients with transmural infarction were reported to have both higher and lower concentrations of platelet EVs compared to patients with subendocardial infarction or unstable angina [43–45], again confirming the need for reliable and reproducible measurements in patients with CAD.

At present, the diagnosis of CCS is based on imaging modalities showing signs of reversible myocardial ischemia [3]. Since none of these modalities ultimately confirm the presence of obstructive CAD, many patients require invasive coronary angiography, which is associated with complications and increases the costs of healthcare. The concentrations of platelet- and endothelial-derived EVs were reported to be higher in blood from patients with CCS compared to healthy controls, but these results were not reproducible [46, 47]. Further, CAD patients with type 2 diabetes mellitus and albuminuria had higher plasma concentrations of EVs from activated platelets, leukocytes, and vascular endothelial cells than CAD patients without diabetes [95]. However, the recent meta-analysis of 34 studies did not confirm these results [96]. Thus, further studies are needed to establish the diagnostic utility of EVs in CAD, and developments in the infrastructure are required to measure and report reliable concentration measurements of EVs.

There are four major obstacles regarding EV analysis in clinical trials: (1) specific patient characteristics (gender, comorbidities, medications), (2) pre-analytics ($>$ 40 variables with regard to blood collection and handling), (3) complexity of blood as a biospecimen to measure EVs, and (4) analytics (please also see: Methodological challenges in EV analysis). In addition, the clinical heterogeneity and relatively small ($n < 100$) number of patients in these mostly single-center studies might have confounded the results [42, 45, 97, 98]. A simplified roadmap to develop EV-based clinically relevant diagnostic tests is shown in Fig. 6.3.

Gender, comorbidities, and medications affect the concentrations of plasma EVs. For example, patients with arterial hypertension, diabetes, and chronic kidney disease were shown to have higher plasma concentrations of EVs, compared to healthy controls [37]. In addition, patients treated with statins or antiplatelet drugs have lower plasma concentrations of EVs, compared to patients not receiving these drugs [42, 99] (please see also: Extracellular vesicles to monitor antiplatelet therapy). Hence, gender, comorbidities, and medications should be taken into account when planning clinical trials to study EVs as biomarkers. Factors including needle diameter, use of tourniquet, a vacuum system, and the choice of anticoagulant may lead to activation and/or fragmentation of platelets and other cells, and thus may affect the measured concentration and the molecular properties of EVs,

resulting in conflicting results depending on the study set-up (see Table 6.1) [100]. In addition, in studies comparing patients and healthy individuals, blood samples are often not collected and handled identically, which in itself may result in differences in the concentrations of EVs present in such samples that are unrelated to the clinical conditions itself, i.e., whether the blood was collected from patients or controls. For challenges related to the complexity of blood as a specimen to measure EVs and analytics, please see paragraph "Methodological challenges in EV analysis." Altogether, the above-mentioned obstacles may contribute to the presence of and/or measuring differences in concentrations of EVs in the same patients. Further studies are needed to verify concentration measurements of EVs.

6.5 Prognosis Prediction

The major risk in patients with CCS is sudden plaque rupture, leading to ACS, whereas the major risk in patients with ACS is the development of chronic heart failure. To determine plaques at high risk of rupture, the term "vulnerable plaque" was introduced [101]. Certain histopathological plaque features, including inflammation, a thin fibrous cap, a large necrotic core, microcalcification, and plaque hemorrhage are commonly found in plaques causing ACS [101]. Recent advances in imaging techniques including intravascular ultrasound, optical coherence tomography, or near-infrared spectroscopy enable detection of individual characteristics of vulnerable plaques [102, 103]. However, the predictive value of a single plaque evaluation for ACS risk has remained poor [104]. Subsequently, it has been suggested that dynamic changes in atherosclerotic plaque composition may be more useful to detect a vulnerable plaque phenotype, compared to a single measurement [101]. Because liquid biopsy-based biomarkers are less invasive than intravascular imaging, biomarkers reflecting plaque vulnerability hold a great promise to identify patients at increased risk of ACS, who require intensive pharmacological and interventional treatment before ACS occurs.

A biomarker of a vulnerable plaque must (1) be released into the circulation after plaque rupture, (2) be specific for coronary plaques, and (3) have established kinetics of release and clearance [105, 106]. Numerous inflammatory molecules and hydrolytic enzymes have been suggested as potential biomarkers of vulnerable plaques, but none of them has hitherto been implemented in the clinics [105].

EVs may fulfill the requirements for a biomarker of a vulnerable plaque, because EVs are thought to accumulate within atherosclerotic plaques, and their concentrations are increased in the coronary circulation following plaque rupture in course of ACS [29, 32, 38, 92]. For example, the concentration of circulating endothelial/platelet EVs ($CD31^+/PS^+$) independently predicted cardiovascular events in stable CCS patients, whereas the concentration of leukocyte EVs ($CD11b^+$) identified patients with vulnerable plaques and those at high risk for recurrent cardiovascular events following PCI [100, 107]. Thus, the concentrations of cell type-specific plasma EVs may reflect plaque vulnerability.

Although no specific biomarker of coronary plaque formation has yet been identified, it has been hypothesized that the cargo of EVs, including proteins and microRNAs (miRNAs), may provide candidate biomarkers. For example, in patients with AMI, and not in healthy controls, several proteins were found to be exclusively associated with EVs, including chymotrypsin C, proto-oncogene tyrosine-protein kinase SRC, and C-C motif chemokine ligand 17. Thus, in the context of AMI, the biochemical composition of EVs may provide additional diagnostic and/or prognostic information compared to plasma proteomics [108]. Another study examined the EV-derived miRNA expression in atherosclerotic plaques and healthy arterial regions from patients undergoing heart transplantation, and showed decreased levels of EV-derived miR-143-3p and

miR-222-3p at lesion sites [48]. Thus, downregulation of miRNAs in the arterial wall may be either the cause or the consequence of atherosclerosis. Measurements of plaque-derived miRNAs in plasma EVs may therefore allow us to diagnose the presence of (vulnerable) plaques. Despite promising preliminary results showing the potential clinical applicability of EV-associated miRNA [109], the extent to which miRNAs are transported by EVs remains controversial. Recently, it was shown that only a small fraction of EVs carries miRNAs, thereby questioning the relevance of EV-associated miRNA released from atherosclerotic plaques as possible biomarkers of plaque vulnerability [51]. More studies are needed to demonstrate whether or not EVs or their cargo are promising biomarkers of plaque vulnerability (Table 6.1).

Once EVs specific for coronary plaques are found, the kinetics of their release and clearance in vivo has to be established. In a recent novel kinetic approach in a mouse model, the half-life time of EVs in the circulation was approximately 7 min after intravenous administration [110]. Traditionally, PS has been considered an "eat-me" signal that facilitates the clearance of cells and EVs by splenic macrophages [111]. However, PS exposure is also sensitive to manipulations in vitro, especially to freeze-thawing [35, 36]. If PS is artifactually due to manipulations in vitro, the clearance rate may seem higher than it truly is in vivo. Further, the concentrations of EVs present in blood are a balance between the production of EVs and their clearance. Hence, the changes observed in EV concentrations in CAD and other diseases, if any, may be due to changes in production, clearance, or both.

6.6 Antiplatelet Therapy Monitoring

Dual antiplatelet therapy (DAPT) with aspirin and P2Y12 receptor antagonists (as clopidogrel, prasugrel, ticagrelor) is the standard of secondary prevention of recurrent thrombotic events in patients after ACS [93, 112]. Despite potent antiplatelet treatment, recurrent ischemic events such as cardiovascular death, recurrent ACS, or stroke occur in ~10% of patients within 1 year after the initial ACS [113], whereas ~11% of patients experience bleeding on DAPT [114]. Hence, the concept of a "therapeutic window" of antiplatelet therapy was introduced, to prevent both too low and too high extent of platelet inhibition since that adversely affects patient outcomes due to an increased risk of thrombotic events and bleeding, respectively [115]. Subsequently, efforts have been made to individualize antiplatelet therapy based on platelet function testing [116]. Aggregometry-based tests, such as light transmission aggregometry, are mostly used in clinical practice. Despite the clear association between the extent of platelet inhibition and cardiovascular events, individualized antiplatelet therapy based on aggregometry has not improved clinical outcome [55, 56]. Hence, there is still no assay to identify patients at high risk for both recurrent ischemia and bleeding.

In contrast to aggregometry, which provides information on platelet functionality in vitro, concentrations or the composition of platelet EVs in plasma may provide a "snapshot" of the platelet activation status in vivo because EVs are released from activated platelets and possibly even originate directly from platelet-rich thrombi in vivo [106]. Accordingly, during the course of antiplatelet therapy, the concentrations of platelet EVs in blood or plasma may reflect the efficacy and safety [117]. Studies on the effect of aspirin on the release of platelet EVs have yielded contradictory results [118, 119]. Aspirin decreased the concentration of platelet-derived EVs in CAD patients, but no decrease was observed in healthy volunteers [118, 120–122]. In contrast, in the presence of antiplatelet drugs, platelets become more difficult to activate, thereby decreasing the production rate of platelet EVs, although the clearance rate of these EVs is not expected to change. Consequently, the measured plasma concentrations of platelet EVs will be lower. Indeed, there is consensus that P2Y12 decreased the concentrations of EVs from platelets [42, 54]. In addition, more potent platelet inhibition with ticagrelor is associated with lower platelet EV concentrations in plasma, compared

to clopidogrel, the previous standard of care in ACS [42]. Finally, in patients non-responsive to antiplatelet therapy with P2Y12 inhibitors, the concentrations of platelet EVs were twofold higher, compared to patients who had normal response to antiplatelet therapy. Based on the results, the concentrations of platelet EVs may potentially be useful to monitor the efficacy of antiplatelet drugs [53]. However, since there is still no consensus regarding the association between platelet EV concentrations and the extent of platelet inhibition, the plasma concentrations of platelet EVs cannot yet be used to monitor the efficacy of antiplatelet therapy in clinical routine (Table 6.1).

6.7 Therapy

New insights into the mechanisms of CAD development and progression allow development of novel treatment strategies, which are important from both health and economic perspectives [123]. EVs may play a role in the treatment of CAD as (1) targets for therapeutic interventions, (2) therapeutic agents, or (3) drug carriers [124].

6.7.1 Therapeutic Targets

EVs are traditionally believed to be mediators of intercellular communication [125], and to be associated with the pathogenesis of many diseases including CAD due to their pro-inflammatory and procoagulant functions [41]. By modulating the turnover of EVs, the EV-mediated pathways involved in the pathogenesis of CAD may be affected, thereby creating a therapeutic option [125].

At present, no drugs are available that specifically target the intercellular signaling pathways thought to be affected by EVs and/or EV cargo. However, different drugs, including antiplatelet drugs, heparin, and statins, which are commonly prescribed to patients with CAD, have been suggested to affect the concentrations of particular EVs in blood. As a result, the pleiotropic effects of different drugs used in CAD, such as inhibition of vascular inflammation or improvement of endothelial function, may at least partly be mediated by the inhibition of pro-inflammatory and/or prothrombotic EV release [126].

The effect of antiplatelet drugs on EVs has been discussed in the paragraph "Extracellular vesicles to monitor antiplatelet therapy." Unfractionated heparin is a standard drug administered during PCI procedures in patients with CAD. Ex vivo addition of heparin to plasma reduces the uptake of EVs by recipient cells, although this effect was EV type- and recipient cell type-dependent [127]. However, since heparin binds also soluble proteins as well as lipoprotein particles, this binding and the subsequent effect(s) are likely not specific to EVs. More research on the effect of heparin on the EV level is thus required to resolve whether it has effects by modulating EV concentrations.

Since the pathogenesis of CAD is associated with an increased level of cholesterol, lipid-lowering therapy, especially statins, is the standard of care in both primary and secondary prevention of acute cardiovascular events. Lipid-lowering drugs interfere with endocytosis, which is also one of the mechanisms by which EVs may enter cells. Endocytosis requires the presence of caveolin, present in cholesterol-rich microdomains in the plasma membrane, and lowering cholesterol levels decreases the availability of caveolin and inhibits endocytosis of EVs [128]. More studies are needed to determine whether the pharmacological modulation of EV concentrations and cargo could be a novel therapeutic strategy in CAD patients.

6.7.2 Therapeutic Agents

EVs can potentially also be used as therapeutic agents to inhibit the mechanisms underlying the pathogenesis of CAD. In CAD, chronic inflammation of the vessel wall is a key factor in initiating and promoting plaque development. Anti-inflammatory therapy, such as anti-interleukin-1β monoclonal antibody, improved cardiovascular outcome [58]. EVs with anti-

inflammatory properties could be used to reduce vascular inflammation. For example, EVs released from healthy endothelium reduced the inflammatory response by suppressing monocyte activation, whereas EVs from atherosclerotic endothelium had pro-inflammatory properties [129]. The authors hypothesized that the anti-inflammatory potential of these EVs is due to transfer of miR-10a, which suppresses the nuclear factor κB-mediated pro-inflammatory pathway [129]. Hence, stimulating the release of endothelial-derived EVs or hampering EV clearance may have a therapeutic effect.

One of the major problems after ACS are the structural and functional changes of the necrotic myocardium, termed post-infarct left ventricle remodeling (LVR), which lead to the loss of contractile tissue and to post-infarct heart failure [130]. Heart failure increases the risk of mortality and deteriorates the quality of life [130]. Hence, therapies to regenerate the necrotic myocardium and reverse LVR are needed to restore the contractile function in patients after ACS. There is increased evidence that not only the different cardiac cells but also EVs released from those cells play a role in the interaction underlying LVR [131]. The EVs can either lead to destructive LVR or have a cardioprotective function [132]. With regard to the latter, especially EVs from mesenchymal stem cells and cardiac progenitor cells may be used as potential therapeutic agents to restore cardiac function after ACS [133].

Mesenchymal stem cells (MSCs), present in bone marrow, suppress the immune response and, hypothetically, penetrate the injured tissue where they differentiate into cardiomyocytes. The strongest beneficial effects were reported for endometrium-derived MSCs [134] and hypoxia-treated MSCs [135]. In studies evaluating the efficacy of MSCs in cardiac regenerative therapy after myocardial infarction, restoration of the heart function occurred faster than differentiation of MSCs into cardiomyocytes [124, 136, 137]. When MSC-derived EVs were administered to rodents and pigs, the infarct size was reduced [137–139]. Based on these observations, it has been hypothesized that the beneficial effect of MSCs on the infarcted myocardium is due to paracrine factors, including EVs and EV cargo such as miRNAs. On the other hand, MSC-derived EVs promote coagulation by exposing TF and PS [140], suggesting acute adverse effects following EV administration. Thus, more research is required to establish whether the MSC-derived EVs are both an efficient and safe therapy.

EVs released by cardiac progenitor cells (CPCs) protect ischemic myocardium from necrosis [120, 136]. In murine and rodent models, injecting human CPC-derived EVs in the necrotic cardiac tissue inhibited apoptosis and promoted proliferation of cardiomyocytes, suggesting that EVs are potential mediators of cardiac regeneration [141, 142]. Similar observations could be made in pigs, where the post-infarct cardiac dysfunction decreased 4 weeks after the intramyocardial injection of EVs [143]. These findings suggest that EVs are also key mediators of CPC-induced myocardial regeneration, which could potentially be used in a cell-free therapy of post-infarct heart failure.

6.7.3 Drug Delivery Vehicles

Besides therapeutic agents and therapeutic targets, EVs can also be used as drug delivery vehicles, by transporting drugs to recipient cells. Synthetic drug carriers as liposomes come with disadvantages such as rapid clearance in the liver and spleen, hypersensitive reactions, and difficulties to cross biological barriers such as the blood–brain barrier [144, 145]. EVs have properties enabling them to circumvent the disadvantages of synthetic options. Although the clearance of EVs remains to be established (please see the paragraph: *Extracellular vesicles to predict prognosis in coronary artery disease*), the structure of autologous EV membranes enables uptake, intercellular trafficking, and delivery of the content to recipient cells, without initiating an immune response [124, 145]. Moreover, the EV membrane itself protects the intravesicular cargo from degradation. Finally, EVs have a relatively large surface area to volume

ratio, which seems to facilitate the interaction of "environmental biomolecules" with the EV surface, which themselves may play a role in intercellular trafficking [145].

Although the idea of EVs loaded with miRNAs might revolutionize the treatment of CAD, many challenges have to be overcome before EV might be clinically useful as drug carriers, including robust isolation of EVs, loading of endogenous cargo on EVs, and distribution of EVs to target organs. In addition, it is a challenge to choose the most appropriate source for isolation of EVs which could be used as drug carriers, including the type of cell and the type of organism [7]. Most likely, the highest level of safety could be obtained by using EVs derived from the patient itself, since these would trigger an immune response in foreign proteins.

The presence of an EV membrane makes it a challenge to load cargo into EVs [145]. In principle, the loading can take place before EVs are being released (endogenous loading), or once they have been isolated (exogenous loading) [146–148]. Several drug loading strategies have been developed, including electroporation, sonication, and freeze–thaw cycles [148]. Although the loading efficacy is cargo-dependent [145], the loading strategies tested thus far have an efficacy of less than 40% [149–151]. Recently, the presence of so-called corona proteins on the surface of EVs, i.e., mostly common soluble proteins present in the environment of the EVs that bind to and associate with EVs, may also play a role in transporting cargo. Plasma EVs are associated with such corona proteins, and because EVs have a relatively large surface versus volume ratio, this may offer an interesting new option for drug "loading" and delivery [18, 152].

The therapeutic cargo loaded into EVs thus far included proteins, miRNAs, and oligonucleotides inhibiting miRNA (anti-miR) function [120]. For example, miR-155 is involved in the activation of monocytes and macrophages, and enhances inflammatory gene expression in murine models, and is therefore a potential therapeutic target in atherosclerosis [76, 153]. In HUVECs treated with oxidized LDLs and in animal models of atherosclerosis, miR-155 was upregulated, suggesting its deleterious function in CAD. In patients with CAD, however, miR-155 was downregulated, contradicting the preclinical results [154]. Thus, the results regarding miR-155-loaded EVs to treat atherosclerosis are inconclusive, possibly due to differences between human and "animal models."

miRNA-126 is associated with the activation of the vascular endothelial growth factor pathway, thereby stimulating vascular regeneration and restoring the ischemic myocardium [120, 136, 155]. Decreased levels of miR-126 are associated with increased levels of placental growth factor, which supports angiogenesis. In CAD patients miR-126 levels were decreased compared to healthy individuals [155–157]. Due to the potential beneficial effects of miR-126 in the course of atherosclerosis, miR-126-loaded EVs may have a therapeutic potential in CAD.

After EVs isolation and therapeutic cargo loading, EVs should be administered and distributed within the body to deliver their cargo. The method of administration, however, may affect the tissues and organs in the human body where EVs will accumulate. EVs can be either injected to a systemic circulation or administrated locally to a specific organ such as the heart [145]. Obviously, the uptake of administered EVs by the recipient cells is important, and the uptake may involve several different pathways, including membrane fusion and endocytic pathways [158, 159]. Thus, although EVs are interesting as drug delivery vehicles, before clinical applications, (1) EV isolation methods should be improved and standardized, (2) there is a need for efficient drug loading, and (3) routes of EV distribution should be investigated [145]. The summary of EVs potential applicability in CAD therapy is shown in Fig. 6.4.

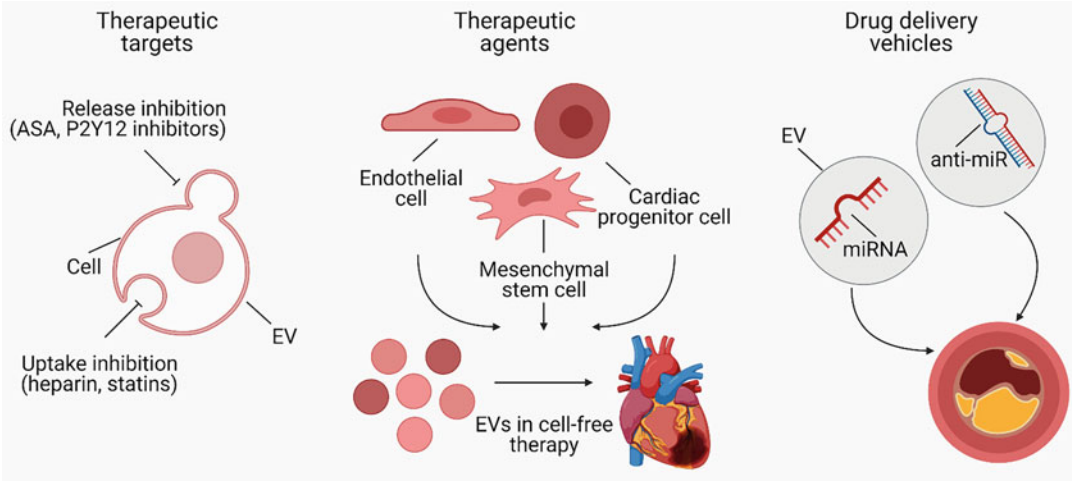

Fig. 6.4 Potential applications of extracellular vesicles (EVs) as therapeutic targets, therapeutic agents, and drug delivery vehicles in coronary artery disease

6.8 Future Directions

Knowledge and insights into EVs and CAD are rapidly evolving. Therefore, the content of this chapter is a snapshot in time rather than a final and complete compendium of knowledge on EVs and CAD. Given the progress that has been made in the EV field during the last decade, including new isolation and detection methods, it is exciting to speculate what the near future may bring. One of the major goals of the EV field is to discover EV-based biomarkers and/or therapeutic targets. Implementation, however, first requires robust standardization so that measurement results of EVs become comparable between institutes, and multicenter studies can be initiated. Only then we may be able to find out whether EVs are truly useful to diagnose CAD, predict prognosis, or monitor therapy.

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Extracellular Vesicles and Vascular Inflammation

7

Pujiao Yu, Shengqiong Deng, Xiaofei Yuan, Jiangqi Pan, and Jiahong Xu

Abstract

Vascular inflammation is the most common pathological feature in the pathogenesis of human disease. It is a complex immune process involved with many different types of cells including platelet, monocytes, macrophages, endothelial cells, and others. It is widely accepted that both innate and adaptive immune responses are important for the initiation and progression of vascular inflammation. The cell–cell interaction constitutes an important aspect of those immune responses in the vascular inflammation. Extracellular vesicles (EVs) are nanometer-sized double-layer lipid membrane vesicles released from most types of cells. They have been proved to play critical roles in intercellular communication in the occurrence and development of multisystem diseases. With the advancement of basal medical science, the biological roles of EVs in vascular inflammation have been clearer today. In this chapter, we will summarize the advance progress of extracellular vesicles in regulating vascular inflammation and its potential application in the clinical.

Keywords

Extracellular vesicles · Vascular inflammation · Endothelial cells · Vascular smooth muscle cells · Monocytes · Macrophage

7.1 Part 1 Background

Vascular inflammation is the process that inflammatory cells are activated and infiltrate the vascular wall, resulting in vascular wall damage, and it is a basic pathophysiological progress involved in multisystem diseases. In cardiovascular system, vascular inflammation is the determinative factor of vascular tension which eventually results in hypertension and atherosclerosis [1, 2]. It is also involved in promoting damage factors diffusion in the acute cardiac injury and regulating angiogenesis in the pathological remodeling. The molecular mechanism underlying vascular inflammation has been gradually improved for the last 3 decades. However, there are still deeper updated studies presented every year refreshing our understanding. Extracellular vesicles are kinds of nanometer-sized double-layer lipid membrane particles released from most types of cells, containing biological information and signaling molecules. Recent studies have proved that extracellular vesicles play a vital role in intercellular communication and participate in the occurrence and development of many diseases,

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including the regulation of the vascular inflammation. In this chapter, we will summarize the advance progress of extracellular vesicles in regulating vascular inflammation and its potential application in the clinical.

7.1.1 Mechanism of Inflammation

Inflammation is widely considered as a host defense mechanism enabling the body to respond to pathological stimulation. In acute inflammation, a rapid recruitment and activation of immune cells, such as neutrophils, eosinophils, macrophages, natural killer cells, and leukocytes, are raised to eliminate exogenous pathogen and endogenous abnormal cells [3, 4]. In this situation, uncontrolled inflammation reaction will also wound the normal surrounding tissues and amplify the damage range. Unlike acute inflammation, chronic inflammation is often described as a long-term pathological progress with a continuous recruitment of inflammatory cells, thereby changing tissue structure slowly [5]. The pathological condition induced by chronic inflammation is difficult to reverse and is usually asymptomatic until onset. A number of cytokines and signaling pathways are involved in the initiation of inflammatory response. On the other hand, inflammatory cells could carry out their function by secreting cytokines and activating signaling pathway. The classic factors include tumor necrosis factor-α(TNF-α), interferon-γ(INF-γ), interleukin-6(IL-6), interleukin-6(IL-8), and transforming growth factor-β (TGF-β), while signaling pathways are NF-κB signaling, Jak/Stat signaling, and Toll-like receptors [6–9]. Recent studies have shown that this complex pathological reaction mediated by multiple cells also has a more precise regulatory mechanism underlying it, including ferroptosis [10], pyroptosis [11], autophagy [12], mitochondrial disorder [13], and others.

7.1.2 Vascular and Inflammation

Endothelial cells (ECs), vascular smooth muscle cells (VSMCs), monocytes, and macrophages are the main cell types implicated in vascular inflammation.

Endothelial Cells

ECs line the luminal of arteries, veins, and capillaries, while its basolateral surface is separated from surrounding smooth muscle cells by a glycoprotein basement membrane. The luminal membrane of ECs is directly exposed to circulation; thus, various inflammatory mediators from plasma or blood cells may stimulate ECs by directly activating the receptors on ECs [14]. The main changes in ECs during the inflammation include dysfunction of vasodilatation and increased permeability. The former change increases systemic vascular resistance and thus leads to the development of hypertension, while the latter promotes plasma exudation, edema formation, and lipid deposition and finally leads to the formation of atherosclerosis [15]. The receptors on endothelium are mainly from three superfamilies including integrins, immunoglobulins, and selectins [16]. Previous studies have confirmed that, except for the classic inflammatory factors mentioned above, the ligands which specifically bind with endothelium receptors could also activate vascular inflammation including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), platelet-endothelial cell adhesion molecule-1 (PECAM-1), vascular adhesion protein-1 (VAP-1), and so on [17–21].

Vascular Smooth Muscle Cells

VSMCs are non-striated, non-voluntary, contractile cells originated from multipotent precursors [22, 23]. The optimal function of VSMCs is to maintain blood pressure through contraction and relaxation in opposition to the heart [23]. The historical view of VSMCs in vascular inflammation is that their migration and proliferation within the intima results in the formation of atherosclerosis plaque. However, since VSMCs are not terminally differentiated, more and more studies have shown that VSMCs could display phenotypic plasticity and change local microenvironment in response to inflammation changes and are capable of regulating contractile proteins, depositing collagen or lipid, forming

calcification, and remodeling the extracellular matrix (ECM) [24, 25]. In the normal artery, VSMCs express a range of smooth muscle cell markers, including smooth muscle cell myosin heavy chain (MYH11), smooth muscle cell actin (ACTA2), smoothelin, calponin, α-smooth muscle actin (α-SMA), and others [26]. Under the condition of inflammation, VSMCs reduce the expression of those markers and increase capacity for migration, proliferation, and secretion of cytokines [27].

Monocytes

Monocytes are differentiated from progenitor cells in the bone marrow and enter the circulation through chemokine receptor 2, representing 2–8% of blood leukocytes. For all stages of atherosclerosis, monocytes are involved and its function on immune response, effects on recruitment of inflammatory factors is the novel components of vascular inflammation [28]. Monocytes can be divided into three well-characterized subsets (classical, nonclassical, and intermediate monocytes) based on the expression of their differentiation antigen, including cluster of differentiation 14 (CD14) and cluster of differentiation 16 (CD16). Classical monocytes are the major components of all monocytes with only expression of CD14 in humans and co-expression of Ly6C, CCR2, CX3CR1 in mice [29]. Nonclassical monocytes are co-expression of CD14 and CD16 in humans and is only expressed CX3CR1 in mice. Intermediate monocytes are the remaining $CD14^{+}CD16^{+}$subset and account for approximately 5% of the total monocyte population. Functionally, the classical monocytes are able to differentiate into macrophages and dendritic cells and evoke inflammatory responses. Nonclassical monocytes circulate longer in blood and have been widely viewed as anti-inflammatory, as they maintain vascular homeostasis. They present more M2-like properties and are a first line of defense in recognition and clearance of pathogens. Intermediate monocyte is the smallest subset population of the total monocytes. It is reported that intermediate monocyte is associated with cardiovascular disease (CVD) events and correlated with plaque thinning, which may be the result of its CD11c integrin expression and stronger capacity to adhere to endothelium [29, 30].

Macrophages

Macrophage is a heterogeneous group of phenotypically and genetically distinct immune cells derived from hematopoietic progenitors and widely distributed in human tissues [31]. As the major immune cell population in the atherosclerosis plaques, macrophages play a vital role in the immune responses and progression of vascular inflammation. They initially display as tissue-resident cells in the absence of inflammatory stimuli, while their recruitment, activation, and local proliferation will be enhanced after injury and pathologic insults [32]. Macrophages express a range of opsonic and non-opsonic membrane receptors for the uptake and detection of pathogen and abnormal cells, including Fc receptors, complement receptors, Toll like receptors (TLR), lectins, and Scavenger receptors [32]. In the progress of atherosclerosis, macrophages take up lipid deposit particles and transform into foam cells and these foam cells further induce a cascade of inflammatory responses that promote more lipoprotein deposition and ECM modification and lead to chronic inflammation [33, 34].

7.2 Part 2 Extracellular Vesicles

Extracellular vesicles (EVs) are nanometer-sized (approximately 50–150 nm for exosomes, 100–350 nm for ectosomes, 100 nm–1 μm for shedding microvesicles, 0.5–2 μm for apoptotic bodies, and 1 μm–10 μm for oncosomes) lipid-containing bilayer membrane vesicles released by budding from the cell membrane surface by different cells [35–38]. They are characterized and identified by integrins and tetraspanins (CD9, CD63, CD89, CD81, CD9, and CD82), by maturation-related proteins flotillin and annexin, and by the heat shock proteins (hsp70 and hsp90) [39]. Rapidly evolving research in the past few decades has revealed that EVs are capable of containing a group of biologically active molecules derived from maternal cells including

proteins, DNA, RNA (mRNAs, miRNAs, circRNAs, and other non-coding RNAs), lipids, and metabolites and serve as a messenger of complex intercellular communications [40–42]. The characteristics of EVs and their contents are changed with the body's internal environment in both physiological and pathological status [43]. Unlike secreted solutes, EV cargos contain proteins and nucleic acids that are enclosed within a lipid bilayer membrane that can be easily transported in the circulation and interstitial space without losing biological activity. EVs have been proved to be accessible in nearly all body fluids and parenchymal tissues and considered a means of horizontal paracrine molecules transfer, delivering microRNA and mRNA between cells of different origin [44]. Apart from their role as paracrine transmitters, EVs also play an endocrine role in communication between adipocytes via vascular tissues, wherein they may act as vectors of atherogenic content [45].

7.3 Part 3 Biological Roles of EVs on Vascular Inflammation

The main biological function of EVs is to act as a carrier for intercellular signal transduction and biological information exchange. Recent studies have proved that EVs play a crucial role in the transmission of inflammatory modulators during vascular inflammation. EVs derived from multiple cells involve in all stages of inflammation development and expand inflammatory response from close to distant tissues. However, those EVs derived from different cells are shown to perform quite different roles in the process of vascular inflammation.

7.3.1 Endothelium Cell-Derived EVs

Endothelium cells have been known as the major functional coordinator in the vascular inflammation and maintain vascular functions. Early studies have reported that endothelial-derived EVs were increased in coronary artery diseases [46, 47]. Basic researches have revealed that EVs derived from inflamed endothelial cells were able to be taken up by monocytes or normal endothelial cells [48]. The functional inflammatory mediators transferred into those host cells would modulate them toward pro-inflammatory condition, increasing the expression of pro-inflammatory markers, such as IL6, IL-8, and ICAM-1 [49]. A subsequent study found that vascular endothelial cells upon inflammation release two heterogeneous size-based populations of EVs which were co-enriched with ICAM-1. The smaller-sized EVs were found to promote the migration of monocytes, while the larger ones were potent to induce the expression of ICAM-1 in host endothelial cells [50]. Moreover, those EVs derived from endothelial cells could also stimulated VCAM-1 expression in the VSMCs and change smooth muscle cell phenotype into pro-inflammatory condition [51]. Caspase-3 is a common apoptosis mediator involved in inflammation. Caspase-3 dependent death of endothelial cells leads to the release of unique EVs which could activate NF-κB signaling pathway and regulate cell death and differentiation in the vascular inflammation [52]. Vascular intima is exposed to a heavy concentration of uremic toxin and indoxyl sulfate in the patients with end-stage chronic kidney disease and the plasma EVs derived from endothelial cells were found to be elevated in those patients [53]. After damage by uremic toxin and indoxyl sulfate, endothelial cell-derived EVs could increase the expression of CCL2 and CCL5 in VSMCs and lead to calcium accumulation which eventually results in vascular calcification [54]. Oxidized low-density lipoprotein is also a kind of continuous stimulators to the vessel. He et al. found that miR-155 was enriched in EVs derived from oxidized low-density lipoprotein induced endothelial cells and could enhance monocyte/macrophage activation and mediate pro-inflammatory responses [55]. A recent study on the effect of endothelial progenitor cell (EPC)-derived EVs also found that EPC-EVs could inhibit cell death, glutathione consumption, ROS production, lipid peroxidation, and iron accumulation in endothelial cells by transferring

miR-199a-3p [56]. EPC-derived EVs could be taken up by VSMCs dominantly through caveolin-dependent endocytosis and increase proliferation/migration activities and cytokine (MCP-1, TNF-α) secretion of Ang II-activated VSMCs [57]. *Alexandru* et al. described a treatment of injecting EPC-EVs separated from healthy origins and found those EPC-EVs could significantly reduce atherosclerosis via transfer of miR-223, miR-21, miR-126, and miR146a [58].

7.3.2 Vascular Smooth Muscle Cell-Derived EVs in Vascular Inflammation

The secretion of EVs is a feature of VSMCs under the state of phenotypic conversion [59]. VSMC-derived exosomes can not only provide the catalytic surface for activating coagulation factors but also packaged with proteins or miRNA for regulating vascular inflammation and vascular calcification [60]. In the physiological condition, EVs derived by VSMCs are loaded with calcification inhibitors, such as matrix Gla protein and fetuin-A [61]. However, inflammatory environment could delete those calcification inhibitors from EVs and enrich them with protein–lipid complex which leads EVs to the messengers of calcifying signal [62]. VSMCs treated with or vessel stress could secrete EVs enriched with miR-143/145 mediating endothelial cells angiogenesis and vascular stability [63]. Likewise, it was found that VSMCs-derived EVs could impair endothelial cell barriers by weakening tight junctions and coherence, lead to increased endothelial permeability, and enhance atherosclerosis via transferring miR-155 [64]. miR92a and miR-195 in EVs released from VSMCs were found to shuttle into macrophages to regulate inflammation and promote atherosclerosis through elevating the expression levels of IL-6 and CXCL1 [65]. Moreover, EVs derived from VSMCs were proved to transfer miR-150 to endothelial cells and to lead to endothelial cells migration by enhancing the expression of vascular endothelial growth factor-A (VEGF-A) and activating the VEFG-A/VEGFR/PI3K/Akt pathway [66]. A recent study also found more novel mechanism of VSMCs-derived EVs on vascular calcification. That ROS in inflammation could promote the release of VSMCs-derived EVs and switched VSMCs phenotype via a mechanism of Ca^{2+} uptake was proved [67]. Endoplasmic reticulum stress could mediate VSMCs secreting glucose-regulated protein-enriched EVs inducing vascular calcification [68]. Specific deletion of Asah1 Gene could enhance VSMCs-derived EV release, mediating arterial medial calcification [69]. Human aortic SMCs were shown to suppress the autophagic activity of endothelial cells through upregulating the levels of miRNA-221/222 in EVs derived from human aortic SMCs.

7.3.3 Macrophage-Derived EVs in Vascular Inflammation

Macrophage-derived EVs mainly express CD40 ligand (CD40L) and were found to stimulate endothelial proliferation and promote angiogenesis within atherosclerosis [70]. Experimental studies have demonstrated that EVs extracted from M1 macrophages could elevate the expression of miR-185-3p in endothelial cells and suppress inflammation by negatively regulating Smad7 [71]. Oxidized low-density lipoprotein-stimulated macrophages could secrete EVs enriched with miR-146a which promoted ROS and neutrophil extracellular traps (NETs) release via targeting superoxide dismutase 2 (SOD2) [72]. Hypertension may result in the infiltration of inflammatory cells, including macrophages. Osada-Oka et al. demonstrated that EVs derived from macrophages in angiotensin II-treated rat was able to regulate the level of ICAM-1 through TLR4 signaling and the level of plasminogen activator inhibitor-1 (PAI-1) via surface receptor activation in the endothelial cells [73]. Likewise, diabetes also displays a higher content of inflammatory cell infiltration and vulnerable atherosclerotic plaque formation with macrophage apoptosis. In the studies related to hyperglycemia model, macrophage-derived EVs promote metabolic reprogramming and cellular proliferation

leading to myelopoiesis, hematopoiesis, and atherosclerosis [74]. miR-19b-3p-containing extracellular vesicles derived from macrophages promote the development of atherosclerosis by targeting JAZF1 [75]. EV derived from M1 macrophages could aggravate neointimal hyperplasia through delivering miR-222 into VSMCs [76], while M2 macrophage-derived EVs could promote the c-KIT phenotype of VSMCs via activation of the c-Jun/activator protein 1 (AP-1) signaling pathway [77].

7.3.4 Monocyte-Derived EVs in Vascular Inflammation

Monocytes are innate blood cells that maintain vascular homeostasis and early response to pathogens in acute inflammation [78]. The role of EVs derived from monocytes in intercellular communication during vascular inflammation and immune response is well established. It has been proved that monocytes are increased in the patients with atherosclerosis [79] and the role of monocyte-derived EVs in vascular inflammation is so diverse. Monocyte-derived EVs display coexisting tissue factor (TF) and activated protein C (APC) and thrombomodulin anticoagulant activity at their surface [80]. Markers for monocyte-derived EVs include CD14, CD11a, and CD18 [81–83]. Hoyer et al. have reported that monocyte EVs could promote leucocyte adhesion to post-capillary venules and T-cell infiltration within the atherosclerosis plaque, while the plaque tissues could also stimulate the release of inflammatory cytokines in monocytes [84, 85]. It was also found that monocyte-derived EVs can promote endothelial cell migration by miR-150 delivery, leading to disarrangement of endothelium [86]. Monocyte-derived EVs were also reported to encapsulate caspase-1 and induce VSMCs death [87]. Further study on smooth muscle cells phenotypes differentiation during vascular inflammation found that EVs from peripheral blood mononuclear cells of atherosclerosis patients were enriched with miroRNA-503-5p which was verified as an activator of inflammatory cytokines and adhesion molecules via downregulating smad family members 1, 2, and 7 [88]. Despite their roles in cardiovascular system, EVs derived from human monocytes could also act as potent carriers of immunomodulatory factors (such as mir-142-39) in vascular inflammation in the progress of liver regeneration after partial hepatectomy [89]. Monocytic EVs could directly induce MCP1, IL-6, and VEGF production which leads to chronic renal vascular inflammation and causes glomerular injury [90].

7.3.5 Platelet-Derived EVs in Vascular Inflammation

Platelet-derived EVs are heterogeneous vesicles released from platelet membranes due to the activation of platelet triggered by multiple factors, including hypoxia, oxidative stress, high shear stress, and endothelial injury [91–93]. Platelet-derived EVs have been widely reported to be elevated in plasma in various conditions associated with vascular inflammation [94–96]. And it is believed that the formation and the release of platelet-derived EVs is also regulated by several molecules and signaling pathway, for example Rho-associated kinase, calpain, and integrin [97, 98]. The function of these EVs has been demonstrated for decades. Some views hold that PLT-derived EVs are more potent at triggering inflammatory reactions [99]. In the progress of acute pneumonia, PLT-derived EVs were proved to carry a concentrated CD40L signal and promote endothelium damage [100]. It was also found that PLT-derived EVs might aggravate the apoptosis level of endothelial cell by deregulating redox metabolism via increased NADPH activity [101]. PLT-derived EVs, which are enriched with miR-320, were found to facilitate endothelial cell motility and reduce inflammation of blood vessel and formation of intramural thrombi via reducing endothelial ICAM-1 expression, which might potentially be a novel means of protection against inflammation injury [102]. Another proposed role of PLT-derived EVs is to act as a delivery system for lipid and fatty acids which could activate

endothelial cells and monocytes via prostaglandin and leukotriene production [103, 104]. Likewise, lysophosphatidylcholine (LPC) was also identified as a major component of PLT-derived EVs, and it could aggregate the formation of inflammatory platelet–monocyte in the progress of vascular inflammation [105]. Other evidence indicates that PLT-derived EVs could facilitate the deposition of the endothelium and monocytes via P-selectin, GPIb, $\alpha_{IIb}\beta_3$, and junctional adhesion molecules A (JAM-A) [106, 107].

7.3.6 Other Cell-Derived EVs in Vascular Inflammation

Neutrophil recruitment is an important characteristic of vascular dysfunction. Several reports have strongly suggested paracrine effects of circulating neutrophil on vascular inflammation, and EVs often act as the main transmitter in those progresses. In the models of atherosclerosis, neutrophil-derived EVs were found to disrupt the endothelial barrier and increased the inflammatory mediators including IL-6, IL-8, and ROS [108, 109]. In response to lipopolysaccharide (LPS), neutrophil from spleen was found to generate EVs to accelerate endothelial senescence through redox-sensitive pathways [110].

Adipose tissue surround vessels play a critical role in regulating vascular and endothelial function during both physiology and pathology condition [111]. It was proved that adipocytes could also release EVs like most cells [112, 113]. During inflammation, adipocytes affected by TNF-α and ROS could release EVs inducing production of VCAM-1 in endothelial cells [114]. Furthermore, adipocyte-derived EVs have been shown to stimulate formation of macrophage foam cells and enhance atherosclerosis [115]. In addition, the cross-talk of adipocyte to endothelial cells or VSMCs has also been shown to contribute to oxidative stress in inflammation [116, 117].

Diabetic retinopathy is the most common cause of blindness and is a consequence of chronic vascular inflammation. EVs derived from umbilical cord mesenchymal stem cells were found to ameliorate inflammatory reaction and oxidative injury in diabetic retinopathy via miR-17-dp/STAT1 axis [118].

Furthermore, *Rautou* et al. found that EVs derived from atherosclerotic plaques could transfer ICAM-1 to endothelial cells to recruit inflammatory cells to promote atherosclerosis progression [119]. Liver-derived extracellular could enhance fatty acid utilization by targeting AGPAT1 in endothelial cells and affected by physical exercise [120]. Tissue-resident mast cells also contribute to processes of vessel remodeling. Mast cells derived from EVs were found to be involved in inflammatory responses via transmit extracellular RNA (eRNA) [121].

7.4 Part 4 Perspective

Vascular inflammation induced by multiple pathological state could increase the risk of cardiovascular disease which has high morbidity and mortality. In most cases, the progress of vascular inflammation, apart from acute infection or vasculitis, is slow and asymptomatic. The consequence of this pathological change is always hard to be reversed and cause permanent damage to organs. Further studies to make clear the comprehensive mechanism of vascular inflammation are the most important way to find a solution for curing these diseases. However, although great efforts have been made in basic and clinical studies to clear the mechanism underlying vascular inflammation, the known regulation mechanisms which have been identified for vascular inflammation are just the tip of the iceberg and still need to be further improved.

As important carriers of signal molecules and material exchange from cell to cell, EVs have been revealed to play vital roles in the occurrence and development of vascular inflammation. What we know about vascular inflammation is that it is a complex progress involved in hundreds of known mediators and unknown number of unidentified molecules released from different cells. As the major carriers of those molecules, EVs undoubtedly could act as the breakthrough point of future studies on vascular inflammation. Furthermore, the treatment based on anti-

inflammatory EVs should also be novel therapeutic targets for those diseases with the pathological basis of vascular inflammatory, such as atherosclerosis, hypertension, and diabetes.

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Extracellular Vesicles for Muscle Atrophy Treatment

8

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Abstract

Skeletal muscle atrophy is a progressive chronic disease associated with various conditions, such as aging, cancer, and muscular dystrophy. Interleukin-6 (IL-6) is highly correlated with or plays a crucial role in inducing skeletal muscle atrophy. Extracellular vehicles (EVs), including exosomes, mediate cell–cell communication, and alterations in the genetic material contained in EVs during muscle atrophy may impair muscle cell signaling. Transplantation of muscle progenitor cell-derived EVs (MPC-EVs) is a promising approach for treating muscle diseases such as Duchenne muscular dystrophy (DMD). Moreover, stem cell-derived EVs with modification of microRNAs (e.g., miR-26 and miR-29) have been reported to attenuate muscle atrophy. Unbiased RNA-Seq analysis suggests that MPC-EVs may exert an inhibitory effect on IL-6 pathway. Here, we review the latest advances concerning the mechanisms of stem cell/progenitor cell-derived EVs in alleviating muscle atrophy, including anti-inflammatory and anti-fibrotic effects. We also discuss the clinical application of EVs in the treatment of muscle atrophy.

Keywords

Extracellular vesicles · Muscle atrophy · IL-6 · Bioengineering · Myogenic progenitor cells

Extracellular vesicles (EVs) produced locally and circulating in the blood play an essential role in cell–cell communication and crosstalk, thereby regulating cell function and metabolism in neighboring and remote tissues [1]. EVs can be divided into several categories according to their sizes and mechanisms of biogenesis. Since current methods cannot separate pure subsets, we prefer the general term "extracellular vesicles." There are two main subtypes of EVs: (1) "cup shaped" exosomes (Exos), which originate from a multivesicular body (MVB) and are released by late endosome fusion with plasma membranes with a size of 30–150 nm [2] and (2) irregularly shaped microvesicles, which originate directly from cell membranes with a size of 100–1000 nm [3]. Exos are implicated to mediate the transfer of microRNAs (miRNAs), proteins, mRNAs, lipids, etc., between cells or tissues [4]. EVs can be taken up by recipient cells via endocytosis, phagocytosis, or direct fusion with the plasma membrane, thus delivering their cargo to regulate cell signaling and function [2, 5].

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8.1 Major Components of Extracellular Vesicles

Exosomes transport proteins, lipids, mRNAs, and miRNAs to regulate the function of recipient cells [6–8]. These molecules are packed into Exos in the host cytoplasm by endosomal sorting complexes required for transport (ESCRT) or with the help of exosome-specific tetraspanin proteins, such as CD63, CD9, and CD81 [6]. Since Exos mediate cellular communication, those derived from diseased cells (such as platelets in septic patients) may elicit pathological responses. In contrast, Exos derived from stem cells may transfer protective factors to recipient cells [9]. The exosomal cargo derived from the cytoplasmic content of the stem cells can also include anti-aging and pro-regenerative particles, which might activate pro-repair and pro-survival pathways in recipient tissues [6].

8.1.1 Proteins

Exosome protein cargoes, such as hypoxia-inducible factor-1α (HIF1α), display activity-dependent phenotypes when transferred to recipient cells [10]. The accumulation of cytoplasmic transactive response DNA binding protein 43 kDa (TDP-43) is a pathological aspect of amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration. TDP-43 was detected in secreted exosomes of neurons, but not of astrocytes or microglia. Protein aggregation and autophagy inhibition are known factors that promote the secretion of TDP-43 via exosomes [11]. Chen et al. [12] observed that the TDP-43 ratio in exosomes isolated from the plasma of ALS patients changed dynamically over time, increasing significantly on follow-up testing at 3 and 6 months.

8.1.2 MRNAs

Many mRNAs are enriched in EVs, especially the 3′-untranslated region (UTR) fragments, which seem to be essential for loading into exosomes [13, 14]. Su X. et al. [15, 16] reported that transplantation with myogenic progenitor cell-derived exosomes (MPC-Exo) increased the expression of the full-length *dystrophin* gene in hearts of MDX (a mouse model of Duchenne muscular dystrophy) mouse recipients. The partial restoration of the *dystrophin* gene expression in MDX mice was associated with a transient improvement in cardiac function. MPC-Exo transplantation into skeletal muscle temporarily increased the full-length expression of dystrophin mRNA and restored the expression of dystrophin protein in the muscle cell membrane of recipient MDX mice. Therefore, intramuscular injection of allogeneic MPC-Exo seems to restore *dystrophin* gene expression in some muscle cells, at least temporarily. This finding is concordant with the study of Aminzadeh et al. [17] showing that injection of cardiosphere-derived cell (CDC)-derived exosomes could provide partial restoration of dystrophin protein expression at 1 week and 3 weeks after delivery. Moreover, the investigators suggested that exosome-mediated miR-148a transfer might be the mechanism of enhanced dystrophin expression. Su X et al. found that MPC also carries dystrophin mRNA, which may also contribute to restore dystrophin expression in MDX hearts. Finally, it is also possible to transfer dystrophin protein directly from donor MPC-Exo into cardiomyocytes of MDX mice, thereby leading to partial restoration of dystrophin expression in recipient cells.

8.1.3 MicroRNAs

Exosomes contain many miRNAs, which differ from the overall miRNA contents of the parent cells [18]. Kosaka et al. [19] demonstrated that incorporating miRNAs into MVBs is controlled by nSMase2, which enhances the shedding of exosomes. nSMase2 inhibition reduces the release of miRNA-containing exosomes. Small GTPases Rab27a and Rab27b are also involved in the output of miRNAs from parent cells via exosomes [20]. Vallarroya-Beltri et al. [21] demonstrated that the miRNA sequence motifs

are essential for loading miRNAs into the exosomes. Using mutagenesis experiments, they demonstrated that the heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) directly binds to miRNAs by recognizing a maturation sequence and controls their sorting in EVs. In exosomes, hnRNPA2B1 is sumoylated, and this post-translational modification promotes its binding to miRNAs. The loading efficiency of EVs is dependent not only on the RNA sequence but also on the cell type and physiological state. Our previous study showed that compared with cardiac MSC (C-MSC), C-MSC-derived exosomes contained high levels of miR-451 [22]. However, miR-144, another miRNA in the miR-144/451 cluster, was absent in C-MSC-exosomes. The mechanism of asymmetric distribution of miRNAs in C-MSC-Exo is unclear, but we postulate that certain small RNAs are preferentially exported to EVs. Atrophy-inducing conditions are associated with selective packaging of miRNAs into exosomes. Hudson et al. [23] reported that administration of glucocorticoids does not change the number of exosomes released into the culture medium, but it appears to shift the miRNA sorting into exosomes (i.e., highly enriched in miR-23a and miR-1). Understanding and exploiting this mechanism might enable the optimization of genetic components inside the exosomes for therapeutic purposes.

8.2 Signaling Pathways Controlling the Secretion of Exosomes

The ESCRT-independent nSMase2 and the small GTPases Rab27a and Rab27b are three crucial proteins that control the production and secretion of exosomes [24]. The pharmacological inhibitor of the nSMase2 pathway, GW4869, effectively blocked exosome biogenesis, while knockdown of nSMase2 using shRNA reduced the release of exosomes in mouse cells [25]. The GTPases Rab27a and Rab27b exert a primary control over membrane microvesicle transport, particularly the secretion of exosomes. Zhang et al. [26] reported that the inhibition of Rab27 reduced the release of exosomes into the culture medium of breast cancer cells, while Jin et al. [27] reported that knocking down Rab27b reduced exosome secretion in MSCs. Ruan et al. [28] reported that preconditioning MSCs with a multi-herb agent containing tetramethylpyrazine (TMP) and borneol (BOR) upregulated Rab27b and increased exosome secretion. In addition, a recent study showed that exosome secretion could reduce cellular stress and maintain cellular homeostasis by exporting various unnecessary or harmful substances [29]. Furthermore, knocking down Rab27b in MSCs increased cellular glycolysis and reduced mitochondrial oxidative metabolism, suggesting that Rab27b may play an essential regulatory role in MSC metabolism. Therefore, in addition to participating in paracrine signaling, the formation and secretion of exosomes may play a fundamental role in controlling intracellular metabolism and stress.

8.3 Linkages Between Extracellular Vesicles and Muscle Atrophy

Extracellular vesicles have recently been recognized as important causal factors of muscle atrophy, and EVs carrying many potential bioactive molecules (such as microRNAs) can act as information transmitters between bone and muscle [30, 31]. He et al. [2] identified four downregulated microRNAs (including miR-24, miR-328, miR-365, and miR-374) and seven upregulated microRNAs (including miR-15b, miR-17, miR-20a, miR-186, miR-221, miR-31a-5p, and miR-99b) in aging bone marrow-MSC-derived EVs. Such dysregulation has been reported to affect bone metabolism and muscle metabolism. Van Pelt et al. [32] also reported that human muscle disuse atrophy leads to a decrease in serum EV-miR-203a-3p, suggesting that miR-203a-3p expression in serum EVs is a sensitive indicator of muscle protein status. Specifically, the expression of miR-203a-3p in serum EVs was related to muscle mass, protein synthesis, and protein degradation in weight-bearing, disuse, and reloading conditions. Aging is associated with significant muscle atrophy in the form of sarcopenia, and the

senescence-associated microRNA miR-34a is elevated with aging in muscle-derived EVs [33]. The increase in miR-34a in aging myocytes appears to result from exposure to elevated levels of oxidative stress [33]. These muscle-derived EVs enriched in miR-34a are therefore thought to comprise part of the senescence-associated secretory phenotype, or SASP, which contributes to additional tissue dysfunction through paracrine communication.

A variety of myokines have been found in secretory intercellular communicating vesicles, especially in exosomes [34]. Myokines are closely related to the regulation of muscle atrophy and skeletal muscle inflammation [35]. EVs released from healthy myotubes can promote the activation or maintenance of muscle differentiation. However, EVs from inflammatory myotubes inhibit the expression of MyoD and myogenin and significantly downregulate p-AKT and p-P70S6K signaling during myogenic differentiation. Cancer-associated sarcopenia is a complex metabolic syndrome characterized by pronounced muscle wasting. Several miRNAs carried by tumor-derived exosomes have been shown to promote inflammatory secretion, activate catabolism, and even participate in regulating cell degradation pathways; these miRNAs can be transmitted to myocytes to affect their function [36]. Interestingly, exosomes can mediate the transfer of transmissible endoplasmic reticulum stress (TERS) from cancer cells to skeletal muscle, which can cause skeletal muscle cell atrophy. Qiu et al. [37] demonstrated that exosomes from oral squamous cell carcinoma (OSCC) cells provoked TERS signaling in myocytes, leading to muscle cell atrophy and apoptosis. Mechanistically, this was linked to delivery of miR-181a-3p to C2C12 myotubes.

8.4 Potential Mechanisms Whereby Stem Cell EVs Can Prevent Muscle Atrophy

EVs derived from stem cells play a major role in the paracrine effects of stem cell transplantation. They can emulate the biological activity of stem cells by transferring proteins, mRNAs, and miRNAs into neighboring cells (such as skeletal muscle cells, endothelial cells) and regulating their function [16, 38, 39]. In order to explore other molecular mechanisms of allogeneic MPC-Exo transplantation for improving cardiac function in muscular dystrophy mice, Su et al. [40] performed unbiased next-generation RNA-sequencing (RNA-seq) and identified differentially expressed genes (DEGs) in hearts of two distinct Mdx mouse strains (D2.B10-$Dmd^{Mdx/J}$ and $Utrn^{tm1Ked}$-$Dmd^{Mdx/J}$). The Venn diagrams depicted 780 genes that were upregulated, and 878 genes that were downregulated, at least two-fold as compared with controls. Gene ontology (GO) and protein–protein interaction (PPI) network analyses determined that these DEGs are linked to various pathways and have complex connections. The Mdx hearts treated with MPC-Exo exhibited upregulation of Bcl-2 mRNA and protein levels, while IL-6 mRNA and protein were downregulated as compared with control treatment. This suggests that anti-apoptotic and anti-inflammatory effects mediate the beneficial effects of MPC-Exo treatment on cardiac function. Consistent with this finding, Zhang et al. [41] reported that adipose stem cell-derived exosomes have a similar effect to inhibit the synthesis of IL-6, thereby improving the histological characteristics of torn human rotator cuff tendons.

8.5 Extracellular Vesicles: Therapeutic Tools for Muscle Atrophy

Recent studies have provided evidence that most of the beneficial effects of stem/progenitor cell therapy are produced by EVs, containing a variety of miRNAs, proteins, and mRNAs. Stem cell-derived exosomes are thus promising therapeutic tools. Although they may be less risky than stem cell transplantation, there are still challenges in optimizing their stability and application. The possibility of using autologous stem cells for exosome purification bypasses the main issues of immune response and the strict regulatory

requirements associated with the use of human stem cells. Exosome therapy can potentially mimic the therapeutic activity of stem cells while avoiding potential harmful side effects such as tumor formation.

EVs carrying higher levels of protective miRNAs can promote muscle function and prevent atrophy, thus producing therapeutic benefits. Zhang et al. [42] reported that intramuscular injection of AAV-miR-23a/27a/24–2 into diabetic mice increased the levels of miR-23a and miR-27a in serum exosomes and kidneys as compared with mice injected with control virus. Consequently, muscle mass and function were improved, in association with upregulation of the insulin signaling pathway and inhibition of the myostatin cascade. Also, Wang et al. reported that compared with control AAV-GFP, injection of AAV-miR-23a/27a/24–2 in tibialis anterior muscle results in higher amounts of miR-23a and miR-27a in serum exosomes [43]. Wang et al. [44] further reported that exosomes with encapsulated miR-26a and muscle-targeting peptide (MSTP) coating reduced skeletal muscle atrophy via regulating the insulin-IGF-1/Akt/FoxO signaling pathway. These findings suggest that exosomes containing these mature miRs might lead to significant improvement in overall muscle function following uptake by myofibers in other muscles.

Interestingly, Zhang et al. [45] reported that the expression of miR-26 was reduced in skeletal muscle and kidney of mice with unilateral ureteral obstruction (UUO). The exogenous delivery of miR-26a-modified exosomes to the muscle inhibited muscle atrophy by targeting the *FoxO1* gene and attenuated renal fibrosis by targeting CTGF and TGF-β1 through exosome-mediated skeletal muscle/kidney communication. Thus, exosome miRNAs may also contribute to antifibrotic effects in remote tissues. Intramuscularly injected miR-29-modified exosomes (Exo/miR-29) can be transported from skeletal muscle to the damaged kidney to ameliorate renal fibrosis [46]. The authors found that intramuscular injection of Exos/miR-29 can protect muscles from wasting in the UUO mouse. Mechanistically, this protection against muscle atrophy appears to be related to reduced expression of the muscle catabolic proteins TRIM63/MuRF1 and FBXO32/atrogin-1, which are upregulated in the UUO model. Moreover, the authors observed that intramuscular injection of Exo/miR-29 can attenuate renal fibrosis in UUO mice by targeting TGF-β3. Wang et al. [47] reported similar findings and showed that the level of miR-29a was downregulated in the kidney and skeletal muscle of UUO mice. However, after AAV-miR-29a transduction, the miR-29a level in serum exosomes was significantly increased in circulating exosomes from the sham and UUO mice. This may facilitate miR-29a uptake up by kidneys, thus producing beneficial effects against renal fibrosis.

It is also known that fibro-adipogenic progenitor cells, or FAPs, represent a cell population in muscle that is an important source of EVs [48]. The FAP secretome plays a key role in muscle repair and regeneration by modulating the proliferation and differentiation of muscle satellite cell populations [49]. Recently it has been shown that histone deacetylase (HDAC) inhibitors can alter the FAP secretome [50]. FAPs treated with HDAC inhibitors are "tuned" to secrete EVs that have elevated levels of the "myomir" microRNA-206. These EVs carrying miR-206 were observed to increase satellite cell proliferation and myogenic differentiation, enhancing muscle regeneration. The work by Sandona and colleagues highlights the potential of exosome-based therapy for improving muscle repair, but also suggests that small therapeutics may be used to improve muscle function via exosome-related mechanisms. A similar strategy using small molecule therapeutics to suppress the secretion of EVs enriched in miR-34a with aging could potentially serve as a novel approach for sarcopenia prevention and treatment.

8.6 Perspective

Muscle progenitor cell-derived exosomes are naturally occurring nanovesicles that can promote muscle regeneration by inhibiting IL-6 signaling and activating anti-apoptotic signaling. Another

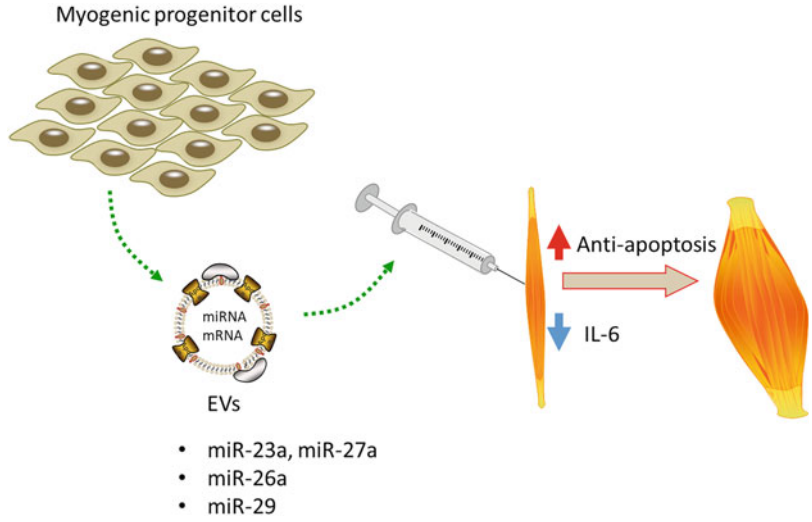

Fig. 8.1 Overview of the application of myogenic progenitor cell-derived extracellular vesicles (EVs) in the treatment of muscle atrophy via optimizing microenvironment

advantage of EVs is that they can carry various defined genetic components (such as miRNAs), which may provide a useful platform for treating patients with muscular atrophy. More research is required to identify key factors involved in restoring muscle structure and function after stem cell therapy in the muscle atrophy model. If miRNAs mediate most of the beneficial effects, fine-tuning of the miRNA cargo will be required to optimize MPC-Exo to confer these beneficial effects (Fig. 8.1). Exosome therapy based on muscle progenitor cells has opened the door to improving the quality of life for patients with muscle atrophy.

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Part III

Extracellular Vesicles in Metabolic Diseases

Extracellular Vesicles and Fatty Liver

9

Xiya Lu, Meiyi Song, and Na Gao

Abstract

Fatty liver is a complex pathological process caused by multiple etiologies. In recent years, the incidence of fatty liver has been increasing year by year, and it has developed into a common chronic disease that seriously affects people's health around the world. It is an important risk factor for liver cirrhosis, liver cancer, and a variety of extrahepatic chronic diseases. Therefore, the early diagnosis and early therapy of fatty liver are important. Except for invasive liver biopsy, there is still a lack of reliable diagnosis and staging methods. Extracellular vesicles are small double-layer lipid membrane vesicles derived from most types of cells. They play an important role in intercellular communication and participate in the occurrence and development of many diseases. Since extracellular vesicles can carry a variety of biologically active substances after they are released by cells, they have received widespread attention. The occurrence and development of fatty liver are also closely related to extracellular vesicles. In addition, extracellular vesicles are expected to provide a new direction for the diagnosis of fatty liver. This article reviews the relationship between extracellular vesicles and fatty liver, laying a theoretical foundation for the development of new strategies for the diagnosis and therapy of fatty liver.

Keywords

Extracellular vesicles · Fatty liver · Alcoholic liver disease · Non-alcoholic fatty liver disease · Exosome

9.1 Introduction

9.1.1 Fatty Liver

Fatty liver is a clinicopathological syndrome characterized by liver steatosis caused by excessive deposition of lipid (mainly triglycerides) and diffuse fatty infiltration in hepatocytes caused by many different causes. With the improvement of living standards and changes in lifestyle of people, the incidence of fatty liver is increasing year by year, and the adult prevalence is about 15%–25% [1–3]. The incidence trends of different races and ages are quite different, with the highest incidence in 40–49 years old. In recent years, the age of onset of fatty liver has shown a younger

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trend [4, 5]. Fatty liver may progress to liver cirrhosis or even hepatocellular carcinoma. At present, fatty liver have become the second largest chronic liver disease after viral hepatitis, seriously endangering people's health all over the world [6].

As one of the most important material metabolism and biotransformation organs in the body, the liver plays an important role in maintaining the balance of lipid metabolism. It is the central organ of triglyceride and fatty acid metabolism and an important place for lipid synthesis, transport, and decomposition. Excessive accumulation of triglycerides in liver caused by excessive fat intake or other factors is the main pathophysiological basis for fatty liver. The proportion of the lipid in liver is 3%–4% under normal circumstances. When the triglyceride content exceeds 5% of the liver weight or liver puncture pathology tests show that >1/3 of the hepatocyte per unit area has steatosis, which can be diagnosed as fatty liver [7, 8].

According to the history of long-term excessive drinking, fatty liver is clinically divided into non-alcoholic fatty liver disease (NAFLD) and alcoholic fatty liver disease (ALD) [9]. Among them, NAFLD refers to the clinical pathological syndrome caused by the exclusion of alcohol and other liver damage factors. The pathological changes of NAFLD are mainly characterized by diffuse hepatocyte bullous fat. According to the degree of liver steatosis, inflammation, and liver fibrosis, NAFLD is divided into simple hepatic steatosis, non-alcoholic steatohepatitis (NASH), fatty liver fibrosis, and fatty liver cirrhosis.

Currently, lifestyle intervention is still the preferred therapy for fatty liver, including alcohol withdrawal, healthy exercise, and balanced diet. Clinical studies have shown that exercise can effectively prevent the occurrence and development of NAFLD [10–12]. However, clinically, some patients cannot adhere to diet or cannot tolerate exercise training, which makes NAFLD poorly controlled. In addition, the drug treatment of NAFLD has greater limitations [3, 10, 13–17]. For example, long-term use of fibrates and statins may cause liver toxicity, and some patients will experience asymptomatic increases in serum ALT; hypoglycemic drugs such as rosiglitazone and pioglitazone are prone to water and sodium retention, weight gain, or other adverse reactions [18–20]. Therefore, actively exploring the pathogenesis of fatty liver and new therapy options has important research significance.

9.1.2 Extracellular Vesicles

Extracellular vesicle (EV) is a lipid-containing bilayer membrane vesicle secreted into the extracellular matrix by different cells. It contains a group of biologically active substances derived from maternal cells including proteins, DNA, RNA (mRNAs, miRNAs, and other non-coding RNAs), lipids, metabolites [21–23]. EV is a kind of nano-sized vesicles widely distributed in various body fluids, including blood, urine, saliva, bile, amniotic fluid, ascites, bronchoalveolar lavage fluid, synovial fluid, breast milk, and cerebrospinal fluid [24, 25]. According to the formation mechanism and diameter of EVs, it can be divided into three subgroups: exosome, extracellular microvesicle, and apoptotic body [26]. Exosomes are vesicles with a double-layer membrane structure of 30 nm–100 nm in diameter, which are formed by multivesicular bodies (MVB) produced by the inward budding of cells [27]. Extracellular microvesicles are directly produced by the cell membrane, usually with a diameter of 100 nm–1000 nm. As for the apoptotic body, the apoptotic bodies are formed and released by vesicles from the cell membrane during the process of cell apoptosis, generally with a diameter of 1000 nm–5000 nm [26].

The main biological function of EVs is to act as a carrier for cell-to-cell signal transduction and material exchange, and the different substances delivered through it participate in the regulation of the pathophysiological functions of target cells, such as protein expression, cell proliferation and differentiation, and immune response [24, 28, 29]. Therefore, EVs play an important role in the occurrence and development of many different diseases. All EVs have surface molecules that enable them to target and bind to recipient cells. Once EV is attached to the target cell, it can

transmit its content to the cytoplasm of the target cell through receptor–ligand interaction, direct fusion with the cell membrane of the target cell, or induce signal transduction through phagocytosis and/or endocytosis, thereby changing the physiological state of the recipient cells [26, 30]. As of July 2017, the EVpedia website (http://evpedia.in-fo) has identified 439,606 proteins, 92,050 mRNAs, 57,592 miRNAs, and 3156 lipids related to EV.

Since EV contains a variety of specific proteins, active nucleic acids, and lipids, it is expected to become a new type of marker for revealing the mechanism of disease and its application in the field of clinical diagnosis and therapy [31]. In addition, the number and composition of EVs can reflect the physiological and pathological process of the body and can be used for disease diagnosis, disease evaluation, and efficacy monitoring. At the same time, because they have the function of delivering biomolecules, they have the potential to develop into clinical drug delivery vehicles [32]. As a rising star of "liquid biopsy," EV abundance is much higher than circulating tumor cells and protected by its double-layer membrane structure, its content is stable, and it overcomes the disadvantages of easy degradation of ctDNA in body fluid samples [33, 34]. Compared with histopathological examination, EV detection based on blood and other samples has the advantages of non-invasive, simple sampling, higher patient compliance, easy disease monitoring, and easy adjustment of therapy plans in time; compared with traditional serum free nucleic acid and protein marker detection, EV detection has the advantages of significant targeting, richer information, and less interference from the detection matrix [33, 35].

More and more studies have confirmed that EVs, especially exosomes, play an important role in cell-to-cell communication. In 1981, Trams et al. first observed nano-sized vesicles in the supernatant of tumor cell culture and named them exosomes [36]. Subsequent studies have confirmed that, except for tumor cells, almost all animal cells can produce EVs, such as blood cells, neurons, astrocytes, dendritic cells, and hepatocytes [37–39]. Recent studies have shown that EV is involved in the occurrence and development of hepatocellular carcinoma, viral hepatitis, liver fibrosis, alcoholic and non-alcoholic fatty liver, and other diseases. It is a potential biomarker for liver disease diagnosis and a new target for liver disease therapy. The number of EVs secreted and the composition of their contents can reflect the pathophysiological state of maternal cells, including liver cells, bile duct epithelial cells, hepatic stellate cells (HSC), natural killer T lymphocytes (NKT), monocyte macrophages, liver sinus endothelial cells, and so on. These maternal cells secrete EVs, which deliver loaded proteins, lipids, and various nucleic acids to target cells through bile, lymphatics, and blood circulation. EVs from other different cell sources in the circulation can also transmit information to the liver at the same time and participate in the occurrence and development of a variety of liver diseases [40]. EVs from different cell sources have different functions and can participate in regulating the proliferation of hepatocytes and bile duct cells, liver detoxification, and immune tolerance [41–44]. In the process of liver fibrosis, EVs derived from sinusoidal endothelial cells can enhance the migration ability of HSC in liver fibrosis through adhesion and participate in liver fibrous tissue deposition [45]. In the pathogenesis of NAFLD, exosomes participate in signal transduction between hepatocytes and inflammatory cells, affecting the activity of the mononuclear macrophage system, thereby regulating the liver's inflammatory response [46, 47]. The main purpose of this review is to summarize the research frontiers of EV in fatty liver.

9.2 Role of Extracellular Vesicles in ALD

Alcoholic liver disease (ALD) is one of the chronic liver diseases caused by long-term excessive drinking and is one of the main manifestations of chronic alcoholism. With the continuous improvement of living standards, the incidence of ALD is increasing year by year in

the world [48]. ALD can manifest as a series of pathological changes, including liver steatosis, hepatitis, liver fibrosis, and liver cirrhosis, and even lead to the occurrence of hepatocellular carcinoma [49]. According to previous studies, 90%–95% of alcoholics suffer from alcoholic fatty liver with 10%–35% of alcoholic fatty liver patients developing alcoholic hepatitis. Among them, 35%–40% of alcoholic hepatitis patients will develop into liver fibrosis and liver cirrhosis, and a small number of malignant changes develop into liver cancer [50, 51].

The liver is the main organ for alcohol metabolism and degradation. Long-term excessive drinking will increase the burden on the liver to metabolize alcohol. When the accumulation of alcohol in the liver exceeds metabolic capacity of the liver, alcohol will directly influence the liver function [52, 53]. The main indicator of histopathological manifestations of ALD is triglycerides. When triglycerides, phosphate esters, and cholesterol accumulate in the cytoplasm, it will cause abnormal lipid metabolism and fatty degeneration of hepatocyte. After absorbed into the circulating blood in the gastrointestinal tract, the alcohol is transported into the liver, where it is converted into acetaldehyde and then into acetic acid under the action of alcohol dehydrogenase and microsomal alcohol oxidase. In this process, NAD is converted to NADH, which leads to an increase in the NADH/NAD ratio and inhibits the tricarboxylic acid cycle in the mitochondria of liver cells, inducing the reduction of the ability of hepatocyte to oxidize fatty acids and causing abnormal accumulation of lipid in the liver. During the metabolism of alcohol in liver, a large amount of reactive oxygen species (ROS) is produced. When the production of ROS exceeds the body's metabolic capacity, it will lead to an imbalance in the production and elimination of ROS in the body, thereby inducing oxidative stress. Oxidative stress interferes with the expression of important lipid transcription factors in the lipid metabolism pathway in mitochondria and at the same time promotes the expression of inflammatory cytokines, which in turn causes abnormal lipid metabolism and lipid peroxidation, inhibits fatty acid oxidation, and promotes other fatty substances accumulate in hepatocytes, like triglycerides, thereby mediating ALD. ROS can also damage the bio-membrane system. The intermediate product of alcohol, acetaldehyde, which has strong lipid peroxidation and toxicity, can destroy the structure of hepatocyte and induce immune responses.

Due to the complex pathogenesis of ALD, there is currently no effective clinical therapy and drugs [54]. In-depth exploration of the pathogenesis of ALD has important scientific research significance and clinical application prospects.

The continuous overactivation of liver macrophages (Kupffer cells) plays an essential role in the occurrence and development of ALD [55]. Using macrophages as a therapy target to inhibit the excessive inflammation of liver in ALD is considered to be an important research direction for ALD treatment [56]. In addition to traditional immune cells and inflammatory factors, various protein molecules and miRNA molecules derived from EVs are also important factors involved in the occurrence and development of ALD.

At present, it has been proposed that both hepatocyte-derived and monocyte-derived exosomes regulate the phenotype of Kupffer cells, leading to the inflammatory response of ALD. Monocytes exposed to ethanol release large amounts of exosomes, which stimulate primitive monocytes to polarize and differentiate into M2 macrophages. Monocytes exposed to alcohol also secrete exosomes containing increased levels of miR-27a, which promotes increased secretion of cytokines, leading to the activation and polarization of other monocytes [57, 58]. CD40 ligand carried by exosomes promotes macrophage activation in the experimental model of ALD [59]. These findings indicate that the level of exosomes secreted by cells increases in disease states and triggers various pathological events.

Based on the effect of intestinal endotoxin in activating macrophages in the liver [60], the use of intestinal antibiotics and probiotics also has a certain therapeutic effect on ALD [61, 62]. Recent studies have shown that exosomes can directly activate liver macrophages in ALD, which is an

important factor in the excessive activation of macrophages [63, 64]. However, the regulation mechanism of alcohol-induced liver exosomes is still unclear. Previous study reported by Saha found that ethanol can stimulate human monocytes to release exosomes rich in miRNA-27a, differentiate primitive monocytes into M2 macrophages, promote the secretion of inflammatory factors, and increase the phagocytosis of macrophages [45]. Another study showed that ethanol stimulates hepatocytes to secrete CD40 ligand-containing exosomes by activating a caspase 3-dependent pathway, promotes the activation of Kupffer cells, and aggravates liver inflammation [59]. Ibrahim et al. found that palmitic acid or lysophosphatidylcholine can activate the mixed lineage kinase 3 of hepatocytes to release exosomes containing the chemokine CXCL10, regulate the activation of Kupffer cells, and accelerate liver damage [65]. Another study showed that lipids can activate the death receptor 5 of hepatocytes, prompting hepatocytes to release exosomes, which in turn activates the inflammatory phenotype of macrophages [66–68]. In addition, lipids can stimulate liver cells to release ceramide-containing exosomes through the endoplasmic reticulum transmembrane kinase 1d pathway and then regulate the chemotaxis of macrophages through S1P-dependent pathways, recruit a large number of Kupffer cells, and aggravate Inflammation of the liver [69].

9.3 Role of Extracellular Vesicles in NAFLD

NAFLD is a metabolic stress liver injury closely related to insulin resistance and genetic susceptibility. It is manifested as an imbalance of metabolism in the body and excessive accumulation of fat in liver [70]. With changes in diet and exercise patterns, obesity is prevalent worldwide, and the incidence of NAFLD is also increasing. The global prevalence of NAFLD is about 25.24%, and it is on the rise [71]. NAFLD is more common in Western countries, with an adult prevalence rate of 10%–30% and an incidence rate of 57%–74% in obese people [72, 73]. In China, the prevalence rate is about 29.81%, which is similar to European countries and American [74]. In the USA, it is estimated that by 2030, NAFLD, NASH, and related hepatocellular carcinoma patients will increase by 21%, 63%, and 137%, respectively [75]. Based on global epidemiological research data, NAFLD is becoming one of the main causes of chronic liver disease, a major global health problem and economic burden [5, 71, 76, 77].

NAFLD is a chronic liver disease caused by a variety of factors such as genetic factors, dietary structure, living habits, metabolic disorders, and so on. Simple hepatic steatosis can develop into steatohepatitis, fatty liver cirrhosis, and hepatocellular carcinoma [78, 79]. In addition, NAFLD also affects the progress of other chronic diseases. It is one of the high-risk factors for cardiovascular and cerebrovascular diseases such as hypertension and diabetes and seriously endangers human health [74, 80].

The pathogenesis of NAFLD is very complicated and has not yet been fully clarified. The "two-hit" hypothesis put forward by Day and James in 1998 has been widely recognized by academia. The "two-hit" hypothesis believes that insulin resistance and abnormal liver lipid deposition caused by various factors are the "first hit," leading to simple hepatic steatosis; then, the "second hit" is mainly caused by oxidative stress and lipid peroxidation, which further promoted the development of fatty liver [81]. During the occurrence and development of NAFLD, insulin resistance enhances the lipolysis activity of adipose tissue, promotes the increase of free fatty acids transported to the liver in serum, and induces the upregulation of the sterol regulatory element-binding protein (SREBP1c) expression. The interaction of these two factors together leads to the deposition of triglycerides in the liver, resulting in simple hepatic steatosis [82]. When the liver is overloaded with triglycerides, the liver undergoes fat mobilization. Fatty acid oxidation produces a large number of highly active molecules, mainly ROS, which induce the occurrence of endoplasmic reticulum stress, and further activates related downstream signaling pathways and causes hepatocyte death and liver damage

[83]. In addition, ROS, including oxygen free radicals (H_2O_2, O_2-), hydroxyl free radicals (OH-) and the derivatives, stimulate hepatocytes to produce inflammasomes, leading to local inflammation in liver lobules and steatohepatitis and then developing into fatty liver cirrhosis, hepatocellular carcinoma [84, 85]. The "two-hit" hypothesis has been recognized by the academic community for a long time. However, with the in-depth study of NAFLD, the "two-hit" hypothesis cannot fully clarify the pathogenesis of NAFLD. Some researchers have found that many pathological changes in the NAFLD process actually occur simultaneously. Therefore, the "multiple hits" hypothesis has gradually become the mainstream view of the pathogenesis of NAFLD [86]. The "multiple hits" hypothesis believes that a variety of factors (including insulin resistance, intestinal mucosal barrier dysfunction, hormone secretion, genetic factors, etc.) are jointly involved in the occurrence and development of NAFLD.

9.3.1 Extracellular Vesicles in Pathophysiology of NAFLD

NAFLD is characterized by hepatocyte dysfunction and death induced by lipid and Kupffer cells-related inflammation. Previous animal model studies have found that inflammation, angiogenesis, and fibrosis are involved in the development of NAFLD. Among them, EVs derived from damaged hepatocytes play an essential role in these biological processes by activating non-substantial cells (such as endothelial cells, HSCs, Kupffer cells) [87, 88].

A large number of studies have shown that EVs play an essential role in the inflammatory response of NAFLD. The mechanism of NASH is very complicated. The damage and death of liver cells induced by lipids and macrophages are the characteristic manifestations of NASH [89]. On the one hand, lipotoxicity injuries hepatocytes which release EVs and promote the activation of Kupffer cells in the liver, thereby aggravating inflammation and fibrosis; on the other hand, EVs derived from fat cells may enter the liver to regulate and metabolize, inflammation and fiber Chemical-related signal pathways, participating in the formation of NASH [26].

EV participates in the innate immune response with steatosis, indicating that EV plays a role in the progression from simple hepatic steatosis to NASH, and may stimulate inflammatory response and cause the deterioration of tissue damage. The levels of EVs derived from Kupffer cells and NKT cells secreted increase in NAFLD patients [90]. In patients with NAFLD, a significant increase in circulating tail vesicles derived from macrophages and NKT cells can be observed [90]. In the NAFLD mouse model, the expression levels of various proteins in microvesicles increased significantly, and the protein expression patterns between exosomes and microvesicles are totally different [91]. Hepatocyte-derived EVs can be detected in the blood of diet-induced NASH mice, and the number of EVs is related to the severity of the disease [87]. The study by Heinrich et al. found that the number of EVs significantly increased in the blood of rats fed by a high-fat diet; EVs derived from plasma can significantly stimulate the production of ROS and induce vascular cell adhesion molecules in primary rat vascular endothelial cells 1 (VCAM1) expression, promote the occurrence of inflammation [92]. Previous studies have shown that lipid deposition induces the release of EVs from primary hepatocytes and activates the inflammatory response of macrophages to participate in the pathogenesis of NAFLD. Death receptor 5 plays an important role in the occurrence of NAFLD as well. It can induce hepatocytes to release EVs containing tumor necrosis factor-like apoptosis-inducing ligand and then activate macrophages through a death receptor 5-dependent process [93]. In addition, EVs rich in ceramide can act as macrophages during NAFLD [69].

When the inflammatory response in the liver persists, NAFLD will continue to progress and then liver fibrosis will occur. Previous studies have shown that EVs also mediate the paracrine pathway between endothelial cells with HSC during liver fibrosis, which EVs derived from endothelial cells can regulate HSC migration [94, 95]. HSC activation is an important sign of

liver fibrosis, and the migration and proliferation ability of HSC after activation are significantly enhanced [96]. In this process, the binding of EVs-bound fibronectin to αVb1 integrin on target cells mediates the adhesion of EVs and promotes the endocytosis of EVs into target cells through the initiation of the protein [97]. Wang et al. found that resting HSC can produce EVs containing Twist-related proteins, drive the expression of miR-214, and thereby reduce the level of connective tissue growth factor [98, 99]. Twist-related proteins can communicate between activated HSCs and resting HSCs [100, 101]. EVs released by damaged liver cells can also be endocytosed by HSC and participate in complex cell-to-cell communication. The miRNA-128-3p carried by EVs inhibits proliferation-activated receptor-γ (PPAR-γ), activates HSC cells, and leads to liver fibrosis [88, 102]. In addition, EVs derived from hepatocytes can regulate the expression of miR-214 in HSC. The adipose tissue-derived EVs of obese patients can increase the expression of matrix metalloproteinase inhibitor-1 (TIMP-1) in hepatocytes and HSC and promote the occurrence of liver fibrosis [94].

9.3.2 Diagnostic Application of Extracellular Vesicles in NAFLD

Previous studies have shown that the secretion of EVs in the circulation of NAFLD patients is significantly higher than that of healthy volunteers, and the nucleic acids and proteins carried by them can be used as specific diagnostic biomarkers of NAFLD [103–106]. In addition, some novel biomarkers compared with traditional diagnostic biomarkers, some biomarkers can change in the early stage of the disease.

Kornek et al. established the correlation between the abundance of immune cell-derived EVs and disease severity, liver aminotransferase levels, biopsy grades, and NAFLD activity scores for the first time in human subjects. The quantification of immune cell particles from serum is used to evaluate the degree and characteristics of liver inflammation in patients with chronic liver disease [90]. The study found for the first time that the number of $CD14^+$ monocyte macrophages and $Valpha24/Vbeta11^+$ NKT cell-derived EVs in NAFLD patients increased significantly, while circulating EVs in patients with chronic hepatitis C were mainly derived from $CD4^+CD8^+$ T cells [90]. The area under curve (AUC) of receiver operating characteristic (ROC) curve for the diagnosis of NAFLD with increased secretion of $CD14^+$ and NKT cell-derived EVs is 0.99 and 0.97, respectively, which is helpful for distinguishing from normal controls or other liver diseases (such as hepatitis C). Another transcriptomics analysis can distinguish NAFLD from CHC by detecting miRNA in EV and revealed that miRNA can be used to distinguish the grade and stage of liver disease [107]. The expression of miRNA122 and miRNA192 in EVs was significantly upregulated in the NASH animal model induced by the choline-deficient-L amino acid (CDAA) diet [91]. In the mouse high-fat diet (HDF)-induced NAFLD model, the secretion of EVs derived from $CD26p^+$ platelets and $CD144^+$ endothelial cells increases, and atorvastatin intervention can reduce the release of EVs [108]. In addition, CXCL10, ceramide, and S1P in EVs all contribute to the diagnosis of NAFLD [65, 69].

Animal and cell models have confirmed that the number of EVs secreted increases with the progression of the disease and is time-dependent. Therefore, EVs can be used not only as a potential biomarker for the diagnosis of NAFLD but also as a tool for monitoring the progression of NAFLD and the severity of histopathology [66, 91]. Povero et al. used CDAA diet to induce NAFLD models in mice for 4 weeks, 8 weeks, and 20 weeks, respectively, and the study has shown that the number of circulating EVs begins to increase at the early stage of disease progression, especially after 8 weeks, showing a time-dependent increase, which is related to hepatocyte apoptosis, fibrosis, and pathological vascular proliferation [91]. Further proteomic analysis of EVs showed that most of the proteins were only present in circulating EVs of mice fed the CDAA diet for 20 weeks, and GO analysis showed that a large

number of proteins involved in inflammation and immunity, cell death, fibrosis, angiogenesis, and cell adhesion were related to NAFLD and NASH occurrence. In addition, previous studies have shown that the increase in circulating EVs in NAFLD patients is associated with alanine aminotransferase (ALT) levels and liver pathological grades [90, 107].

The in vitro experimental study by Povero et al. showed that in the NAFLD mice model constructed using CDAA and a high-fat diet, compared with the control group, the number of EVs in the blood circulation increased in the NAFLD group, and proteomics analysis reveals the difference in EV protein components between the NAFLD group and the control group. The liver is the main source of miR-122 [109]. The level of miR-122 carried by EV in the blood circulation of NAFLD group was significantly increased, and it was proved that miR-122 in NAFLD was mainly encapsulated in EV, suggesting that hepatocyte-specific miRNA was released by EV in blood circulation during the progression of NAFLD [91]. In addition, Csak et al. found that an increase in the level of miR-122 in EVs in the blood circulation was accompanied by a decrease in the level of miR-122 in the liver [110]. The decreased level of miR-122 in the liver was conducive to the upregulation of tissue remodeling regulators and was also associated with liver fibrosis in NASH. Plays an important role in [110]. Yamada et al. used a rat model of NAFLD to observe the level of miR-122 in the blood circulation and found that serum miR-122 levels can indeed be used to assess early NAFLD and may be superior to traditional biomarkers for detecting liver conditions [111]. These animal studies laid the foundation for understanding the role of EV in NAFLD.

9.4 Perspective

EVs are important carriers of signal transduction and material exchange between cells. A large number of studies have revealed the role of EVs in fatty liver and are involved in the occurrence and development of fatty liver. Some EVs have specificity and can be used as biomarkers for clinical diagnosis, disease evaluation, and efficacy testing of fatty liver. Some exosomes can become carriers for drug delivery, so as to achieve specific targeted therapy of fatty liver, which provides a new direction for fatty liver associated research. In addition, EVs also play an important role in the diagnosis of fatty liver. The diagnostic efficacy of specific nucleic acids or proteins in EVs still needs to be verified by large sample clinical studies. In terms of treatment, the large-scale preparation, separation, purification, and preservation methods of EVs are still immature. The mechanism of EVs targeting cells is still unclear, so the communication between cells mediated by EVs is still the focus of future research.

In conclusion, EVs have great potential in the diagnosis, therapy, and prognosis of fatty liver and provide new ideas for the further diagnosis and therapy of fatty liver. It is expected that with the advancement of technology, EVs can achieve clinical transformation as soon as possible and provide a new direction for the diagnosis and therapy of fatty liver.

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Extracellular Vesicles and Obesity

10

Kai Wang and Cong Zeng

Abstract

Extracellular vesicles (EVs) are a group of vesicles with membrane structure released by cells, including exosomes, microvesicles, apoptotic bodies, and oncosomes. EVs are now recognized as important tools of cell-to-cell communication, allowing cells to exchange proteins, lipids, and genetic material to participate in physiological and pathological processes. It has been reported that EVs regulate host–pathogen interactions and participate in pathological processes of infectious disease, neurological diseases, cancer, cardiovascular diseases, etc., it also plays an important role in the process of growth and development. EVs have a bright future in clinical application. They can be used to monitor clinical status, therapeutic effect, and disease progression. At the same time, EVs have the potential to be developed as clinical drug delivery vectors due to their ability to deliver biomolecules. However, it is still unclear whether EVs are reliable and useful markers for the diagnosis or early detection of obesity, and whether they can be used as drug vectors for the treatment of obesity. In this review, we summarize the research progress of EVs and obesity. It is hoped that EVs may become a new target in the diagnosis and treatment of obesity.

Keywords

Extracellular vesicles · Obesity · Target

10.1 Introduction

Extracellular vesicles (EVs) are circulating nanoparticles derived from cells and contain cytoplasmic components such as proteins, mRNAs, microRNAs, and DNAs that bind to and modify local and remote cellular targets [1, 2]. According to their biosynthesis or release pathways, EVs can be classified as exosomes (30–150 nm, originated from the endocytic pathway), microvesicles (100 nm–1 μm, released directly from the plasma membrane), apoptotic bodies (1–5 μm, produced by apoptosis), and oncosomes (1–10 μm, observed in cancer cells) [2–4] (Fig. 10.1). For a long time, the main goal of cell biology has been to understand the importance of intracellular vesicles to the organization and function of eukaryotic cells. However, the biological study of EVs has been largely ignored. Our understanding of EVs has advanced considerably since the late 1960s, and we now know that most cell types can release vesicles originating from distinct subcellular membrane compartments into the interstitial space [5]. Secretion of EVs was originally thought to be a method of removing unwanted

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compounds from cells [6]. Until recently, EVs have been found to regulate host–pathogen interactions and participate in infectious disease, neurological diseases, cancer, cardiovascular diseases, and other diseases, and it is crucial to the development and function of organism in the whole process of evolution [7–10]. EVs have the ability to alter the activity or properties of specific target cells and tissues. Moreover, they have been proved to stimulate or inhibit target cells by inducing cell signaling or by inducing genetic or epigenetic changes [11, 12]. Nowadays, EVs have aroused wide interest in multiple biomedical fields as a new disease diagnosis and treatment strategy [13]. EVs are now emerging as new regulators of intercellular communication. They can mediate local communication between cells of the same origin, namely endothelial, liver, immune, or pancreatic cells, and cross organ systems by release into peripheral blood (28878126). Therefore, EVs play an important role in the prognosis and diagnosis of metabolic diseases [14, 15].

Normal weight is defined as a body mass index (BMI) of 15–24.9 kg/m^2, overweight as 25–29.9 kg/m^2, obesity as $\geq$30 kg/m^2, and extreme obesity as $\geq$40 kg/m^2 [16, 17]. Obesity is a complex disease with multiple etiologies. It is currently believed that obesity is caused by excessive food intake or changes in the body's metabolism, which leads to increased energy storage in the form of fat in multiple organs [17, 18]. Although obesity was officially recognized as a disease long ago, it has not received the attention it deserves in the medical community [19]. Obesity is currently a serious health problem, which not only increases the cost of medical care for patients, but also causes a great physical and social psychological burden [20]. Since 1970, rates of obesity in developed countries have risen sharply [20]. It is estimated that 39.6% of adults and 18.5% of children and adolescents in the USA suffer from obesity [21]. Now the incidence of obesity is also increasing rapidly in developing countries. Excess adipose tissue increases the workload of an organ and causes changes in its anatomy. It can adversely affect health by altering heart, lung, endocrine, and immune functions. Obesity is related to the occurrence and development of numerous diseases, such as hypertension, dyslipidemia, insulin resistance, stroke, diabetes, fatty liver, coronary heart disease, and cancer [22, 23]. Current therapies mainly focus on suppressing calorie intake and increasing exercise, but these methods are not sufficiently effective. Therefore, the clinical management of obesity is currently facing huge challenges, and it is of great importance to explore new targets for early detection and treatment of obesity.

An increasing number of studies have shown that EVs have become a key participant in obesity, with several molecules described as EVs cargo altered [17, 24]. However, it remains unclear whether EVs are reliable and useful markers for the diagnosis or early detection of obesity. At the same time, it is also necessary to clarify whether EVs can be a reliable carrier for drug treatment of obesity. In this review, we summarize the research progress of EVs and obesity, focusing on their value as biomarkers and drug carriers.

10.2 Extracellular Vesicles

EVs are nano-scale, lipid membrane-separated particles released by almost all cells in the world of life. They play an important role in both normal physiology and disease pathology [25]. The first observation of EVs and their relevance occurred simultaneously in various physiological environments, without realizing that this form of function or communication is a universally shared cell biology characteristic. Specifically, EVs were first discovered by Chargaff and West as accelerating platelet-derived particles in normal plasma, and reported in 1946 [26], and then called "platelet dust" by Wolf in 1967 [27]. In the subsequent decades, the understanding of EVs is increasing, but their important role in cell biology has not been recognized. Until 2006–2007, with the discovery that EVs contain RNA (including microRNA), they began to receive great attention as a medium of communication between cells [28, 29].

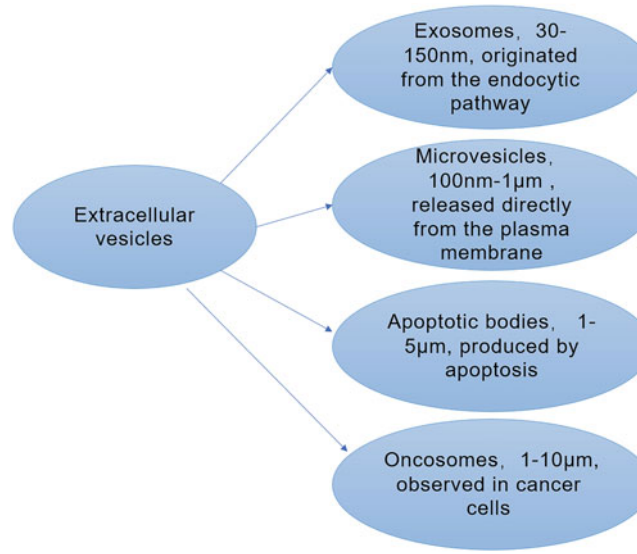

Fig. 10.1 Classification of extracellular vesicles

EVs are currently classified mainly by size. For example, exosomes are defined as having a diameter of less than 150 nm, while microvesicles are defined as having a diameter of up to 1000 nm. With the continuous deepening of research, the current consensus of experts has recognized that understanding the biological origin and molecular content of different sizes of EVs may be relevant [30]. Microvesicles seem to originate directly from the plasma membrane, forming outward buds at specific points in the membrane, then fission and subsequent release of vesicles outside the cell. In contrast, exosomes are generally thought to derive from the endosomal compartment [31]. Apoptotic bodies are formed during apoptotic cell death or programmed cell death (a planned and highly regulated mechanism) [32, 33]. Oncosomes, a relatively new category of EVs, mainly originate from the plasma membrane shed by aggressive cancer cells [31, 33]. In normal circulation, a large number of EVs may originate from platelets or megakaryocytes, although most cells are thought to release EVs [34]. In addition, EVs also exist in many different biological fluids, such as breast milk, saliva, urine, cerebrospinal fluid, nasal and bronchial lavage fluid, plasma, serum, and semen [4, 35]. From the perspective of function, EVs are considered to be transmitters of genetic information and involved in the occurrence and development of various diseases as they contain proteins, metabolites (including lipids), and nucleic acids (such as RNA) that reflect the origin and function of cells [1, 5, 36]. With the rapid development of high-throughput transcriptome, proteomics, and lipidomics technology, the molecular composition of EVs has been analyzed and identified in greater depth. Multiple comprehensive and influential databases including Vesiclepedia, ExoCarta, and EVpedia have been established in order to fully integrate and utilize the high-throughput data of EVs research, greatly contributing to research progress in this field [13].

The regulation of target cells by EVs involves a series of extremely complex regulation processes. Interactions between different types of recipient cells and EVs vary greatly, possibly depending on the specific characteristics of the recipient cells [13]. EVs transmit genetic information to recipient cells through three main processes: endocytosis, receptor–ligand interaction, and direct fusion with the plasma membrane [13]. Multiple studies have reported that EVs play an important role in cancer, muscular dystrophy, cardiovascular disease, regenerative medicine, and neurodegenerative diseases through these strategies [37–40]. Over the past few

decades, the application of drug delivery systems has been limited due to low efficiency, cytotoxicity, and/or immunogenicity. At the same time, traditional synthetic delivery systems face difficulties in effectively crossing biological barriers, including tissue barriers, cellular barriers, and intracellular barriers. Therefore, in recent years, the field of natural drug carrier systems has developed rapidly. One of the most preferred options for this natural vector is EVs, which can effectively cross biological barriers and induce functional changes in target cells [41, 42]. The observation that EVs can transfer RNA and protein cargo to recipient cells has greatly stimulated translational research, focusing on EVs as an effective vector for therapeutic delivery [43]. In addition, studies have shown that ligands on the surface of EVs can bind to cell receptors to activate cytoplasmic signaling pathways. In view of their importance in intercellular signaling and intercellular communication, there is growing interest in their potential role as non-invasive biomarkers for disease detection and prognosis. EVs have been increasingly recognized as functional biomarkers for a variety of diseases [4].

10.3 Obesity

Current statistics show that the epidemic trend of obesity is not optimistic. More than two billion people worldwide are overweight, accounting for about 30% of the world's population, of which more than 603.7 million adults have reached obesity [23, 44]. Since 1980, the prevalence of obesity in most countries around the world has doubled and continues to rise [44].

Obesity is the result of increased fat deposition due to the imbalance between calorie intake and energy expenditure [45]. Previously, obesity was thought to be caused mainly by low physical activity (a sedentary lifestyle) or excessive consumption of high-energy foods. However, the etiology of obesity is actually quite complex [46] and requires consideration of a complex interplay of behavioral, environmental, physiological, genetic, social, and economic factors, all of which affect food intake, nutrient turnover, thermogenesis, lipid utilization of fatty acids, and fat storage [45, 47]. Adipose tissue occupies an important position in the formation of obesity. There are two morphologically different types of adipose tissue: white adipose tissue (WAT) and brown/beige adipose tissue (BAT). The evolution of multicellular organisms is also accompanied by the development of specific cells or organs whose function is to store excess nutrients as lipids. Efficient storage during periods of food abundance is the basis for survival during periods of food scarcity. WAT plays an important role in this process, strictly regulating energy homeostasis by acting as a critical energy reservoir for other organs. High-calorie food intake and low-cost activities shift the energy balance into storage mode. In adipocytes, free fatty acids (FFA) are esterified into triglycerides, which are then encapsulated in lipid droplets containing regulatory proteins to ensure lipid storage or mobilization [48, 49]. There is significant heterogeneity among obese individuals. In the human body, WAT is organized into two main fat depots: subcutaneous fat depot and visceral fat depot. Correspondingly, in obesity, excessive subcutaneous fat found around the buttocks and thighs is commonly known as subcutaneous obesity, which is most common in women. Visceral obesity, however, refers to the concentration of fat in the abdominal organ area. Visceral obesity is more common in men and tends to be more harmful to health, particularly affecting cardiovascular risk [45]. Compared with WAT, BAT has a smaller cell volume and is rich in mitochondria and uncoupling protein 1 (UCP1), which is mainly found subcutically in specific parts of the newborn, but less in adults [50]. BAT not only protects against cold, but also burns excess fat and sugar, generates heat, and prevents excess fat from being stored in the body.

Studies have suggested a possible link between obesity and death. The mortality rate increases when BMI is lower than 18.5 kg/m^2 and BMI is 30 kg/m^2 or higher. However, the mortality rate is lowest when the BMI is about 25 kg/m^2 [20]. Obese individuals are also more prone to a variety of health complications that

lead to premature death [51]. Common complications include type 2 diabetes, sleep apnea, cardiovascular disease, cancer, and immune impairment [22, 23, 44]. In addition, obesity can negatively affect not only psychological and emotional problems, but also cognitive function. Exercise has long been considered one of the best ways to treat a variety of diseases, including obesity [52]. Moderate exercise can enhance immune surveillance, which plays a key role in the development of some chronic diseases [53]. However, the treatment of obesity is a process of comprehensive management. Relying solely on strengthening exercises is sometimes difficult to achieve good results. Pharmacotherapy for obesity may be considered for patients who have not achieved weight loss goals through lifestyle changes alone [54]. Lack of efficient, safe, and precise drug carriers is one of the main factors limiting drug therapy. With the application of nanotechnology in the medical field, it is expected to provide better, more effective, and more targeted treatments for obese patients [55]. The appropriate drug carrier has also aroused great interest among researchers. EVs have been widely recognized as the most promising natural drug carriers. Meanwhile, in view of the limitations of BMI in the diagnosis of obesity, the development of biomarkers for early detection of obesity, especially visceral obesity, is essential for the prevention and treatment of obesity and its complications.

10.4 Extracellular Vesicles in Obesity

As an alternative to paracrine communication, EVs have emerged as key players in metabolic disorders. EVs play an important role in the development of obesity by serving as a mode of intercellular communication between adipocytes, skeletal muscle cells, hepatocytes, and immune cells [2, 15]. Plasma levels of EVs often correlate with disease severity, conferring potential value on EVs as biomarkers. Studies have shown that obesity is associated with an increase in the number of EVs, especially those secreted by adipose tissue. There is strong evidence for the presence of adipose tissue-secreted EVs at circulating levels [56, 57]. Stepanian et al. reported higher numbers of plasma EVs in severely obese women compared with normal-weight controls [58]. In a mouse obesity model, the production of circulating EVs is increased during obesity. The authors describe perilipinA, detectable at circulating levels by immunoblotting, as a biomarker for adipose tissue EVs. It was conclusively demonstrated that circulating EVs-perilipinA is increased in obesity in mice and humans and is also associated with insulin resistance. Furthermore, circulating EVs-perilipinA amounts were significantly reduced after caloric restriction [57]. Amosse et al. found that plasma microvesicles and exosomes increased significantly with increasing BMI in patients with metabolic syndrome [59, 60]. Lwitczak and collaborators focused on adipocyte markers (adiponectin, FABP4, and PPARγ) in obese patients before and after bariatric surgery and concluded that alterations in adipose tissue homeostasis are mediated by circulating EVs and plasma-derived FABP4 after bariatric surgery [61]. Camino et al. found that EVs carrying candidate biomarkers of obesity were released by adipose tissue of obese patients. Results show that circulating EVs-TFGBI may be useful in monitoring glycemic status in obese patients, while EVs-mimecan may be helpful in tracking obesity [62]. EVs shed from adipose tissue have been shown to play a key role in obesity and its associated comorbidities [63, 64]. EVs-cystatin C have been shown to be positively associated with metabolic complications of obesity, including low systemic inflammation, low HDL-cholesterol levels, and metabolic syndrome. Conversely, EVs-CD14 were negatively associated with adipose tissue abundance and dyslipidemia, as well as with a reduced relative risk of developing type 2 diabetes [65].

Adipose tissue-derived EVs protein content varied between healthy and obese subjects. In obese subjects, matrix metalloproteinase-2 (MMP-2), transforming growth factor-beta-inducible protein (beta)ig-h3, thrombospondin-1,

fatty acid binding protein-4, mimecan, and visceral adipose tissue-derived EVs ceruloplasmin increased [66], while leptin, septin-11 levels decreased [67]. Circulating exosomal miRNAs can be detected earlier in serum than many traditionally used protein biomarkers during obesity progression [68]. Many circulating exosomal miRNAs have been found to be altered in obese subjects and are involved in the onset and progression of obesity [69]. Circulating exosomal miRNAs are mainly derived from adipose tissue, and it has been found that in genetically engineered mice lacking miRNA processing enzymes (Dicer) in adipose tissue, the levels of circulating exosomal miRNAs are significantly reduced, which can be reversed after transplantation of adipose depots [70]. Exosomal miRNAs are potential biomarkers of obesity. Compared with normal volunteers, RNA-sequencing revealed that 72 exosomal miRNAs were differentially expressed in obese patients [71]. In addition, exosomal miRNA profiles in obese patients were altered after weight loss by surgery, with 41 miRNAs differentially expressed in postoperative versus preoperative blood [71]. The researchers finally found that 9 miRNAs were highly expressed in patients before surgery compared with healthy volunteers and were involved in WNT, neurotrophic factor, and insulin signaling [71]. Studies have suggested that exosomal miRNAs shed by visceral adipocytes may be involved in regulating key inflammatory and fibrotic signaling pathways. A total of 55 differentially expressed miRNAs were detected by comparing obese versus lean visceral fat donors, and qRT-PCR confirmed downregulation of miR-148b and miR-4269 and upregulation of miR-23b and miR-4429. TGF-β signaling and Wnt/β-catenin signaling were also determined to produce corresponding changes through pathway analysis [72]. Moreover, EVs-miRNAs may also be involved in the pro-metabolic effects of aerobic exercise on obesity, which may be related to the communication between liver and white adipose tissue. It was confirmed in mice that aerobic training altered the circulating EVs-miRNA profile in obesity, including reduced levels of miR-122, miR-192, and miR-22 [73]. Alterations in cholesterol efflux capacity in adolescent obesity may be driven in part by adipocyte-derived EVs-miRNAs, including miR-3129-5p, miR-20b, miR9-5p, miR-320d, miR301a-5p, miR-155-5p [74]. EVs-miRNAs are also involved in the regulation of obesity-related diseases, especially cardiovascular diseases. Exosomal miRNA-29a and miRNA-122 mediate cardiac dysfunction and mitochondrial inactivation in obesity-related cardiomyopathy [75, 76].

Inflammation plays an important role in the development of obesity. During obesity, the number of M1 macrophages in adipose tissue increases, which is associated with adipose tissue inflammation and insulin resistance. In contrast, M2 macrophages releasing anti-inflammatory cytokines were reduced [77]. Cheng et al. showed that a decrease in BMI at 1 month after bariatric surgery was associated with an improvement in blood glucose and a reduction in endothelial, platelets, and monocyte derived MPs, reflecting a reduction in inflammation [78]. EVs were initially considered to be simple biomarkers; however, with the advancement of research, EVs are now believed to be involved in the development and maintenance of metabolic diseases, especially obesity, in a positive manner. EVs are involved in regulating inflammatory immune responses during obesity. Monocytes are stimulated by exosomes released from adipocytes to differentiate into active macrophages, and pro-inflammatory cytokines such as IL-1 and TNF-α are also induced to increase secretion [79]. As half of MIF in plasma circulates in microvesicles, its tautomerase activity triggers rapid activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) in macrophages, which also play a critical role in inflammatory disease [59, 80]. Ying et al. found a novel role for adipose tissue macrophages in mediating obesity-induced β-cell adaptation by releasing miRNA-containing EVs. The ability of miR-155 released by EVs to regulate β-cell function by directly inhibiting mafb expression was finally demonstrated [81]. Pan et al. found that exosomal miRNA-34a secreted by adipocytes transmits a signal of nutrient excess to adipose macrophages and exacerbates obesity-induced systemic

inflammation and metabolic dysregulation by inhibiting M2 macrophage polarization [82].

Currently, it is difficult for most obese patients to achieve good results through exercise and diet control. There is an urgent need to find good treatments for obesity. EVs may serve as an important member of the treatment of obesity. One study found that muciniphila-derived EVs resulted in a more significant decrease in body and fat mass in mice fed a high-fat diet [83]. It was further found that muciniphila-derived EVs contain various biomolecules that can positively affect obesity by affecting related genes [83]. At the same time, considerable attention has been paid to the potential of RNA medicine. Given the important value of miRNAs in obesity, it is possible to use miRNAs as drug candidates to treat obesity. MiR-122 is highly expressed in obese livers and adipose tissues and has been identified as one of the circulating miRNAs associated with obesity [84]. The first miRNA drug candidate, miravirsen, is currently in clinical trials for the treatment of hepatitis C. However, the reason for limiting the effectiveness of obesity drug treatment may be the lack of precise drug delivery. EVs promise to solve this dilemma. EVs derived from intestinal flora can be used as a mucosal delivery vector to improve obesity in mice [83]. Milbank et al. found that small extracellular vesicles selectively deliver therapeutic agents from the blood to the brain by targeting neurons in the ventromedial hypothalamus of mice, resulting in increased BAT thermogenesis and energy expenditure without affecting food intake, which ultimately has a therapeutic effect on obesity [85].

Whether used as biomarkers, targets, or drug vectors, some promising effects are provided by EVs in the treatment of obesity. In this strategy, cell-derived EVs are used as shuttles of active molecules to improve the specificity and efficacy of the treatment. With the deepening of research, the potential role of EVs in the pathogenesis and treatment of obesity has gradually been revealed.

10.5 Conclusion and Future Perspective

It is thought that obesity may be related to imbalance of energy intake and expenditure or metabolic disorders. Cell-derived EVs can be used as vehicles to transport cargoes including proteins, lipids, and nucleic acids between local and distant cells. EVs have been found to act as novel mediators of cellular communication in metabolic diseases, and their role in obesity is also an emerging area of research [2, 15, 17]. EVs in the body come from different sources, vary in size, and contain cargoes that can be used as indicators of the metabolic state of the source cells. Therefore, EVs have long been mainly used as potential markers of disease, and research has also focused on detecting their amount in plasma, which can be used to assess the severity of disease. Numerous studies have uncovered obesity-related EVs, especially circulating exosomal miRNAs, and intervention studies have enabled us to identify biomarkers with prognostic and diagnostic value, as well as potential therapeutic candidates. While the field of EVs research is an exciting area, especially in the field of cancer biomarkers, several challenges remain before they can be translated into the clinic. First, technical difficulties remain in the efficient isolation and quantification of EVs from plasma, and the specific tissue origin of EVs in vivo cannot be precisely identified. Therefore, it is necessary to develop a more efficient and specific way to isolate EVs, so as to provide technical support for EVs to be used as biomarkers for early diagnosis of obesity and assessment of obesity in the future. Second, studies have found that the content of circulating EVs is altered in obese patients, but the specific mechanism of this change is still unclear, and it is difficult to determine whether EVs are only biomarkers of obesity or are triggered by homeostasis disorders. Furthermore, despite emerging evidence in adult obesity, relatively few studies have been conducted on EVs in obese

adolescents. Adolescent obesity is a special population, manifested by less confounding variables such as other chronic diseases, drugs affecting inflammation, etc. Therefore, research on obesity in adolescents provides a new opportunity to explore the role of EVs in obesity and how it is affected by diet and physical activity, and most importantly, whether EVs can be used as a new marker for evaluating treatment effects in obese patients [86]. So far, EVs studies have mainly focused on EVs-miRNAs or EVs proteins alone, and few people have explored the synergistic effect between RNAs and proteins carried by EVs. This combined approach may become a new strategy for obesity diagnosis and treatment. Although the methods for the isolation, purification, quantification, and analysis of EVs still need to be optimized, EVs have shown great potential in the occurrence and development of obesity, which can not only become an important marker for the early diagnosis and severity assessment of obesity but also can be used as an effective drug carrier for the treatment of obesity in the future.

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Extracellular Vesicles in Bone Remodeling and Osteoporosis

11

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Abstract

Osteoporosis is a systemic disorder characterized by bone mass loss, leading to fractures due to weak and brittle bones. The bone tissue deterioration process is related to an impairment of bone remodeling orchestrated mainly by resident bone cells, including osteoblasts, osteoclasts, osteocytes, and their progenitors. Extracellular vesicles (EVs) are nanoparticles emerging as regulatory molecules and potential biomarkers for bone loss. Although the progress in studies relating to EVs and bone loss has increased in the last years, research on bone cells, animal models, and mainly patients is still limited. Here, we aim to review the recent advances in this field, summarizing the effect of EV components such as proteins and miRNAs in regulating bone remodeling and, consequently, osteoporosis progress and treatment. Also, we discuss the potential application of EVs in clinical practice as a biomarker and bone loss therapy, demonstrating that this rising field still needs to be further explored.

Keywords

Extracellular vesicles · Osteoporosis · Bone remodeling · Exosomes · Bone · MiRNA

11.1 Background

Osteoporosis is considered a severe public health problem [1, 2] with high personal and social costs [2, 3] associated with sustained disability, physical limitations, psychosocial impairment, and reduced quality of life [4]. The most recent estimated prevalence of any frailty fracture, defined as the number of individuals with disabilities, was 56 million people worldwide in 2000 [2, 5]. The clinical significance of osteoporosis relies on fractures' risk. Then, osteoporosis diagnosis is based on imaging and measurement of bone mineral density quality, which is a significant determinant of bone strength [6]. In this disease, the proximal femur, vertebral bodies, and wrists are the most commonly affected sites [6, 7].

Although osteoporosis has been considered a disease predominant in women, with aging, the increase in bone loss is also evident in men [8]. In women, the decline in estrogen levels related to menopause causes trabecular and cortical bone loss due to a high bone remodeling rate, leading to osteoporosis [9, 10]. The decrease in testosterone levels in men also contributes to age-related bone loss [11, 12]. The reduction of sex steroids

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hormones levels in women and men is the primary cause of osteoporosis, but indirect effects also play a significant role in its pathogenesis. The secondary causes of osteoporosis include alcoholism, smoking, sedentarism, hypogonadism, hyperparathyroidism, hyperthyroidism, gastrointestinal disorders, systemic diseases, pharmacotherapy, obesity, among others [7, 11, 13, 14].

A failure of bone homeostasis is the cause of bone loss during the pathological process of osteoporosis, involving cellular and molecular mechanisms with the induction of a gap between bone resorption and formation [15, 16]. The mid-twenties is the period of achievement of the peak of bone mass for both genders. After that, a plateau followed by a hormone-dependent process accelerates bone mass loss in the first years in postmenopausal women and andropausal men, associated with an impairment in bone cells' function [5]. The critical cells involved in bone metabolism are osteocytes, osteoblasts, osteoclasts, and T cells [17, 18]. The increase in bone resorption is associated with upregulation of osteoblast and osteocyte apoptosis, oxidative stress, and osteoblast nuclear factor kappa-B (NF-κB) activity; decrease in osteoclast apoptosis with an increase in its differentiation induced by the receptor activator of NF-kB/receptor activator of NF-κB ligand/osteoprotegerin (RANK/RANKL/OPG) system; and T-cell indirect regulation of the osteoclasts activation [10, 17, 18]. Osteoporosis's general and pharmacological management are weight-bearing exercises, adequate dietary intake (calcium and vitamin D supplementation), and oral bisphosphonates for individuals at increased fracture risk [6]. However, emerging therapeutic opportunities arise, such as extracellular vesicles (EVs), becoming a promising tool for osteoporosis treatment [19].

EVs are vesicular structures surrounded by a lipid bilayer containing transmembrane proteins delivered in the extracellular environment [20]. Their classification depends on the biogenesis, size, and intracellular site of origin, which is categorized into three main sub-classes: exosomes (10–100 nm), microvesicles (100–1000 nm), and apoptotic bodies (1000–5000 nm) [21]. Exosomes originate from the membrane invagination of internal compartments such as endosomes and are released through exocytosis of multivesicular bodies; microvesicles are produced by bud from the plasma membrane; apoptotic bodies are formed by fading cells during the later processes of apoptosis [22]. EVs trigger critical intercellular signaling and transportation of membrane and cytosolic proteins, lipids, microRNAs (miRNA), and messenger RNAs (mRNA) from the donor cell to a target cell [23]. EVs' binding to the destination cell characterizes their mechanism of communication, and it may (1) prevail linked to the plasma membrane, (2) berth at the plasma membrane, or (3) be endocytosed and then (4) fuse with the membrane of an endocytic compartment [24, 25]. EVs can be dissociated from many biological fluids such as human plasma, bronchoalveolar fluid, serum, saliva, urine, semen, bile, cerebrospinal fluid, amniotic fluid, tumoral effusions, ascites, and milk [26].

EVs are also released by many cell types, such as bone cells [27–30]. Recent studies have demonstrated that osteoclasts [27], osteoblasts, osteocytes, and their precursors deliver exosomes that participate in the physiological processes of bone remodeling [28, 31]. Their function is related to the stimulation of osteoclast and up- or downregulation of osteoblast differentiation [27, 29, 30], bone regeneration, inhibition of osteoblastic activity [32, 33], and play a crucial morphogenetic role in the growth plate and endochondral bone formation [34]. Bone-derived exosomes also may trigger pathological conditions such as osteosarcoma [35] and prostate cancer genes [36]. Albeit mechanisms underlying bone metastasis have yet to be thoroughly clarified, new studies have raised the possibility that EVs participate significantly in cancer progress and cancer bone tropism [37]. Similar to the function of cytokines and soluble factors, EVs derived from other body fluids may also regulate the differentiation of osteoclasts and osteoblasts

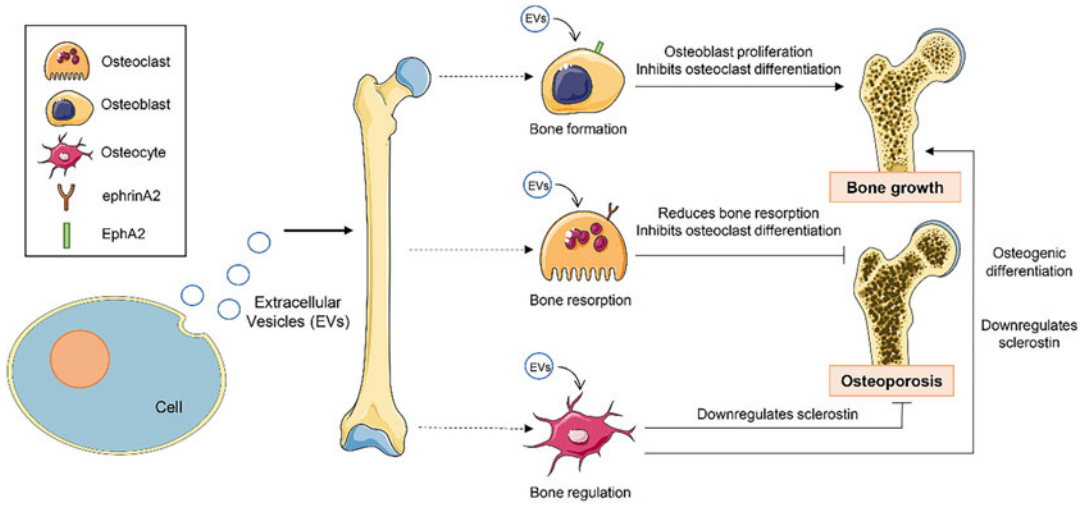

Fig. 11.1 Effect of extracellular vesicles (EVs) in bone remodeling. EVs derived from a secretory cell can act on bone through osteoblasts, osteoclasts, and osteocytes. In contact with these cells, the vesicles transfer their content and act by modifying the metabolic responses. In osteoblasts, EVs increase their proliferation through the positive regulation of genes such as Runx2 and Wnt/β-catenin, decreasing the RANKL/OPG ratio and activating osteoclasts, contributing to bone growth and maintenance. In osteoclasts, EVs decrease these cells' differentiation and activity via RANKL/OPG, preventing excessive bone resorption seen in osteoporosis. In osteocytes, miRNAs transported by EVs may contribute to the reduction of sclerostin production or promote osteoblasts' differentiation into osteocytes, favoring bone growth. (The figure was created using Servier Medical Art according to a Creative Commons Attribution 3.0 Unported (CC BY 3.0) (https://creativecommons.org/licenses/by/3.0/). The figure is a remix of figures from http://smart.servier.com/)

[27, 29, 30]. According to its origin, bone-derived exosomes show different mechanisms in regulating bone remodeling (Fig. 11.1).

As a result of their biological property and function, EVs are prone to perform an encouraging tactic to achieve pathogen identification, be used as a vaccine or drug delivery devices, determination of therapeutic targets, and work as specific biomarkers for a variety of diseases, such as autoimmune diseases and osteoporosis [26, 38, 39]. This chapter focuses on the biology of EVs and how they regulate osteoporosis progress and treatment. We also discuss the potential application of EVs in clinical practice.

11.2 Proteins from EVs in Bone Loss

Proteins derived from exosomes can contribute to cell signaling and participate in critical metabolic processes, such as bone cell activity and tissue remodeling [40]. The content of EVs varies according to the cell from which it originates. Bone cells secrete EVs that act through cell signaling to carry out the process of bone resorption and formation. RANKL, RANK, osteopontin (OPN), OPG, ephrin-B1, and stomatin proteins are packaged in bone cell exosomes and regulate the tissue remodeling [41, 42].

Exosomes derived from osteoclasts are rich in proteins, such as ephrin-A2 (EFNA2), located on the surface of vesicles secreted by these cells. The osteoclast-derived exosomes permeate the communication between osteoclasts and osteoblasts through EFNA2 to deliver their content and downregulate the osteoblast differentiation [29]. Semaphorin 4D (sema4D) is another protein identified in the EVs upregulated upon osteoclasts differentiation and acts as an inhibitor of bone formation via osteoblast signaling [43]. Likewise, actins and integrins, which are essential in forming the sealing zone and the process of cell

adhesion and signaling, respectively, have been identified in the cytoskeleton structure of osteoclast-derived exosomes and are part of osteoclasts' action mechanism in bone resorption and cell signaling [44, 45].

On the other side, transforming growth factor-beta receptor II interacting protein 1 (TRIP-1) is associated with an augmentation of osteoblast differentiation. TRIP-1 is expressed in osteoblast-derived exosomes and can influence bone matrix mineralization by increasing type 1 collagen production and calcium phosphate deposition [46, 47]. Also, osteoblast-derived EVs contain regulatory proteins that stimulate the formation of osteoclasts, such as RANKL. These EVs then merge with osteoclasts to deliver RANKL and signal for bone resorption [48]. In addition to the proteins mentioned above, osteoblast differentiation and mineralization markers, such as bone sialoprotein and osteonectin, respectively, as well as vascular endothelial growth factor (VEGF) and bone morphogenetic protein (BMP), were also found in the content of EVs derived from bone tissue and are strongly related to the proliferation and action of osteoblasts, osteocytes, and osteoclasts [34, 49]. VEGF contributes to bone formation by stimulating angiogenesis and enhancing osteoblast maturation and mineralization and is considered essential for the healing of fractures and bone repair [50, 51]. BMPs induce bone formation by stimulating osteoblast proliferation from mesenchymal progenitor cells [52].

Cell signaling mediated by proteins in EVs may help study osteoporosis's pathological processes [53]. The differences between the content of serum exosomes from healthy elderly individuals with osteoporosis were evaluated. A study demonstrated a negative regulation of bone remodeling proteins, such as integrins β1 and β3, in osteoporosis patients [54]. These proteins are transmembrane structures that mediate the cell adhesion process, coupling the extracellular matrix's cytoskeleton [55]. Specifically, β1 integrin is responsible for osteocyte mechanosensation [56] and β3 integrin perceives mechanical traction and translocates Wnt/β-catenin, inducing the expression of osterix, an important osteoblast transcription factor [57]. Proteins associated with the transforming growth factor-beta (TGF-β) cascade, such as TGF-β1 itself, the latent-transforming growth factor beta-binding protein 1 (LTBP1), and the signal transducer and activator of transcription (STAT), were also reduced in volunteers with osteoporosis, resulting in a more significant bone loss due to impairment of bone resorption and formation [54]. In the bone, proteins related to bone homeostasis can be dysregulated in humans, contributing to osteoporosis onset as bone morphogenetic protein receptor type II (BMPR2), programmed cell death protein 4 (PDCD4), osterix (OSX), runt-related transcription factor 2 (RUNX2), versican core protein precursor (VCAN), affecting bone deposition when reduced. In contrast, the increase in c-Fos protein and RANKL, related to osteoclast activity, may contribute to bone resorption [19, 58–60]. Interestingly, a study with vesicles derived from the plasma of young and elderly individuals has shown that the protein galectin-3, related to osteoblastogenesis, is reduced in the elders compared to young individuals, demonstrating less osteogenic capacity in these individuals. Thus, the destructive process that involves increased bone resorption in patients during the pathological process of osteoporosis yields differences in the vesicles' proteomics content, increasing the proteins related to bone disease markers.

Body fluids also secrete exosomes with meaningful content. A set of bone remodeling proteins has been identified inside the exosomes in healthy individuals' urine [61]. Among these molecules, the collagen triple helix repeat-containing protein 1 (CTHRC1), a Wnt family member 5A (WNT5A), and OPG are related to pro-osteogenic and anti-osteoclastic functions [62]. In an animal model of osteoporosis, an intravenous administration of 100 μg of exosomes derived from human urine samples reversed the loss of trabecular bone volume/total volume (BV/TV), the number of trabeculae (Tb.N), trabecular thickness (Tb.Th), cortical thickness (Ct.Th), and endosteal perimeter (Es.Pm). However, when silencing the RNA of the CTHRC1 and OPG proteins, the vesicles' effects

were not observed, resulting in lower BV/TV, Tb.N, Es.Pm, Ct.Th, and smaller periosteum perimeter (Ps.Pm) [63]. These data reveal the influence of proteins on exosomes' protective action in osteoporosis.

Moreover, EVs isolated from bodily fluids, such as milk, also have a crucial role in bone remodeling. In breast milk exosomes, β-casein, β-lactoglobulin, elongation factor-1alpha, OPN, and TGF-β have been identified and may influence the milk EVs on bone loss reversal [64, 65]. Studies with EVs from commercial bovine milk have been shown to accelerate osteoblasts' differentiation, increasing osteocytes' presence in vitro [66]. Another study observed that the same vesicles' treatment promotes small osteoclasts' formation but reduces bone resorption [67]. In addition to these studies, several EV proteins as integrins, growth factors, proteins involved in ribosome and cytoskeleton structure, calcium ion binding and metabolism, derived from bone marrow and human umbilical cord were strongly related to physiological processes such as angiogenesis, motility and cell activation, and the immune response [68]. Studies with both cell lines, bone marrow and human umbilical cord, have demonstrated osteogenic effects in cell culture [69] and experiment with mice [70, 71] for acting in the proliferation of osteoblasts and decreasing the activity of osteoclasts.

Therefore, EVs' effect on bone diseases still needs to be further explored concerning their content to highlight their function since proteins transported via exosomes play an essential role in cellular communication.

11.3 Biomarkers: The Influence of miRNA Derived from EVs on Bone Loss

EVs may be released by all resident bone cells, such as osteoblasts [72], monocytes/macrophages [73], osteoclasts [74], and osteocytes [75]. The vesicles themselves and the mediators released by them may act as bone loss biomarkers, supplying cell-to-cell communication function [46]. Circulating EVs and their components can be monitored and reflect the body's physiological and pathological characteristics. Because of these characteristics, EVs can be considered biomarkers for identifying and diagnosing several pathologies, such as neurological diseases, neoplasms, autoimmune or immunological diseases, and even bone diseases [19].

However, these vesicles' identification and isolation are still tricky due to their low blood concentration, despite their tremendous medical and biological interest [76, 77]. It is necessary to the immunoselection of exosomes based on their plasma membrane protein content to isolate specific EVs released by a given population of cells [78]. This method seems interesting for diagnosing bone diseases since bone mineral density alone may not identify the disease's initial stages, as it does not have sufficient sensitivity [19]. Also, serum bone turnover markers such as procollagen I N-terminal extension peptide (P1NP) and C-telopeptide breakdown products (CTX), which are nowadays the most informative ones for the monitoring of osteoporosis, have several limitations for diagnosing [79]. Therefore, new biomarkers are needed, and miRNAs and EVs are robust candidates for bone loss progression.

Some biomarkers are released by resident bone cell EVs or activated by the vesicles' precursors. It has already been demonstrated [80] that mononuclear cells and granulocytes release EVs that carry lncRNA JPX, and interestingly, JPX has been reported as an activator of the Inactive Specific Transcript X (XIST), which is abundant in the serum of patients with bone loss, acting as an inhibitor of osteogenesis [81]. Although this molecule was described as a biomarker for bone loss, miRNAs as biomarkers are more explored in the literature because they are one of the main content present in the EVs, which are short single-stranded RNAs responsible for regulating post-transcriptional gene expression by suppressing mRNAs, thus altering the function of the vesicle receptor cell.

Nine miRNAs (miR-93, miR-24, miR-23a, miR-124a, miR-122a, miR-21, miR-125b, miR-100, and miR-148a) were increased in the

serum of patients with osteoporosis compared to healthy control [59]. Three miRNAs previously reported during osteoporosis [82] and detectable in plasma (miR-21, miR-133a, and miR-146a) [83] were evaluated. Two of these miRNAs in plasma from postmenopausal osteoporosis patients were correlated with bone loss. It has been seen that miR-21 is downregulated, and miR-133a is upregulated in postmenopausal patients compared to healthy patients [84]. Serum EVs have also been evaluated as biomarkers of bone loss during antiretroviral therapy for HIV [85]. There was a change in the profile of the vesicles, with an increase in EVs that express important markers of bone metabolism such as osteocalcin, RANKL, RANK, and, consequently, a reduction in bone mineral density (BMD). Interestingly, changes in these vesicles were early and before the bone loss occurred, suggesting that serum EVs may serve as biomarkers of bone remodeling [85].

MiRNAs are also considered critical components for bone tissue homeostasis [86]. Several miRNAs are related to bone remodeling regulation via action on osteoblasts and progenitor cells, such as mesenchymal stem cells (MSCs), that have differentiation and autologous renewal capabilities [87]. Depending on the autocrine and paracrine signals they receive, they can differentiate into osteoblasts, chondrocytes, and adipocytes [33], influencing the bone formation and resorption processes. A series of exosomatic miRNAs are present during the differentiation of MSCs into osteoblasts, being their overexpression (let-7a, miR-199b, miR-218, miR-148a, miR-135b, miR-203, miR-219, miR-299-5p, and miR-302b) or inhibition (miR-221, miR-155, miR-885-5p, miR-181a, and miR-320c) important for osteoblastogenesis [87]. Also, it has been reported that miRNA let-7a is responsible for the increase in osteogenesis while suppressing adipogenesis [88], and miR-199b is involved in controlling osteoblast differentiation via Runx2 signaling [87]. In contrast, miR-221 and miR885-5p, which are negative regulators of osteoblast differentiation, act by suppressing the expression of Runx2 and are downregulated in bone marrow cells [28].

Beyond the miRNAs secreted during osteogenesis by MSCs, some are also secreted by the osteoblasts to induce their differentiation. The exosomal miRNAs miR-1192, miR-680, and miR-302a activate the Wnt B-catenin pathway, which is an essential regulator of bone marrow cell differentiation [30]. The miRNA mentioned above stimulates the osteogenesis pathway by suppressing adipogenic factors via stimulation of Runx2 and osterix [89]. Moreover, miR-218 activates the Wnt signaling cascade and triggers increased osteoblast activity by inhibiting sclerostin, an inhibitor of osteoblast differentiation [90]. The action of Wnt is mediated by BMP2, a glycoprotein that recruits osteoprogenitor cells to the bone formation site [91, 92]. On the contrary, MiR-140-3p is highly expressed in osteoblast EVs and can inhibit osteoblast differentiation via repression of BMP2 expression [28].

Conversely, concerning bone resorption, EVs secreted by osteoclasts can suppress osteoblast action through miRNA-mediated gene regulation. Osteoclasts are giant cells that perform bone resorption and participate in bone loss progression [93]. Higher levels of miR-214, which is secreted by osteoclasts to inhibit molecules for osteoblast differentiation, were verified in elderly postmenopausal women (60–90 years old). MiR-214 upregulated in osteoclasts induces a more significant resorption activity of these cells and less bone mineral density in vivo [94] and is transferred to osteoblasts to inhibit its function [29]. Also, patients with osteoporosis and ovariectomized (OVX) mice demonstrated higher exosomal miR-214 and EFNA2. Mice that overexpress miR-214 specific for osteoclasts had less osteoblast activity [29]. Blocking the production of the exosomes osteoclast-derived miR-214 inhibited its transfer to the osteoblasts and consequently increased bone formation in mice [29]. It was proposed that suppressing the action of miR-214 could attenuate osteoporosis in aging women [95]. The indirect activity of miR-214 delivery was also described. EFNA2 protein was present in EVs of osteoclasts, and this protein can be used as a biomarker of osteoporosis progression since it is in higher concentrations in the

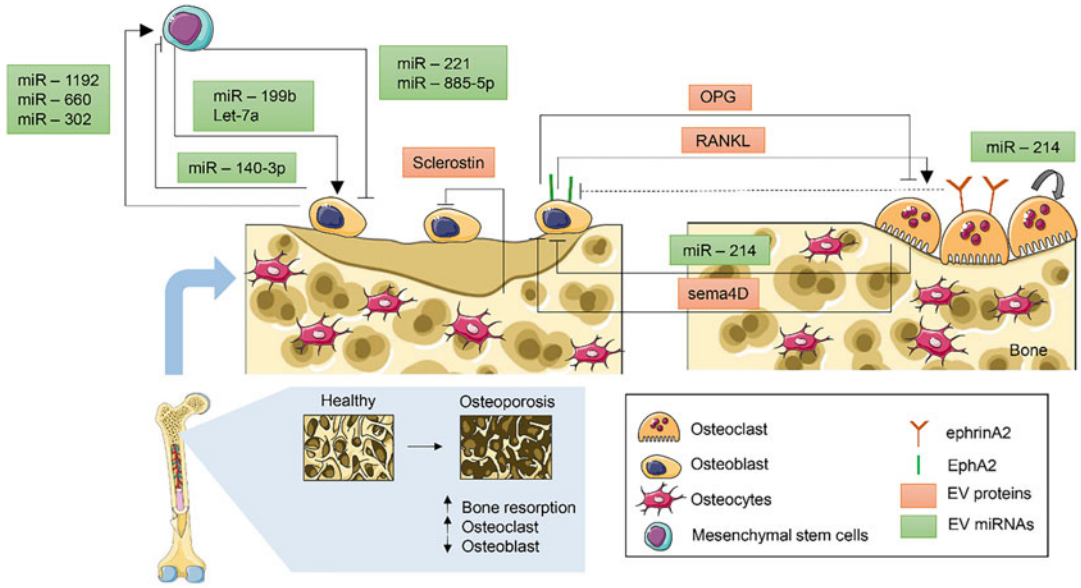

Fig. 11.2 Exosomal miRNAs and proteins up- or downregulated in the differentiation and activity of bone cells. Proteins and miRNAs derived from exosomes can contribute to cell signaling and participate in tissue remodeling, being regulated positively or negatively during bone loss. Osteoclasts secrete EVs enriched with sema4D and ephrinA2 proteins and miR-214, negatively regulating osteoblast differentiation. MiR-214 can also positively regulate the action of osteoclasts. In contrast, osteoblasts secrete EVs containing RANKL and OPG proteins that stimulate or inhibit osteoclast activity, respectively. EV-derived osteoblasts also contain miRNA that acts on mesenchymal stem cells to stimulate osteogenesis (miR-1192, miR-660, and miR-302) or inhibit this process (miR-140-3p). The mesenchymal stem cells release EVs containing miRNAs that induce (miR-199b and Let-7a) or inhibit (miR-221 and miR-885-5p) the differentiation of osteoblasts. Finally, osteocytes can secrete exosomal sclerostin, which inhibits osteoblast differentiation. (The figure was created using Servier Medical Art according to a Creative Commons Attribution 3.0 Unported (CC BY 3.0) (https://creativecommons.org/licenses/by/3.0/). The figure is a remix of figures from http://smart.servier.com/)

serum of patients and mice with bone loss [29]. Exosomal EFNA2 secreted by osteoclasts can be a critical protein that mediates the communication between osteoclasts and osteoblasts and enables incorporating EVs into the target cell to release miR-214 for their action [29, 96].

Therefore, EVs are an important biomarker of bone loss and effectors of signaling for bone remodeling (Fig. 11.2). The study of vesicle isolation techniques and aimed at their application in the treatment of osteoporosis or as precocious markers of bone loss are critical and represent a significant advance in the study of bone diseases.

11.4 Effect of EVs in Animal Models of Bone Loss

Osteoporosis is a complex health condition characterized by decreased mineral density and bone fragility [97]. EVs are being administered in animal models to assess their potential effects on osteoporosis to treat and reverse bone loss [19].

Ovariectomy (OVX), the surgical removal of ovaries, is commonly used to induce osteoporosis in animals, reducing the hormone estrogen as occurs in postmenopausal women [98]. In a study, OVX mice were treated with one weekly injection in the tail's lateral vein of 3 mg/kg vesicles derived from osteoblasts with different stages of maturation for 4 weeks. The results showed an improvement in bone parameters of the femur evaluated by computed microtomography such as BV/TV, Tb.N, Tb.Th, and trabecular separation (Tb.Sp) compared with the control group. The significant effects were demonstrated mainly between the second and third week of osteoblast differentiation, showing how different the vesicles' response is, according to the different osteogenesis stages [99]. Similar

results were found in a study with OVX mice that received 100 μg of exosomes derived from human exfoliated primary teeth (SHED) intravenously 2 days after the surgery. Bone loss was recovered and improved by evaluating BMD, BV/TV, Tb.N, and Tb.Th in the femur [100]. SHED-derived exosomes are composed of miRNAs, small RNAs, and proteins associated with osteoblast differentiation, such as Wnt3a and BMP2 [100, 101]. SHED cells also have osteogenic potential, regulating ALP, Runx2, dentin sialoprotein (DSP), and OCN, and are immunoregulatory, being able to influence the production of regulatory T cells (Tregs) and inhibit helper T cells 17 (Th17) [102, 103]. Another study observed that OVX mice's treatment with bovine milk EVs in a concentration of 14.3×10^6 particles/mL in the drinking water for 4 weeks improved bone microarchitecture. Among the results found, the increase in BV/TV and Ct.Th was associated with increased treated animals' mechanical resistance compared with the control OVX group. Besides, a reduction in osteoclast presence in the femur and the RANKL/OPG ratio in the serum was observed [104].

Moreover, senile osteoporosis was also evaluated in an experiment with EVs. Aging can lead to decreased bone turnover, with decreased bone formation and increased resorption [97]. In study with 16-week-old elderly mice, they administered 100 μg exosomes derived from the human umbilical cord by injection into the lateral vein of the animals' tail. Treatment with exosomes improved BV/TV, Tb.Th, Tb.Sp, Ct.Th, and Es.Pm of the mice's femur [71]. Besides, osteogenic genes such as Runx2, bone gamma-carboxyglutamate protein (Bglap), and collagen type I alpha 1 (Col1a1) were elevated in mice's femur that received the exosomes compared with the untreated group. According to these data, genes related to osteoclast activity such as tartrate-resistant acid phosphatase (Trap), nuclear factor of activated T cells 1 (Nfatc1), and cathepsin K (Ctsk) were decreased in the treated group [71], indicating that exosomes derived from the human umbilical cord have potential to improve osteoporosis.

Other models of bone loss have already demonstrated some effects of EVs as a potential treatment for osteoporosis. Obesity, a multifactorial chronic disease characterized by adipose tissue expansion and increasing inflammatory markers, can lead to high rates of bone turnover and consequent osteoporosis [14, 105]. Mice fed a diet rich in refined carbohydrates to induce obesity and bone loss were treated for 4 weeks with 14.3×10^6 EV particles/mL derived from bovine milk in the drinking water, and bone parameters were analyzed. Data showed a reversion of bone loss associated with a better RANKL/OPG ratio in treated mice [104]. In addition to estrogen deficiency, aging, and diet quality, osteoporosis can also be induced due to oral drugs such as glucocorticoids [97, 106]. A study evaluated the effect of EVs derived from bovine milk colostrum on bone remodeling influenced by prednisone [39]. This study administered 7.5 mg/kg of the drug in the subcutaneous tissue in the shoulder area of 4-week-old males' mice using slow-release pellets and offered, through oral gavage, the concentration of 0.15 mg/kg (low dose) and 1.5 mg/kg (high dose) of EVs during 8 weeks of treatment. The results showed that BMD values and the percentage of bone volume were significantly higher in the femur of mice that received the high dose of EVs than in mice that received prednisone without treatment [39]. Furthermore, in the same study, less differentiation of osteoclasts was observed in cell culture exposed to concentrations of 150 ng/mL or 50 ng/mL of vesicles derived from bovine colostrum [39], demonstrating that exosomes act not only in bone microarchitecture but also in at the molecular level.

Although this is a new approach, using EVs in osteoporosis models has become increasingly explored and can be considered a therapeutic strategy for managing osteoporosis and other bone diseases.

11.5 Role of EVs on Human Bone Loss

Although EVs' action on bone loss was demonstrated in animal experiments, clinical

trials have not yet been conducted. However, studies have already shown their potential to influence human cells and proteomic profiles in patients' bone loss. A hypothesis that EVs derived from stem cells could induce mesenchymal stem cells' differentiation in the osteogenic lineage was evaluated using human stromal cells from the bone marrow cultured in a medium with exosomes secreted from bone marrow cells. It was shown that these exosomes could induce the differentiation of mesenchymal stem cells into mature osteoblasts [107]. Another study with cell culture showed that exosomes derived from bovine milk are uptaken by human macrophages [108]. Also, human MSCs treated with bovine milk EVs have been demonstrated to uptake these molecules and accelerate their differentiation into osteoblasts/osteocytes [66], suggesting that individuals are prone to be influenced by the use of these vesicles.

The exosomes of healthy patients or with pathological conditions differ mainly in their protein content and miRNA [109]. An increased presence of miRNA-21 and decreased expression of important bone formation markers, such as alkaline phosphatase, Runx2, and osteocalcin, were observed in EVs isolated from adult patients' bone marrow with osteoporosis [60]. The serum-derived exosomes' content from elderly patients with increased bone loss was also characterized by increased expression of bone resorption markers [54]. Proteins associated with osteoblast cell proliferation and signaling pathways, such as from the Wnt/β-catenin pathway, were also reduced in the same patients [54]. The affected molecules were β1 integrin proteins and β3, CD34, and the transforming growth factor-beta (TGF-β), which regulates MSCs differentiation and controls bone resorption D. Another study demonstrated that 17 protein levels from serum-isolated exosomes were significantly dysregulated in the osteoporosis and osteopenia groups, including integrin β3, integrin α2β1, Talin 1, and Gelsolin [110]. A characterization of microvesicles in the serum of individuals with osteopenia and osteoporosis showed that among the proteins evaluated, 19 were upregulated compared with healthy individuals. Among them, vinculin, profilin-1, and filamin A are bone resorption-related [58]. These data demonstrated that the serum proteins could be indicators for patients' bone loss disease evaluation and diagnosis.

11.6 Conclusion and Future Perspectives

Studies with EVs are essential, mainly to elucidate their clinical applicability. The research advancement related to these molecules may provide new uses for them and the mechanisms associated with their action, both in animals and humans. These components are already explored as therapeutic agents in several pathologies such as breast cancer, kidney disorders, arthritis, bone diseases, among others [111–114]. To date, proteins and miRNA were already identified and associated with bone remodeling and, consequently, bone loss related to osteoporosis. Both molecules were associated with their presence in EVs secreted by cells or tissues and tested as biomarkers or treatments for osteoporosis. However, clinical studies evaluating the effect of EVs in treating patients with bone loss are still needed to expand the knowledge about these molecules and improve the quality of life of those who could benefit from them. Thus, the study of EVs is a promising field of research with a possible impact on broad biomedical areas related to health diseases.

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Part IV

Therapeutic Implications

Diagnosis of Extracellular Vesicles in Cardiovascular and Metabolic Diseases

12

N. Eichner-Seitz

Abstract

Early detection and identification of those with or at increased risk for cardiovascular disease (CVD) and metabolic dysfunction is crucial for improving disease management and prognosis. Given the complex, multifactorial nature of the pathogenesis of the CVD and metabolic dysfunction, it is essential to have biomarkers that encompass the multiple facets of disease development. The uniquely ubiquitous nature and functionality of extracellular vesicles (EVs) in various disease pathologies can provide novel insight into both diagnosis and prognosis while further improving assessments used in clinical and research practice. Herein we summarize the use of EV count and content (including miRNA and protein) in diagnosis of CVD, obesity, metabolic syndrome, and type 2 diabetes (T2D), as well as highlight the potential utility for enhancing determination of prognosis and long-term complications in these clinical populations. Although the results are promising, future work is needed in both methodology and in relation to other factors such as sex and medications, in order to apply these findings in clinical practice.

Keywords

Microparticles · Extracellular vesicles · Bioeffector · Metabolic health · Heart disease

12.1 Background

The World Health Organization (WHO) estimates that nearly 31% of all worldwide deaths are attributed to cardiovascular disease (CVD), a rate which has increased significantly over the past two decades. Simultaneously, the WHO also estimates global deaths from diabetes have exploded by 70% within the past two decades. Alarmingly, individuals with type 2 diabetes (T2D) are 2–3 times more likely to develop CVD compared to patients with normal glucose regulation, suggesting that impaired glucose metabolism plays a key pathogenic role in CVD development [1]. Recent interventions aimed at improving CVD risk through aggressive glucose management, however, failed to show significant improvements [2] suggesting that disease development and progression is multifactorial. Other mediators of metabolic dysfunction, including obesity, hypertension, dyslipidemia, and insulin resistance, may likely also play key roles in the combined pathogenesis of CVD in those with metabolic dysfunction.

Early detection and identification of those with or at increased risk for CVD and metabolic dysfunction is crucial for improving disease

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management and prognosis [3]. Given the complex, multifactorial nature of the pathogenesis of the CVD and metabolic dysfunction, it is essential to have markers that encompass the multiple facets of disease development in order to most accurately identify and treat. Identification of markers that can encompass multiple etiologies remains challenging, both in research and clinical practice. The American Heart Association estimates that the current biomarkers utilized to assess CVD risk, such as blood pressure and cholesterol, account for only 40–50% of known risk [4]. Although other typical blood-based biomarkers to assess CVD and metabolic dysfunction used in clinics worldwide, such as CRP, glucose, and HbA1c allow clinicians to quickly identify those at risk or with the disease, there are limitations [5, 6]. Other markers to assess metabolic dysfunction that are more likely to be used in research, such as inflammatory cytokines (IL-6, leptin, ghrelin) are not easily accessible to those in a clinical setting. To date, no marker has been identified that is able to accurately identify disease development and prognosis that can be utilized in both a clinical and research setting. Taken together, these findings suggest the need for identification of a biomarker (or bioeffector) that can be used to assess multiple etiologies of pathogenesis in CVD and metabolic dysfunction .

First described as merely "cell dust" by Wolf in 1967 [7], the unique characteristics of extracellular vesicles (EVs) provide a potentially novel way to enhance diagnosis and prognosis in cardiovascular and metabolic diseases. Derived from various cell and organ types directly involved in the pathogenesis of these diseases (such as the endothelium, platelets, leukocytes, hepatocytes, and pancreas), EVs are membrane-bound particles generated in response to stimuli including cell activation, injury, or apoptosis. EVs are unique biomarkers that carry transferrable content including proteins, lipids, and nucleic acids acting as facilitators of communication or "cross-talk" between cells and across organ systems [8, 9]. Surface proteins on EVs including intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) allow for EV targeting, binding, and eventual uptake into the target cell via endocytosis, phagocytosis, or micropinocytosis [10, 11]. Impairments in this communication have been implicated in the development and progression of diabetes [12], metabolic syndrome [13], and cardiovascular disease [8, 10]. Much work within the last decade has shown EVs to be elevated in a wide range of both cardiovascular [11, 14, 15] and metabolic diseases [16–18] (Fig. 12.1) although some recent work has suggested that there are many other factors beyond EV count to be considered, including changes in EV size and function. In this chapter, we hope to summarize the research progress of diagnosis and prognosis of extracellular vesicles in cardiovascular and metabolic diseases, considering clinical implications for both healthcare providers and researchers .

12.2 Extracellular Vesicles in Cardiovascular Disease Diagnosis

Given the enormous societal and economic cost of CVD worldwide, public health initiatives have focused on promotion of both primary and secondary prevention in CVD. It is well-documented the disease onset begins much earlier than presentation of clinical symptoms and is in fact an accumulation of risk factors and injuries over time [19, 20]. In fact, overt disease presentation is associated with a greater increase in recurrent cardiac events, morbidity, and mortality [21, 22]. Therefore, identification and utilization of sensitive biomarkers can play a key role in mitigating these increases in morbidity and mortality. Unfortunately, traditional biomarkers have limitations, especially in more complex patient cases [6, 23]. Combined with economic concerns for both direct and indirect costs of disease, the American Heart Association has called for further evaluation of potential novel biomarkers to improve diagnosis, prognostication, and treatment strategies [24]. The most useful CVD biomarker is one that can be used as both a diagnostic marker for acute cardiac disease (such as a myocardial infarction) and for

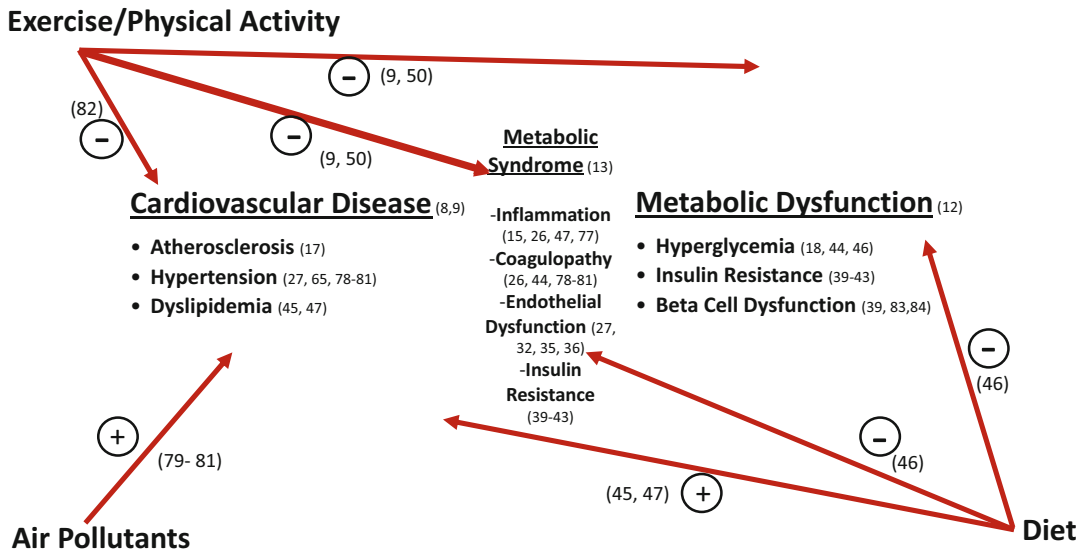

Fig. 12.1 Extracellular vesicles (EVs) in diagnosis of cardiovascular disease and metabolic dysfunction. Previous work demonstrating the diagnostic roles EVs may play in both cardiovascular disease (CVD) and metabolic dysfunction, as well as highlighting the potential interactive pathways by which EVs might modulate traditional risk factors for disease. Note that there is significant overlap in mechanisms for disease development, further highlighting the unique role EVs can play in enhancing diagnostic practice of these complex etiologies

monitoring disease progression or response to therapy [23]. Given the ubiquitous nature of specific subtype EV release, as well as its clear fundamental relationship with disease pathogenesis and progression, it stands to reason that its utilization in both diagnosis and prognosis of patients with CVD can improve treatment and management.

CVD encompasses various disease pathologies, including stroke, hypertension, coronary artery disease (CAD), and peripheral artery disease (PAD), each commonly linked by the process of atherosclerosis. Not only are circulating EVs released in response to vascular damage of the endothelium, they have also been shown to accumulate in atherosclerotic plaques [16], promote inflammation and coagulation [25], and have been associated with impaired endothelium-dependent relaxation [26], reduced flow-mediated dilation [27], and increased carotid intima-media thickness [28]. Recent work also shows that changes in EV function might also be key to understanding the implications for EVs in CVD development, highlighting the necessity to move beyond EV count. They have been implicated in eliciting changes in gene expression of cardiomyocytes implicated in cardiac hypertrophy as a result of elevations in ACE II [29]. Alterations in EV content, such as miRNA, in response to exposures such as air pollution have also been implicated in the development of CVD [30]. Interestingly, elevations in EV shedding from endothelial progenitor cells may contribute to increased aortic stiffness by decreasing the circulating levels of endothelial progenitor cells, independent of other traditional risk factors [31].

12.3 Extracellular Vesicles in Metabolic Disease Diagnosis

Metabolic disease refers to a cluster of conditions or physiological risk factors which include hyperglycemia, hypertension, increased waist circumference (i.e., obesity), and dyslipidemia. Insulin

resistance is also typically reported. Patients with this clustering of risk factors are at increased risk for both CVD and T2D, accounting for up to 1/3 of CVD cases in men and nearly half of newly reported T2D within a decade [32]. The exact mechanism by which this constellation of risk factors promotes disease development is unclear and likely has a multi-system combined pathogenesis. This pathogenesis may be linked together, in part, through increased blood pressure and dyslipidemia in people with hyperglycemia via insulin resistance [33]. In conjunction with this pathophysiologic process, elevations in inflammation due to visceral adiposity and endothelial damage have been reported in patients with metabolic disease [34, 35] indicating the presence of a prothrombotic and proinflammatory state. Given the complexity and overlap of this state, it remains difficult to assess two key drivers of metabolic dysfunction: insulin resistance and inflammation. Herein we will discuss the relationship of EVs to these key mediators of metabolic dysfunction and how they can provide insight into early diagnosis of metabolic dysfunction.

12.3.1 Evs in Insulin Resistance, Hyperglycemia, and T2D

Insulin resistance (IR) is a result of reduced responsiveness of skeletal muscle, pancreatic tissue, liver, adipose, and vasculature tissue to insulin which is needed for tissue metabolism. The precise cause of IR is uncertain and is most likely multifactorial, although disruption of the endothelium is a leading candidate [36] Insulin and a measure of insulin resistance (IR), HOMA-IR [37] have previously been shown to be closely related to EVs in vivo [38, 39] and recent work demonstrates that macrophage and adipocyte [40] EVs can reduce insulin-stimulated glucose uptake in both the liver [41] and skeletal muscle [42]. EVs derived from macrophages have also been shown to interfere with GLUT-4 translocation in human adipocytes by decreasing Akt-phosphorylation [39]. EVs also carry relevant cargo such as eNOS [43] and bioactive lipids [44] that may further mediate IR. Additionally, EV count has also been shown to correlate with factors altered in metabolic disease, such as blood pressure, body mass index, and HOMA-B (a measure of beta cell function) [38] illustrating both a direct and indirect relationship between various subtypes of EVs and IR.

Hyperglycemia is a potentially key modulator of EV release and function. Multivariable analysis in a large cohort of patients with varying degrees of glucose tolerance showed hyperglycemia to be the primary metabolic deficit associated with release of endothelial-derived EVs, after adjustment for other potential mediators such as BMI and blood pressure [17]. In vitro work has shown hyperglycemia to increase endothelial-derived EV propagation and greater EV oxidative stress and pro-coagulant activity compared to normal glucose conditions [43]. In vivo data also support the idea that EV release may be modulated in response to changes in diet, as endothelial EVs were lowered following carbohydrate restriction in adults with type 2 diabetes [45]. Additionally, although other various modulators of EV release include the potential to be modified with diet stimuli, such as lipids, and have been suggested to impact EV release [46], hyperglycemia is likely a crucial mitigator of EV release, especially in state of chronic hyperglycemia, such as T2D.

Extensive research has been done to elucidate the role EVs might play in future diagnosis of diseases related to IR and hyperglycemia, such as T2D. In fact, a recent meta-analysis of 48 studies has reported that endothelial, platelet, and monocyte EVs were significantly increased in patients with T2D [47]. Other work also suggests a dose–response to states of IR and hyperglycemia, as endothelial EVs (CD62E+) were significantly elevated in individuals with prediabetes and T2D when compared to normal glucose tolerant patients, while AV+ CD62E+ and AV+ EVs were only elevated in T2D, suggesting increased apoptosis only in the patients with T2D [17] Furthermore, the content of EVs also varied based on glucose tolerance, with a significant differential reduction in miRNA-126-3p in EVs from patients with prediabetes and T2D compared to normal glucose tolerant [17]. These

findings highlight EVs as an early marker of endothelial dysfunction and activated endothelium in patients with impaired glucose regulation early on in the process of metabolic dysfunction, i.e., "prediabetes."

12.3.2 Obesity-Related Inflammation and EVs

Obesity is known to play a key role in pathogenesis of both cardiovascular and metabolic disease [48]. Given the known relationship between these diseases and EVs, it stands to reason that EV profile would be altered in patients with obesity. However, even in patients with simple obesity, platelet EVs have been elevated in conjunction with reduced fibrinolytic activity [49]. Again, in a cohort of severely obese women compared to a lean control, total EVs, platelet EVs (AV + CD41), and endothelial EVs (AV+ CD31+/CD41−) were associated with obesity, independent of metabolic abnormalities. Interestingly, there were no significant changes in EV count following massive weight loss [50]. It is important to note that some individuals in this cohort underwent bariatric surgery, which may differentially impact EVs in comparison to weight loss via caloric deficit [50, 51]. This is contrary to previous work reporting decreased platelet EVs correlating with decreased BMI, fat mass, and visceral fat area following a 12-week weight loss intervention with or without exercise in patients with obesity but no diabetes [49]. Taken together, these results suggest that weight loss may reduce platelet derived EVs, in conjunction with fibrinolytic abnormalities, through decreased adipose tissue. Given these associations of EVs with an increased prothrombic state [52] independent of the presence metabolic abnormalities such as diabetes, these findings emphasize the significance of EVs as a relevant parameter for identification of high-risk patients with simple obesity.

At present, we lack a complete understanding of the mechanism by which obesity-related complications develop; however, inflammatory processes, the development of insulin resistance, and vascular dysfunction most likely all play a key role [48, 53]. As EVs are markedly elevated in individuals with obesity [54] and play a role in systemic cross-talk among metabolic organs including the liver, pancreas, skeletal muscle, and adipose tissue [55], they most likely will provide mechanistic insight into the process by which obesity induces metabolic and cardiovascular complications. For example, through transfer of adipokine content, adipocyte-derived EVs (CD14+) have been shown to interfere with insulin signaling in both the liver [41] and skeletal muscle [42]. Additionally, other work has suggested macrophage-derived EVs (M0 THP-1) interfere with GLUT-4 translocation in human adipocytes by inducing IR [39]. Although the exact mechanism by which this induces IR is unclear, activation of NfK-B was present, highlighting potential inflammatory pathways. Over 50 visceral adipocyte-derived exosomal miRNAs are reported to be differentially expressed in obese versus lean individuals, including downregulation of miRNA-1483b and miRNA-4269 in conjunction with upregulation of miRNA-23b and miRNA-4419. These differentially expressed miRNA mediators were predicted to play a key role in regulation of end-organ inflammatory and fibrotic signaling pathways [56].

12.3.3 Metabolic Syndrome

Metabolic syndrome (MetS) is the accumulation of interrelated risk factors for both T2D and CVD, presenting a unique lens when considering diagnostic approaches (Fig. 12.1). Although several international associations have slightly different criteria for the definition of MetS syndrome, insulin resistance and obesity (measured as central adiposity) are widely accepted as the most important contributors to this pathology [57]. As discussed above, EVs have been extensively documented in relation to both of these crucial drivers of MetS; therefore, it stands to reason that EVs also hold the potential to aid in diagnosis of MetS. In fact, EVs derived from the endothelium (CD146+), platelets (CD41+), leukocytes (CD11a), and erythrocytes (CD235+) are

increased in patients with MetS [14, 58, 59]. Interestingly, in addition to its relationship with MetS, increases in leukocyte-derived EVs (CD11a) were positively related to number of MetS criteria, Framingham risk, inflammation (indicated by C-reactive protein), and the presence of preclinical atherosclerosis in a cohort of over 200 patients without CVD [14]. These results highlight the potential utility of EVs as effective diagnostic tools, especially in MetS patients who are asymptomatic for associated long-term sequelae of MetS, such as CVD.

EVs might also aid in our understanding of disease progression in MetS. In one cohort of MetS patients, circulating endothelial EVs CD31+ were significantly higher compared to healthy controls. During states of apoptosis, endothelial EVs typically express CD31+. However, endothelial EVs CD62E, typically elevated in states of activation, did not differ between the two groups [60]. This suggests that increased levels of endothelial cell apoptosis might play a key role in the development of atherosclerosis in patients with MetS. Given that endothelial apoptosis can be triggered with high glucose conditions [61] and that hyperglycemia induces endothelial apoptosis via increases in NADPH oxidase activity of endothelial-derived EVs [62], EVs provide a unique understanding of MetS as both potential diagnostic biomarkers and "bioeffectors."

12.4 EVs in Assessment of CVD and Metabolic Disease Prognosis

12.4.1 EVs Increase Accuracy in CVD Prognosis Assessment

Elevations in circulating EVs have widely been reported in stroke, hypertension, coronary artery disease, and peripheral artery disease [26, 63], as well as other types of CVD including heart failure, post-myocardial infarction, and cardiac myopathies [64]. As recent data has also shown EVs to promote vascular dysfunction through various potential mechanisms including direct damage to the endothelium and content transfer to the endothelium cells themselves, it stands to reason that the number of circulating EVs might hold prognostic value and improve prediction of future course of both metabolic and cardiovascular diseases. Endothelial EV count has been shown to predict future CVD events in high-risk but asymptomatic individuals, independent of other factors [65]. Addition of EVs to traditional risk stratification assessment tools has previously been shown to improve predictability. In fact, when assessing the Framingham risk score in patients deemed "high-risk," addition of endothelial-derived EVs (in conjunction with other traditional parameters) significantly improved prediction of future CVD events [65]. This study of 378 "high-risk" individuals (defined as having >2 risk factors for coronary artery disease without having overt disease) is one of the largest studies to date highlighting the prognostic role of endothelial-derived EVs. Similarly, addition of CD31+/annexin V+ EVs to traditional risk factor models independently predicted risk of coronary revascularization and CVD death in patients with stable CAD during a 6-year follow-up [66]. Although less studied, other subtypes of EVs such as leukocyte-derived EVs might hold prognostic value as well. In a cohort of 42 patients with high-grade carotid stenosis, leukocyte-derived EV CD11b/CD66b independently predicted plaque vulnerability, highlighting the potential role of EVs for identifying those who are asymptomatic but at most risk for complications such as neurologic events [67]. Chironi et al. have also shown leukocyte EVs to predict subclinical atherosclerosis in asymptomatic patients [14]. Other interventions have shown that participants who suffered from a cardiovascular event within 1 year of the intervention had increased lymphocyte (CD3+/CD45+) and smooth muscle (SMA-alpha) EVs compared to participants who did not have an event within the same 1-year follow-up and improved predictive power of the Framingham score [68]. Ability to accurately and sensitively identify these patients might allow for earlier initiation of treatments. These results highlight the potential prognostic utility of EV count in

both asymptomatic and symptomatic clinical populations.

12.4.2 Proteins from EVs in CVD

It is important to consider other factors besides circulating count when discussing the potential prognostic role EVs might play in disease management. After adjustments for conventional risk factors, elevation of proteins in EVs such as Cystatin C, Serpin G, Serpin F2, and CD14 are reported to be related to increased risk of secondary cardiovascular events such as myocardial infarction [69] and acute coronary syndrome [70]. Interestingly, in a cohort of 404 patients presenting with dyspnea at an emergency department, EV protein levels of Serpin G1, Serpin F2, and CD14 were also associated with the occurrence of heart failure, suggesting that heart failure and vascular events such as acute coronary syndrome might share a common underlying pathophysiological mechanism [71]. These results highlight not only the potential prognostic utility of these EV proteins but also perhaps a better insight into the complex but interrelated pathophysiology of heart failure and vascular disease.

12.4.3 miRNA from EVs in CVD

Other EV biological content that must be considered in CVD prognosis is miRNA expression as various EV miRNA subtypes have been associated in the pathogenesis of CVD including atherosclerosis, heart failure, myocardial infarction, and even cardiac arrhythmias [72]. Endothelial EVs have been shown to be a major source of circulating miRNA-126-3p and miRNA-199-5p [73] and low levels of these miRNA in patients with stable CAD were associated with higher risk for future cardiovascular events [63]. Similarly, others have noted that miRNA 192, miRNA 194, and miRNA 34a could also predict the development of HF a year after an acute MI [74]. The mechanism by which this occurs is unclear, but many speculate that EVs enable "cross-talk" between systems. In vitro studies have shown that EVs transfer miRNA from injured cardiomyocytes to a vast array of other cell types including endothelial and immune cells, as well as fibroblasts, thereby promoting and regulating inflammation and angiogenesis at the site of injury [75]. The association of certain high-level risk factors typically seen in CVD, such obesity, smoking, and lack of physical activity, may in part be explained by their influence on EV miRNA. The response of EV miRNA can be up- or downregulated based on the stimuli and can be altered in patients with obesity [76], following exposure to particulate matter [77–79] and exercise [80]. These alterations in EV miRNA also correlated with increased blood pressure and coagulation [76–79] (Fig. 12.1). Taken together, these findings help to elucidate the exact cellular mechanisms by which well-known risk factors such as obesity, smoking, and physical inactivity exert influence in the development of disease (Table 12.1).

12.4.4 EV Content in Assessment of Metabolic Disease Prognosis

Given that EVs have the unique ability to "communicate" via changes in content and are produced by various key organs within the metabolic system including the liver, skeletal muscle, adipose tissue, and pancreas, a deeper understanding of changes in EV content in metabolic disease might improve prognosis assessment. In healthy individuals, transfer of endothelial EV miRNA-126 promotes vascular endothelial cell repair. However, in patients with T2D, lower levels of miRNA126 are present and may, in part, be implicated in the endothelial dysfunction seen in T2D [62]. Additionally, miRNA carried by EVs has been shown to mediate intracellular communication between skeletal muscle and beta cells of the pancreas in vitro. Specifically, insulin resistant EVs derived from muscle transferred miRNA16 to a beta cell receptor that is thought to be involved in the development of insulin resistance. In conjunction with this cross-talk, beta cells can also internalize skeletal muscle EVs, perhaps contributing to the

Table 12.1 Extracellular vesicle (EV) count and content as prognostic biomarkers in cardiovascular disease (CVD)

Author	Disease	Sample size	Subtype EV	Source of EVs	Method of quantification	Proteomics analysis	Count or content?	Results
Nozaki et al. [65]	Asymptomatic	378	Endothelial-CD 144	Fresh plasma	Flow cytometry	n/a	Count	CD144 independently predicted future CV events in high-risk patients. Addition of EVs to Framingham score improved predictability
Sinning et al. [66]	CAD	200	Endothelial-AV+ CD31+	Fresh plasma	Flow cytometry	n/a	Count	Circulating AV+ CD31+ was independent predictor of CV event in stable CAD
Sarlon-Bartoli et al. [67]	CAD	42	Leukocyte-EV CD11b/CD66b	Frozen plasma	Flow cytometry	n/a	Count	Circulating CD11bCD66b + independently predicted plaque instability
Chironi et al. [14]	Asymptomatic	216	Leukocyte-EV AV+ CD11a	Frozen plasma	Microplate affinity capture assays	n/a	Count	AV + CD11a was increased in patients with 2–3 site subclinical atherosclerosis compared to those with 0–1 site disease
Chiva-Blanch et al. [68]	Asymptomatic	25	Smooth muscle cell-EV AV + SMA alpha; lymphocyte EV AV + CD3+/CD45+	Frozen plasma	Flow cytometry	n/a	Count	Addition of AV + SMA alpha and CD3+/45+ significantly increased prognostic value of Framingham risk score in patients with moderate-to-high risk at 1-year follow-up
Kanhai et al. [69]	Overt CVD, CAD, PAD	1060	n/a	Frozen plasma	n/a	Multiplex immunoassay	Content (protein)	EV levels of CD 14+ were related to increased risk for CV events, all-cause mortality, and ischemic stroke; increase in cystatin C, serpin F2, EV level was related to increased risk for CV events and all-cause mortality
de Hoog et. al. [70]	ACS	471	n/a	Frozen plasma	n/a	Multiplex immunoassay	Content (protein)	Sex-dependent associations of EV pIgR, cystatin C, and C5a with ACS in patients presenting with suspected CAD
Zhang et al. [71]	Acute HF	404				Quantitative immune assay	Content (protein)	EV levels of CD 14+, serpin F2, and serpin G1 were associated with occurrence of HF in patients suspected of acute HF

Jansen et al. [73]	CAD	181	Total AV+, endothelial-AV+ CD31+; platelet CD42b	Frozen plasma	Flow cytometry	Reverse transcription PCR	Count and content (miRNA)	Increased expression of miRNA-126 and miRNA199a in circulating endothelial and platelet EVs was associated with lower major CV event rates. Higher levels also predicted major CV event-free survival and PCI
Mautsumoto et al. [74]	ACS	21	Exosome CD63	Frozen plasma	n/a	Western blot analysis	Content (miRNA)	Increased miRNA 192, miRNA 194, and miRNA 34a from exosomes released in post-MI patients predicted onset of HF within 1 year

CAD coronary artery disease, *PAD* peripheral artery disease, *ACS* acute coronary syndrome, *HF* heart failure, *CV* cardiovascular, *PCI* percutaneous intervention, PCR polymerase chain reaction

changes in beta cell mass that is seen during the development of insulin resistance [81, 82]. Further work is needed, however, to better characterize the role EV miRNA play in systemic cross-talk.

Less research exists examining the role EV cargo in patients with metabolic disease such as T2D [83]. Insulin signaling proteins carried by circulating EVs, such as leptin receptor and phosphor-insulin receptor are decreased in patients with T2D [12], suggesting EV proteins are related to impaired insulin signaling seen in metabolic dysfunction. Additionally, EV protein levels may also provide insight into future development of diabetes. Elevations in the EV protein CD14 were associated with a 16% lower risk for development of T2D in a cohort of patients with CVD [84]. Although these studies are promising, further work utilizing larger sample populations and improvements in proteomics is needed [83].

Besides CVD, other long-term complications from metabolic dysfunction also have significant morbidity and mortality. Since the clinical diagnosis of T2D may occur much after the onset of disease, microvascular complications such as diabetic nephropathy can be present at diagnosis [85]. In fact, nearly 45% of end stage renal disease cases are attributed to T2D, requiring extensive and invasive treatments such as dialysis [86]. Identification of these high-risk patients is crucial in order to prevent the development of these irreversible and burden-some complications. EVs found in urine present a unique opportunity to assess for diabetic nephropathy. Elevations in urine EVs have been found in patients with diabetes plus renal disease versus those with diabetes alone. These findings correlated with decreased glomerular filtration rate [87]. Differing levels of miRNA profiles in urine EVs have been reported in patients with and without diabetic nephropathy, although there is little overlap in the findings, most likely due to small populations and differences in methodology [88–90]. Other EV cargo contained in urine, such as protein cargo, might also hold prognostic value as well, given that higher levels of the glycoprotein C-megalin are documented in the progression of diabetic nephropathy [87, 89] (Table 12.2).

Table 12.2 Extracellular vesicle (EV) count and content as prognostic biomarkers in metabolic dysfunction

Author	Disease	Sample size	Subtype EV	Source of EVs	Method of quantification	Proteomics analysis	miRNA analysis	Count or content?	Results
Kraenondoeck et al. [84]	CVD	1012	n/a	Frozen plasma	n/a	Multiplex immunoassay	n/a	Content (protein)	EV-CD14 levels were associated with a RRR of 16% for development of T2D during a median follow-up of 6.5 years
De et al. [87]	T2D	56	Exosome CD63, CD81	Fresh urine	Nanoparticle tracking analysis	Bicinchoninic acid protein assay	n/a	Count and content (protein)	UEVs correlated with the progression of the albuminuric stages; C-megalin content per UEV with the corresponding numbers of UEVs increased along with the development and progression of DN
Jia et al. [89]	T2D	80	n/a	Frozen urine	Transmission electron microscopy	n/a	Reverse transcriptase PCR	Content (miRNA)	miR-192 levels were significantly higher than the miR-194 and miR-215 levels in UEVs and all three miRNAs were significantly increased in the MIA group compared with the normoalbuminuric
Delic et al. [88]	T2D	8	Exosome	Frozen urine	n/a	n/a	Reverse transcriptase PCR	Content (miRNA)	Deregulated miRNA in MIA DN patients but not in normoalbuminuric DN patients
Prabu et al. [90]	T2D	160	n/a	Fresh urine	n/a	n/a	Reverse transcriptase PCR	Content (miRNA)	Increased UEV miRNA signature levels of let-7i-3p, miRNA-24-3p, and miRNA-27b-3p, and decreased levels of miRNA-15b-5p in MIA. Modifications in miRNA-30a-5p in patients with macro but not MIA
Ferrante et al. [56]	Obesity	7	Exosome: CD63; Adipocyte EV FABP4; macrophage CD14	Adipose tissue	Nanoparticle tracking analysis	n/a	Reverse transcriptase PCR	Content (miRNA)	Exosome miRNA-23b, miRNA-148b, miRNA-4269, and miRNA 4429 differentially expressed between lean vs. obese. TGF-β and Wnt/β-catenin signaling pathways targeted by these miRNAs may be important in the development and progression of chronic inflammation and fibrotic disease

CVD cardiovascular disease, *T2D* type 2 diabetes, *PCR* polymerase chain reaction, *RRR* relative risk reduction, *UEVs* urine extracellular vesicles, *DN* diabetic nephropathy, *MIA* microalbuminuria

12.5 Application in Clinical Practice

12.5.1 Future Work

Despite these promising results, much more work is needed before EVs can be utilized in a meaningful and cost-effective way in clinical practice. Larger, randomized controlled trials examining other known modulators of EV release and content, such as sex [70] and medications [71] must be conducted in accordance with standardized and clearly reported methodologies that would allow for accurate comparison among studies. Although this chapter highlights that EVs have the potential to aid in diagnosis and prognosis assessment of patients with complex CVD and metabolic dysfunction, the practicality of implementation in clinical practices without access to the equipment and personnel necessary to properly implement EV analysis must be considered. As such, future work must consider the potential cost-benefits of implementation of EVs in clinical practice in conjunction with improvements in patient outcomes.

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Therapeutics of Extracellular Vesicles in Cardiocerebrovascular and Metabolic Diseases

13

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Abstract

Extracellular vesicles (EVs) are nanoscale membranous vesicles containing DNA, RNA, lipids, and proteins, which play versatile roles in intercellular communications. EVs are increasingly being recognized as the promising therapeutic agents for many diseases, including cardiocerebrovascular and metabolic diseases, due to their ability to deliver functional and therapeutical molecules. In this chapter, the biological characteristics and functions of EVs are briefly summarized. Importantly, the current state of applying EVs in the prevention and treatment of cardiocerebrovascular and metabolic diseases, including myocardial infarction, atrial fibrillation, myocardial hypertrophy, stroke, diabetes, Alzheimer's disease, fatty liver, obesity, thyroid diseases, and osteoporosis, is discussed. Lastly, the challenges and prospects related to the preclinical and clinical application of EVs receive a particular focus.

Keywords

Extracellular vesicles (EVs) · Cardiocerebrovascular disease · Metabolic disease · Therapeutics

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13.1 Introduction

Extracellular vesicles (EVs) are the general term for nanoscale microscopic vesicles with membrane structure released by cells under physiological and pathological conditions [1], which are secreted by almost all types of cells and are widely found in body fluids, such as blood, saliva, cerebrospinal fluid, urine, sweat, ascites, emulsion, and amniotic fluid [2]. EVs are functional

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phospholipid bilayer vesicles carrying biological information of donor cells, including lipids, DNAs, RNAs, and proteins [3], that can be taken up by recipient cells and affect the physiological processes of recipient cells. EVs can be divided into mainly two types based on their biogenesis:(1) Exosomes (diameter 40–160 nm) generated by the endocytic pathway; (2) Ectosomes (diameter 50 ~ 5000 nm) shed directly from the plasma membrane [4]. Recently, due to the promising therapeutical potential of EVs in treating various diseases, the translational application of EVs, including natural and engineered EVs, is receiving extensive research focus. Natural source of EVs has biological origins that can be derived from stem cells, platelets, endothelial cells, red blood cells, and other cells [5], while engineered EVs, including artificially constructed lipid bilayer vesicles and modified natural EVs, are also commonly used as either therapeutical reagents or drug vehicles.

Recently, increasing studies have indicated the promising potential of the translational and clinical applications of EVs, particularly in the field of cardiocerebrovascular [6–8] and metabolic diseases [9–11]. Some of the traditional chemosynthetic and genetic drugs have unsatisfied performance due to their poor solubility, low stability, and large side effects [12]. The use of organic or inorganic carriers to delivery drugs can improve their solubility and bioavailability, but has bad specificity and may cause immune rejection [13, 14]. Therefore, extracellular vesicles have emerged as a novel drug delivery method and been actively explored. Their natural origins can escape immune response, hydrophobicity enables easier membrane penetration, antiphagocytic surface markers prevent them from phagocytosis by macrophages [15, 16]. More importantly, using EVs as drug carriers can decrease toxicity and improve solubility and stability of drugs.

13.2 Extracellular Vesicles as Therapeutics: Natural or Engineered?

13.2.1 How the Natural EVs Can Be Used for Therapeutics

Native EVs are cell-derived vesicular nanoparticles (30–5000 nm in diameter) that can be released by almost all type of cells and have been found in most cellular microenvironments. Aiming to facilitate intercellular communication, EV secretion is either spontaneously or be triggered by stimulations including cell activation, oxidative stress, oncogenic transformation, radiation damage, cell death, and/or apoptosis [17]. Natural EVs possess diverse endogenous properties that offer stability and facilitate crossing of biological barriers for the delivery of molecular cargo to cells, acting as a form of intercellular communication to regulate function and phenotype. The endogenous properties of EVs enable them as excellent therapeutic tools mainly due to their: (1) ability to delivery and protect bioactive cargo, (2) biocompatibility and membrane penetration, (3) nanoscale, (4) specificity of targeting. However, it is worth noting that there are still some problems with natural EVs: despite their specificity, they are easily trapped in non-specific tissues, especially liver and lung [18], resulting in insufficient targeting in vivo. The heterogeneity and composition complexity of natural EVs may reduce the therapeutic effect and even bring safety problems. Due to the lack of efficient separation methods, industrial production, and accurate monitoring of

drug load, the use of natural EVs in clinical practice is still challenging.

13.2.2 How the Engineered EVs Can Be Used for Drug Delivery Vehicles or Therapeutics

Engineered EVs comprise two different types based on the drug loading methods [4, 19, 20]: one is integrating the drug into the producing cells and collecting the drug-loaded EVs through the natural biosynthesis process, and another one is collecting EVs from different sources, such as stem cells, human blood, bacteria, and other microorganisms and loading drugs into them through different biotechnologies. Engineered EVs possess improved functions in harboring specific pharmaceutical contents, enhancing drug stability and loading rate, and having better tropism and target specificity if modified surface epitopes.

Endogenous Loading

Drugs such as proteins or RNAs can be sorted into EVs through intracellular expression. This method can reduce the difficulty of drug loading and realize drug loading simply and effectively. For example, Dr. Dooley's group identified two "scaffold" proteins, PTGFRN and BASP1, which are preferentially sorted in EVs served as primary candidate to facilitate cargo loading [21]. Exoil-12, currently equipped with PTGFRN, is the first engineered EVs that enter clinical trials in September 2020 by Codiak BioSciences.

External Loading

After purification, the membranes of EVs can be temporarily opened by mechanical or chemical techniques to allow the compounds to diffuse into vesicles. The most common methods include ultrasound, electroporation, saponin treatment, and incubation [22, 23]. These drug delivery methods are more complex than the endogenous expression method and have some disadvantages such as uncontrollable drug delivery efficiency and the need to remove unloaded drugs [21]. Incubation is a very simple method, and many early drug delivery schemes use this method. Saponins are mild surfactants that can cause transient instability of the membrane, thus making the drug enter EVs, but the subsequent removal of excess saponins is required. Liposome fusion is also an ideal method for drug delivery. The fusion lipids of liposome can fuse with exosomes to pass their cargos. Furthermore, instantaneous delivery of CRISPR-based genome editing machinery is important for reducing off-target effects and immune responses. EVs are explored as vehicles to deliver Cas9 or dCas9 ribonucleoprotein (RNP) [24]. However, the lack of a mechanism to enrich RNP into EVs limits the application of EVs as RNP delivery tools. Recently, Dr. Lu's team described a mechanism for active enrichment of RNP into EV [24], which indicates that the efficiency of gene editing using EVs as delivery vector can be improved. In the future, with improved EVs delivery efficiency, targeting ability, and therapeutic effect, engineered EVs can be a promising therapeutic strategy for cardiocerebrovascular and metabolic diseases.

13.3 EV Therapeutics in Cardiocerebrovascular Diseases

In cardiovascular and cerebrovascular diseases, atherosclerosis and ischemia-reperfusion injury can cause damage to vascular endothelial cells, cardiomyocytes, and neurons [25]. It is well established that EVs can cross the blood–brain barrier, remain cell-targeted properties after loading drugs, protect the loaded drugs from degradation, and sustain their stability in circulation [26, 27]. Increasing evidence has shown that natural or engineered EVs carrying different surface markers and contents can interact with target cells and play different roles in the prevention and treatment of cardiovascular and cerebrovascular diseases (Fig. 13.1). A large number of studies and clinical trials have proved that EVs derived from mesenchymal stem cells or cardiac

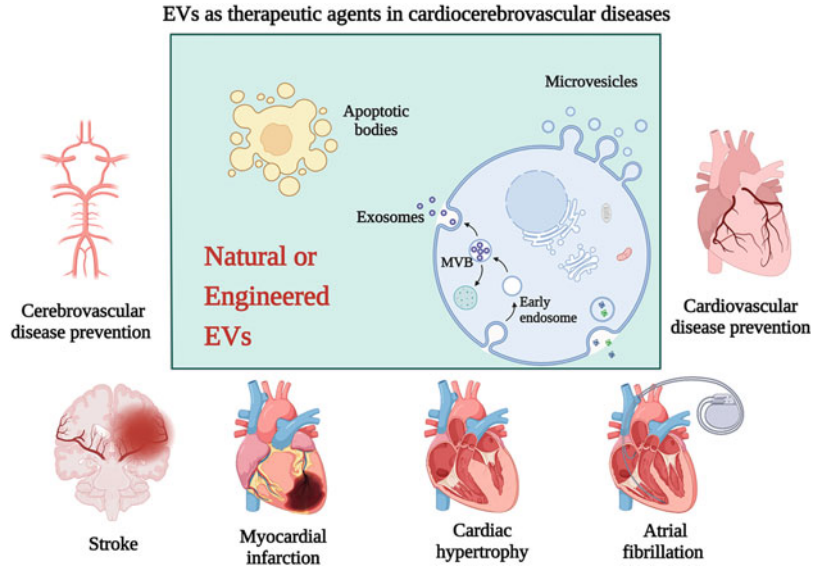

Fig. 13.1 EVs as therapeutic agents in cardiocerebrovascular disease

progenitor cells can protect myocardial tissue, inhibit apoptosis of myocardial cells, and improve cardiac function after myocardial infarction [28–30].

13.3.1 Cardiovascular Ischemia/Reperfusion

Cardiovascular ischemia–reperfusion injury is the main pathological process in the early stage of most cardiovascular diseases [25, 31]. Dr. Yellon's group found that the plasma endogenous exosomes from healthy patients can powerfully protect the heart tissues from cardiac ischemia–reperfusion injury through activating a pro-survival signaling pathway involving toll-like receptor (TLR4) and various kinase [32]. Activation of these extracellular signal-regulated kinase and p38 mitogen-activated protein kinase signaling pathway induces phosphorylation of HSP27, thereby enhancing myocardial tolerance to ischemia–reperfusion injury and achieving myocardial protection. Besides, abundant microRNAs (miRNAs) in EVs have been found to play important roles in cardiac protection in ischemia–reperfusion injury, such as miR-24, miR-486-5p, miR-182-5p and miR-5187-5p [33–36].

One of the main goals in the treatment of ischemic heart disease is to stimulate vascular repair. Angiogenesis is therefore an important biological process in terms of cardiocerebrovascular disease treatment. It is found that the treatment with the conditional medium from human mesenchymal stem cells or its derived exosomes had angiogenic properties, which resulted in reduced myocardial infarct size and preserved systolic and diastolic performance [37, 38]. Exosomes derived from endothelial progenitor cells carry pro-angiogenic miRNA-126 and miRNA-296, which can upregulate angiogenesis factor, promote the proliferation and differentiation of vascular endothelial cells, thereby exerting its anti-apoptotic effect [39, 40]. In addition, some studies suggest that EVs are involved in the prevention of cardiovascular diseases by inhibiting thrombosis. It has been shown that both high and low blood flow will increase the level of tissue factors in white blood cells and EV released, leading to the formation of thrombosis [41, 42]. CD36, a platelet-expressed molecule, is thought to promote thrombosis in a ligand-dependent manner. Studies have shown that platelet-derived EVs containing polyubiquitin can reduce platelet aggregation and inhibit platelet CD36 expression through ubiquitination, thus

inhibiting the formation of atherosclerotic thrombosis [43, 44].

13.3.2 Myocardial Infarction

Myocardial infarction is one of the most common types of ischemic cardiovascular disease, which is most often caused by plaque rupture with thrombus formation in an epicardial coronary artery, resulting in an acute reduction of blood supply and thereby myocardial cell death. Although the clinical treatment using early coronary angioplasty and thrombolytic drug therapy can preserve the damaged heart function to a certain extent, the mortality rate is still high [45, 46]. There is increasing evidence showing that paracrine factors play an important role in myocardial infarction and that changes in circulating microRNAs (miRs) accurately reflect myocardial injury in vivo [47, 48], especially the EVs derived from stem cells or progenitor cells [28–30]. For example, EVs isolated from human induced pluripotent stem cell-derived cardiovascular progenitor cells (hCPC-EVs) were found to be able to decrease the adaptive allogeneic immune response, including the number of cardiac pro-inflammatory Ly6C-high monocytes and circulating levels of pro-inflammatory cytokines, in myocardial infarction mouse models, suggesting the cardioprotective effects of the hCPC-EVs [49]. In addition, the injection of hCPC-EVs derived from human embryonic stem cells into myocardium during acute myocardial infarction effectively promoted myocardial repair, significantly reduced myocardial apoptosis, promoted angiogenesis, and reduced fibrous scar formation after myocardial infarction [50]. Moreover, hypoxic pretreatment of hCPCs can significantly improve the cardioprotective effect of hCPC-EVs.

MicroRNAs delivered by EVs have also been showed to play various roles in cardiovascular disease. It has been shown that miR-93-5p-containing exosomes from adipose-derived stromal cells (ADSCs) have a cardioprotective effect after acute myocardial infarction (AMI). Further mechanism study demonstrated that miR-93-5p can suppress hypoxia-induced autophagy and inflammatory cytokine expression by targeting Atg7 and TLR4, which attenuates AMI induced myocardial damage [51]. Our recent study also showed that miR-30d is abundantly enriched in EVs that derived from cardiomyocytes and inhibits fibroblast proliferation and activation by directly targeting integrin α5 in cardiac fibroblasts [52].

13.3.3 Cardiac Hypertrophy

Cardiac hypertrophy is the abnormal enlargement, or thickening, of the heart muscle, generally caused by hypertension and hemodynamic overload. Hypertrophy growth is thought to be accompanied with many forms of heart disease, including ischemic disease, hypertension, heart failure, and valvular diseases. In response to the high-pressure stress conditions, intracellular microRNA levels are associated with dysfunctional gene expression profiles in many cardiovascular diseases. It has shown that miRNAs have emerged as paracrine signaling mediators to affect intercellular communications and other cellular behaviors by EVs. For example, miR-21-3p is an identified paracrine-acting RNA delivered by cardiac fibroblast-derived exosomes, which can induce cardiomyocyte hypertrophy and using pharmacological inhibition of miR-21-3p in a mouse model of cardiac hypertrophy can attenuate pathology [53]. Therefore, fibroblast-derived miR-21-3p as a paracrine signaling mediator of cardiomyocyte hypertrophy has potential to be a therapeutic target.

The increased angiotensin II (Ang II) level in the heart is considered to be an important factor in inducing rational hypertrophy of heart. Ang II increases EVs release by activating Ang II type 1 receptor and type 2 receptor, which in turn activates mitogen-activated protein kinase (MAPK), Akt protein kinase B (PKB), and renin angiotensin system and eventually exacerbates pathological cardiac hypertrophy [54–57]. It is found that inhibiting cardiac fibroblast-derived EV release by GW4869 and dimethyl amiloride (DMA) decreases the cardiomyocyte hypertrophy

progression, which indicates targeting Ang II-induced exosome release could serve as a novel therapeutic approach to treat cardiac pathological hypertrophy [58].

13.3.4 Atrial Fibrillation

Atrial fibrillation, an irregular and often very rapid heart rhythm (arrhythmia), is one of the most common clinical tachyarrhythmias, which often causes stroke, coronary heart disease, heart failure, limb arterial embolism [59–61]. EVs have also been found to be closely related to the occurrence and development of atrial fibrillation. Abnormal expression and function of ion channels are often occurred in atrial cardiomyocytes, leading to irregular changes of atrial ventricular conduction velocity and resulting in the formation of local focal excitation and reentry of atrial muscle, which is a crucial factor in inducing atrial fibrillation [62, 63]. Interestingly, the abnormal expression of ion channels can be induced by paracrine cells. Coculturing with myofibroblasts decreased the expression of L-type calcium channel Cav1.2 in atrial myocytes, which is thought to be mediated by EVs since inhibiting the myofibroblasts exosome release restored the downregulated Cav1.2 expression [64, 65], suggesting the potential of using exosome release inhibitor as a treatment against atrial fibrillation.

Inflammation is another important risk factor for atrial fibrillation and activated inflammatory cells and complement systems are usually associated with the occurrence and persistence of atrial fibrillation and atrial fibrillation-associated thrombosis [7]. Previous studies have shown that the expression levels of various complement factors, including C1q, C3, C5, C7, C8, complement factor B, and complement factor H, are upregulated in patients with atrial fibrillation [66, 67]. EVs are always considered to reflect the dynamic changes of parent cells and tissues under pathological conditions. Quantitative proteomic analysis of plasma exosomes in patients with paroxysmal atrial fibrillation showed significant enrichment of C7 and C8 in plasma exosomes from patients with atrial fibrillation [68]. These studies suggest that EVs play important roles in pathogenesis of atrial fibrillation, which can act as potential therapeutical targets.

13.3.5 Stroke

The EV-mediated intercellular information exchange also plays important roles in the occurrence and development of stroke disease. Transient or more persistent focal ischemic stroke involves a series of pathophysiological processes, in which brain damage generated and accumulated toxic proteins can be degraded by Nedd4 family interacting proteins 1 (Ndfip1) and ubiquitin ligase Nedd4 [69–71]. Dr. Tan's team found that the exosomes derived from neurons contained Ndfip1 and Nedd4 [72], which could rapidly sequester and remove toxic proteins in the recipient cells after injury. It was also found that overexpression of miR-5121 in stretch-injured microglia-derived exosomes can partly reverse the traumatic brain injury by promoting neurite outgrowth and synapse recovery [73]. Dr. Wang's group also showed that primary human endometrial stromal cell-derived exosome is internalized by human umbilical vein endothelial cells and dorsal root ganglion neurons to enhance neuroangiogenesis [74]. Additionally, exosomes secreted by neural progenitor cells in the stroke patients can promote the migration and tubulogenesis of endothelial cells and exosomes secreted by cerebrovascular endothelial progenitor cells in the stroke patients can improve the proliferation and differentiation ability of neural progenitor cells [75]. These results suggest that cerebrovascular endothelial cells and neural progenitor cells can coordinate with each other and promote neurogenesis and angiogenesis through secreting exosomes after stroke, thus playing a protective role in ischemic tissue.

At present, in vivo and in vitro experiments have confirmed the therapeutic efficacy of exosomes derived from MSCs in brain remodeling after stroke. Currently, more than 700 miRNAs have been found in the exosomes secreted by MSCs, and these miRNAs can

execute different functions in recipient cells [76–78]. Hongqi et al. found that the treatment of miR-133b-enriched exosomes derived from MSCs significantly improved neural plasticity and functional recovery in the ischemic boundary area after stroke [79].

13.4 EV Therapeutics in Metabolic Diseases

Emerging studies have demonstrated that EVs are abundantly produced by cells within metabolic tissues, such as adipose tissue, pancreas, muscle, and liver, and are actively communicating with other cells and organs [10]. Metabolic dysfunction is associated with changes in circulating EV levels as well as the alterations in their EV cargo. EVs have therefore been proposed as novel therapeutical targets or regents against various metabolic diseases (Fig. 13.2).

13.4.1 Diabetes

It has been found that EVs can be used to repair or prevent damage caused by diabetes. Their immunomodulatory and repair properties can protect or even restore the original islets of patients with early diabetes from autoimmune damage and may inhibit the rejection of islet transplantation. Studies have shown that MSC-derived exosomes can promote islet endothelial cell angiogenesis in mice under hypoxia, possibly through the upregulation of HIF1-α, the regulation of VEGF expression, the activation of mTOR signaling pathway, and the promotion of islet endothelial cell angiogenesis [80–82]. A recent study showed exosomes loaded with BAY55–9837, a potential therapeutic peptide in the treatment of type 2 diabetes mellitus (T2DM), significantly increased the plasma half-life of BAY55–9837 27 times longer than that of BAY55–9837 alone and markedly increases insulin secretion, thereby leading to the alleviation of hyperglycemia, when coupled with superparamagnetic iron oxide nanoparticle (SPIONs) with pancreas islet targeting activity and an enhanced blood glucose response with the help of an external magnetic force (MF) [83]. The chronic administration of BAY-exosome-SPION/MF significantly improves glycosylated hemoglobin and lipid profiles.

Skin trauma is a common complication of diabetes that can damage the skin barrier. Chronic diabetic wound complication, represented by diabetic foot, is one of the most serious and costly chronic diabetic complications with the highest

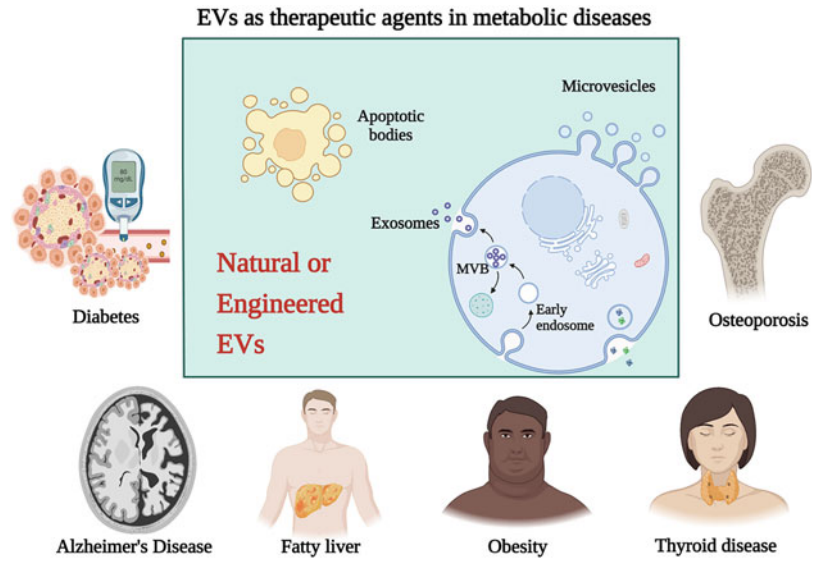

Fig. 13.2 EVs as therapeutic agents in metabolic disease

mortality and disability rate. Traditional treatments involve local debridement dressing and negative pressure wound therapy. However, these existing treatments are inefficient, time-consuming, and expensive [84, 85]. Therefore, improving diabetes chronic wound healing, reducing the amputation rate, and improving the survival rate of patients have become an important area to be addressed. Recent studies have shown that stem cells have great therapeutic potential in regenerative medicine by secreting anti-inflammatory, anti-fibrosis, and pro-angiogenic factors, such as growth factor cytokines or extracellular vesicles, showing a strong skin repair effect [86, 87]. Dr. Hu's group found that adipose-derived mesenchymal stem cells derived exosomes (ADSC-Exos) enhanced the proliferation and migration of human dermal fibroblasts and promoted the proliferation and tube formation of HUVECs in vitro and accelerated the wound healing in a diabetic skin wound model by regulating inflammation, stimulating vascularization, and promoting the production of extracellular matrix [88].

Diabetic nephropathy or chronic kidney disease is another one of the most common complications of diabetes mellitus. EVs are also being proved to play an effectively renal protective role against the renal injury in the T2DM rat model, which is manifested as increased serum creatinine, urea nitrogen, and urinary microalbumin, and alleviated glomerulosclerosis, renal fibrosis, and other pathological changes [89], although the underlying mechanisms still remain unclear [90, 91]. Current studies have found possible mechanisms including: promoting neovascularization, nutrition absorption, and immunomodulatory and reducing inflammatory and oxidative stress [92–95].

It has been shown that obesity significantly increases the risk of diabetes, and obesity is closely related to the metabolic status of adipose tissue or adipose tissue-related cells such as macrophages [96]. Macrophages secrete exosomes containing miR-155 that can target adipocytes to downregulate obesity-related genes, including PPARγ [97]. In addition, circulating exosomal miRNAs, such as miR-122, miR-192, miR-27a-3p, and miR-27b-3p, also inhibit the expression of PPAR-α in white adipose tissue [98], thus alleviate diabetes.

13.4.2 Alzheimer's Disease

Alzheimer's disease (AD) is a common age-related neurodegenerative disease, which causes cognitive decline in the elderly and affects self-care ability and quality of life. Still now, due to the lack of a complete understanding of its etiology and pathogenesis, AD remains a big challenge without effective drugs. The extracellular accumulation of Aβ amyloid proteins in brain is considered to be one of the major causes of AD; thus, removal of Aβ protein accumulation is thought to be a potential direction for AD treatment [99, 100].

As early as 2013, Dr. Ochiya's team for the first time found that exosomes secreted by human adipose-derived MSCs could significantly reduce the accumulation of intracellular and extracellular Aβ protein in neurons [101]. Next, Mijung Lee et al. found that EVs from adipose-derived stem cells can significantly decrease the apoptosis of AD neuronal cells and promote axonal regeneration in damaged neurons [102]. Shan-Shan Wang et al. then demonstrated the beneficial effects of MSC-derived EVs in a mouse model of AD, which is manifested as improved cognitive behavior, rescued impairment of CA1 synaptic transmission, and long-term potentiation [103]. Victor Bodart-Santos et al. also found that EVs derived from human Wharton's jelly mesenchymal stem cells can protect and promote regeneration of neurons by keeping the number of synapses in hippocampus neurons stable and promote synaptic growth by inhibiting the reduction of synaptic protein PSD-95 caused by Aβ protein accumulation [104]. One of the pathological conditions during AD progression is hypoxia, and the composition of stem cell-derived EVs is significantly affected by different culture conditions. It had been found that the expression level of miR-21 in MSC-EVs was significantly

increased after hypoxia treatment, and these miR-21 enriched MSC-EVs significantly improved the learning and memory capabilities and lowered the plaque deposition and Aβ levels in APP/PS1 AD mice when compared with normoxic MSC-EVs [105]. These studies demonstrate that EVs derived from MSCs can be utilized as promising therapy in repairing cognitive impairment of AD.

In addition, EVs also have a regulatory effect on the microglial immune response. Studies found that EVs can promote microglia polarization to anti-inflammatory M2 subtype in AD transgenic mice, and upregulate the expression of anti-inflammatory factors, including TGF-β and IL-10, in brain tissues, which can improve the neuronal survival microenvironment [106–108]. This immunomodulatory effect of EVs is also confirmed in transgenic mouse AD models. Together with above discussed therapeutic effects of degrading Aβ clusters and promoting cognitive function of brains, EVs hold great potential of significantly slowing the progression of cognitive impairment in AD patients.

13.4.3 Fatty Liver

As one of the most important solid organs of human body, liver composes a variety of cells, including hepatocytes, hepatic stellate cells, Kupffer cells, hepatic sinusoidal endothelial cells, and other immune cells [109]. The communication among these different cells is crucial for maintaining the homeostasis of liver function and their malfunction will lead to the disorder of lipid metabolism in liver, which eventually results in the occurrence of fatty liver [110]. The most common types of fatty liver disease are non-alcoholic fatty liver disease (NAFLD) and alcoholic fatty liver disease (ALD). EVs serve as an important communication medium among these liver cells, which can be produced and absorbed by a wide range of liver cells, including all of the above cell types. Liver is recognized as an ideal target organ for EV-based therapy due to the inherent regenerative capacity of liver tissue and its tendency to trap nanovesicles in the circulation [111]. There is increasing evidence suggesting that EVs have great potential in the treatment of fatty liver [112, 113].

EV-based therapy, including natural and engineered EVs, has become a hot research topic in the treatment of liver diseases [114], although most of the EVs-based fatty liver therapies are still in the preclinical stage and most EVs in these studies were derived from mesenchymal stem cells. Ohara et al. found that EVs isolated from amniotic-derived mesenchymal stem cells can deactivate stellate cells and Kupffer cells by suppressing the lipopolysaccharide (LPS)/TLR4 signaling pathway, which ameliorate inflammation and fibrogenesis in a rat model of non-alcoholic steatohepatitis (NASH) and hepatic fibrosis [115]. Bruno et al. found that the human liver stem cell-derived EVs can significantly reduce liver fibrosis and inflammation, demonstrated by the downregulation of fibrosis-associated genes, indicating the anti-fibrotic and anti-inflammatory effects of EVs-target therapies for fatty liver [116]. Hou et al. demonstrated that miR-223-enriched EVs released from IL-6-stimulated myeloid cells can target liver cells and attenuate NAFLD-related fibrosis [117]. In addition, EVs derived from induced pluripotent stem cells can be internalized by hematopoietic stem cells and inhibited fiber formation through exosomal miRNAs, including miR-92A-3p and miR-302-3p [118].

Compared to its application in NALD treatment, EV-based therapies have not been extensively studied in the treatment of ALD. Momen-heravi et al. found that miRNA-122 inhibitors delivered by engineered EVs can be used to prevent the release of pro-inflammatory EVs produced by alcohol-treated liver cells [119]. Increased circulating EV concentrations have been reported in patients with ALD and alcohol-fed mice compared with healthy individuals [58]. Alcoholic hepatitis (AH), the most severe form of ALD spectrum, has higher plasma concentrations in several EV subgroups when compared with heavy drinkers without AH

[120]. In addition, hepatocyte-derived EVs in the AH mice are enriched with miR-27a and miR-181 [121]. Therefore, strategies targeting biogenesis, release, and transport of EVs are of potential interest in treating alcohol-related liver disease.

13.4.4 Obesity

Obesity is a complex disease characterized by excessive fat accumulation, which leads to metabolic abnormalities, such as T2DM, fatty liver, and cardiovascular and cerebrovascular diseases [122]. The mechanisms linking obesity to metabolic disorders are complex, but the dysregulation of adipose tissue-derived EVs has been identified as an important pathogenic factor in the obesity-induced diseases [123–125], within which exosomal miRNAs have been shown to play critical roles.

Although the mechanism of obesity-related diseases is quite complicated, EVs have given us an alternative hint that is to look at the communications between different cells such as adipose cells and immune cells to get a better understanding of the intricate signaling pathways. In fact, increasing evidence has shown this adipose cells–immune cells interplay plays critical roles in the pathogenesis of obesity-related diseases, which also acts as potential therapeutical targets. One of the recipient cells of adipocyte-derived EVs is macrophage. The adipose tissue of mice on chew diet is dominated by M2-type macrophages, which helps in maintaining the systemic metabolic homeostasis, partly due to the EV-mediated signaling and nutrient exchange between adipocyte-resident immune cells and metabolic organs [126]. However, high-fat-diet-induced obesity disrupted this balance by increasing the level of miRNA-34a in adipocyte-released EVs. As a result, the shift of macrophages from M1 to M2 was inhibited and adipose inflammation was activated. Notably, the transfused EVs that isolated from obese mice were mainly taken up by peripheral blood monocytes in lean mice and then promoted cytokine release and macrophage activation, leading to systemic insulin resistance [127]. Therefore, targeting adipocyte-derived exosomal miR-34a could be promising therapeutics to treat chronic obesity-related diseases [128]. Furthermore, it is also found that the exosomes secreted by M2 macrophages can enhance insulin sensitivity in obese mice through delivering functional miR-690 [129]. The administration of exosomes derived from ADSCs can reduce adipo-inflammation by driving M2 polarization of adipose tissue macrophages, thereby preventing diabetic liver steatosis and the progression of obesity [130]. Another study also found that the treatment of ADSC-derived exosomes can improve the metabolic homeostasis in obese mice, including improved insulin sensitivity, reduced obesity, and alleviated liver steatosis [131]. These findings described a novel exosome-mediated ADSC-macrophage cross-dialogue mechanisms that promote immune and metabolic homeostasis in white adipose tissue, thus providing potential therapies for obesity.

It is also worth noting that other organs may also produce extracellular vesicles associated with insulin resistance during obesity progression. For example, hepatic exosome-containing miR-130a-3p suppresses adipogenesis and attenuates glucose intolerance by targeting PHLPP2/AKT/GLUT4 axis in adipose cells [132]. As an insulin-secreting organ, the islet is also involved in regulating insulin sensitivity. miR-26a in pancreatic β-cell-derived exosomes can alleviate obesity-induced insulin sensitivity and hyperinsulinemia [133]. Although current researches on EVs as therapeutics for obesity-related diseases are just the tip of the iceberg, advanced technologies and methods will greatly advance the therapeutical application of EVs in obesity.

13.4.5 Osteoporosis

Osteoporosis is a common orthopedic disease characterized by low bone mass and damaged bone microstructure after bone metabolism disorder, resulting in decreased bone strength, increased bone fragility, and fracture risk [134]. Dysfunction of mitochondria caused by

intercellular signal transduction is the main cause of this disease, and the therapeutic effect of EVs through transport of substances is an interesting research area. EVs is an intercellular communicator that can deliver encapsulated substances, alter the phenotype and function of target cells, and mediate intercellular communication [135]. Therefore, EVs is of great value in the treatment of osteoporosis.

As one of the most important cargo in EVs, miRNAs play pivotal roles in bone homeostasis repair and regeneration. Chen et al. found that the miR-503-enriched EVs that derived from peripheral blood mononuclear cells can inhibit RANKL-induced osteoclastogenesis and prevent bone loss in ovariectomy mice [136]. Meanwhile, Deng et al. found that osteoblasts can promote the osteoclast formation by shedding microvesicles that contain functional RANKL and being transferred to osteoclast precursors [137]. In order to better understand the role of EVs in osteoblast–osteoclast communication, Cappariello et al. loaded osteoblast-derived EVs with osteoclast suppressor drugs, including zolenic acid and dasatinib, and found that osteoblast EVs can shuttle osteoclast suppressor drugs to inhibit osteoclast activity both in vivo and in vitro [138], which opens up a way for the application of EVs in the treatment of bone diseases. Additionally, Song et al. showed that EVs derived from vascular endothelial cells have a more effective bone-targeting effect than EVs derived from osteoblasts or bone marrow mesenchymal stem cells on inhibiting osteoclast activity and differentiation through miR-155 [139]. Therefore, EVs containing miR-155 may be potential targets for the treatment of osteoporosis. Fang et al. found that peripheral blood mononuclear cell-derived EVs could significantly restore the decreased osteogenic differentiation of bone marrow mesenchymal stem cell in steroid-induced femoral head necrosis [140], which is a potential treatment strategy for steroid-induced femoral head necrosis. Interestingly, some tumor cells can influence osteoclast function by secreting EVs. Guo et al. found that the secretion of EVs containing miR-20a-5p by breast cancer cells can promote the proliferation and differentiation of osteoclasts [141]. Xu et al. found that increased expression of miR-21 was observed in EVs derived from lung adenocarcinoma cells and promoted osteoclast generation by targeting programmed cell death protein 4 [142]. These studies revealed that EVs from a variety of cells can regulate osteogenic differentiation and hold great clinical potential to be applied in the treatment of osteoporosis.

13.5 Challenges and Future Directions

EVs contain abundant biological information about the origin of donor cells, which can reflect the specific state of parent cells. Meanwhile, EVs can mediate intercellular communication and regulate the function of adjacent or distal recipient cells, thus regulating the pathophysiological process of diseases and playing important roles in the treatment of cardiocerebrovascular and metabolic diseases. Existing studies have proved that EVs containing functional biological cargo can be used in the treatment of myocardial infarction, atrial fibrillation, diabetes, Alzheimer's disease, and dyslipidemia at the level of expression in the body [10, 94, 143–146]. However, EVs research is still in its infancy, and our understanding of the outer membrane structure of the cell is far from enough. These vesicles are released into the extracellular environment and are not immediately devoured by surrounding cells, which is their characteristic for remote signal communication. Advances in understanding these vesicles will challenge current conventional ideas about cell–cell communication and open up new opportunities for translational medicine, such as diagnosis and therapy. Moreover, the therapeutic effects of EVs are mostly animal experiments and cell experiments, and the conclusions obtained are not convincing due to the limited sample size of clinical studies. In addition, there is no unified standard for the separation, detection, and purification of exosomes and particulates, which leads to insufficient recognition of EVs in clinical

application. It is still lack of efficient and reliable EVs classification and separation methods. Therefore, a unified, efficient, and reliable EVs separation method needs to be established before clinical application.

Second attention should be paid to the complexity of the relationship between EVs and cardiocerebrovascular diseases and metabolic diseases: most of the existing studies mainly focus on the biological effects of EVs on target cells, associated with corresponding phenotypes and diseases. EVs are highly heterogeneous, and their contents are related to cell types, cell states, and stimulation of cells. EVs contain hundreds of proteins, as well as a variety of RNA, lipids, and metabolites. These characteristics lead to a complex relationship between EVs and target cells in cardiocerebrovascular and metabolic diseases, which requires a combination of proteomics and modification omics, lipidomic, and other research methods. On the basis of multiple omics results, the corresponding target molecules are selected to improve the mechanism research and find new targets for disease prevention and treatment.

Thirdly, the targeting mechanism of EVs is not clear: the forms of EVs targeting involve surface ligand receptor binding, direct membrane fusion, and cellular uptake. However, the specificity of EVs is still an important question for its translational implications in clinical studies.

In conclusion, as a non-classical cellular communication mediator, EVs have important physiological and translational functions, especially in this review's focus of cardiocerebrovascular and metabolic diseases. The components of EVs are of great significance in elucidating the pathophysiological mechanism of cardiocerebrovascular and metabolic diseases, and EV itself is great therapeutic tool of delivering drugs. EVs have promising potentials of understanding the pathogenesis of cardiocerebrovascular and metabolic diseases and translational applications. Therefore, it is urgent to establish a standardized and high-purity EVs separation method and combine multiple omics research with big data analysis to open up more possibilities for developing EV-based therapeutics in the treatment of cardiocerebrovascular and metabolic diseases.

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Gene Therapy of Extracellular Vesicles in Cardiovascular and Metabolic Diseases

14

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Abstract

The ultimate and most complex form of treating human diseases is embodied by gene therapy. For an effective gene therapeutic product we need to hack the cellular plasma membrane entry-system, then escaping degradation in the cytosol and in most cases, we need an efficient hacking of the nuclear membrane-system, achieving the delivery of genetic construct into the central stage of the target cells: nucleoplasm or chromosomal DNA found in this highly controlled space. These steps need to be performed in a targeted, ordered, and efficient way. Possessing intrinsic ability of nucleic acid and protein delivery, extracellular vesicles can bypass biological barriers and may be able to deliver a next-generation platform for gene therapy. Fine-tuned genetic constructs included in (synthetic) extracellular vesicles may provide an upgraded approach to the current gene therapeutical technologies by significantly upgrading and improving biosafety, versatility, and delivery, thus evoking the desired therapeutic response. This chapter addresses the main types, vectors, challenges, and safety issues of gene therapy. Afterwards, a brief introduction and beneficial roles of extracellular vesicles are given. The concept of engineering vesicles for gene therapy is also discussed. A snapshot of most relevant clinical trials in the field of cardiovascular and metabolic diseases is shown. Finally, a wrap-up and outlook about gene therapy are presented.

Keywords

Gene therapy · Extracellular vesicles · Synthetic EVs · Genetic constructs · Metabolic diseases · Cardiovascular diseases

14.1 Background

DNA is the most effective and reliable form for storage of information. Cells are continuously encrypting–decrypting the information stored in the 25 types of DNA molecules (22 autosome, 2 heterosome linear chromosomes, and 1 mitochondrial circular chromosome) and make use of the acquired information based on intra- and extracellular stimuli. Around 1.15% of the human genome encodes for proteins [1]. DNA sequence variations in the protein encoding genes and/or other regions, which impact the transcription of the protein coding genes are responsible for inherited disorders and greatly influence the development of most human diseases. According to the ClinVar database the most common pathogenic variations are single

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nucleotide variations (SNV), representing around 50% of the alterations found in the background of human diseases [2].

The concept of gene therapy was introduced in 1947 by Clyde Keeler, even before the depiction of the DNA structure [3]. In the third decade of twenty-first century human gene therapy is starting to become a reality for treating/healing inheritable genetic disorders [4], infectious diseases, and acquired cancer diseases, so far the United States (US) Food and Drug Administration (FDA) and/or the European Medicine Agency (EMA) has approved several gene therapy products (Table 14.1). On the clinicaltrial.gov website there are 5627 listed studies involving gene therapy, the US leads with 2777 registered studies, followed by European Union and UK with 1506 studies. Currently, in the European Union Clinical Trials Register there are 1358 registered clinical trials involving gene therapy (DOA: 08.04.2023). Most gene therapy-involving trials target cancer; however, around 10% of the trials are addressing rare inheritable disorders (including metabolic disorders) and around 6–8% address cardiovascular diseases. Pharmacologically, gene therapy is a nucleic acid-based complex drug. It is a fascinating and one of the most complex approaches to treat or cure human diseases. The delivery of genetic information to diseased target cells is quite complex and requires transport through complex cellular and tissue barriers [7]. Gene-corrected cells must survive in the long-term (or transmit the modification upon cell division to their daughter cells), reverse the condition, and at the same time escape immunological recognition. Since the number of treated people is relatively low, the spectrum and frequency of short- and long-term side effects of gene therapy are not yet fully uncovered. Delivery vehicles of genetic constructs are imperfect, each with many downsides and/or limitations. Extracellular vesicles, as natural nano-sized shuttle messengers of extracellular communication may contribute to the development of next-generation vehicles for gene constructs. Currently, EV-based gene therapies are at preclinical stage, designing and optimizing both the EVs and the genetic constructs need to be finished before proceeding into clinical phase. The goal is to have highly efficient and safe gene therapies on the market in the near future.

14.2 Basic Principles

The aim of gene therapy is to provide optimal gene expression in order to restore phenotypic balance. Gene therapy can directly manipulate the genome, can target transcripts with oligonucleotides and exon splicing events may be altered by small molecules [1]. To achieve the genetic modification some artificial **genetic construct** is required: often a DNA molecule or an RNA molecule or a synthetic oligonucleotide [8], which is frequently accompanied by other accessory molecules. Moreover, recent advances in genome editing techniques push the boundaries and perspectives for gene therapies. Nucleases, that are able to induce double-stranded DNA breaks (DSB) at target sites are clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) nuclease systems, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and engineered meganucleases, as well genome editing without DSBs through base editing, such as prime editing and death editing hold potential for methodological improvement.

14.2.1 Forms of Gene Therapy

Gene therapy nowadays is a major and hot topic in human genetics, due to its potential impact upon human diseases; however, many barriers need to be passed before real clinical transition will happen. There are two main therapeutic strategies: either diseased cells are targeted to alleviate the disease or diseased cells are targeted for selective destruction. The gene construct can induce short- or long-term effects. If the genetic construct contains DNA that is designed to be introduced into nuclear chromosomal DNA, the genetic alteration will have a long-term effect. The transgene becomes a stable part of the

Table 14.1 Approved gene therapeutic products by U.S. Food and Drug Administration (FDA) and/or the European Medicine Agency (EMA) [5, 6]

Name	Active ingredient	Indication	Vector	Target	Route of Adm	Year of approval
VITRAVENE (Novartis)[a]	Fomivirsen sodium	CMV retinitis		CMV IE-2	Intravitreal	1998
MACUGEN (Valeant)[b]	Pegaptanib sodium	AMD		VEGF via ASO	Intravitreal	2004
GLYBERA (UniQure)[c]	Alipogene tiparvovec	Lipoprotein lipase deficiency	AAV1	LPLS447X	Intramuscular	2012
KYNAMRO (Kastle)[d]	Mipomersen sodium	hoFH		Apolipoprotein B-100	Subcutaneous	2013
IMLYGIC (Amgen)	Talimogene laherparepvec	Metastatic melanoma	HSV-1	GM–CSF–HSV-1	Intratumoral	2015
STRIMVELIS (GSK)	Autologous CD34+ cells	ADA-SCID	RV (ex vivo)	ADA gene	Intravenous	2016
EXONDYS 51 (Sarepta)	Eteplirsen	DMD		Dystrophin	Intravenous	2016
SPINRAZA (Biogen)	Nusinersen sodium	Spinal muscular atrophy (SMA)		SMN mRNA	Intrathecal	2016
ZALMOXIS (MolMed)	Allogeneic T cells	Melanoma	RV	RV-ΔLNGFR+ HSV-TK Mut2	Intratumoral	2016
KYMRIAH (Novartis)	Tisagenlecleucel	B-cell lymphoma B-cell ALL		CD19+ cancerous B-cells via CAR-T cells	Intravenous	2017
LUXTURNA (spark therapeutics)	Voretigene neparvovec-rzyl	Retinal dystrophy	AAV2	*RPE65*	Subretinal injection	2017
YESCARTA (kite pharma)	Axicabtagene ciloleucel	B-cell lymphoma		CD19+ cancerous B-cells via CAR-T cells	Intravenous	2017
ONPATTRO (Alnylam)	Patisiran sodium	hATTR	Lipid nanoparticle	Transthyretin	Intravenous	2018
TEGSEDI (Akcea therapeutics)	Inotersen sodium	hATTR		Transthyretin	Subcutaneous	2018
ZOLGENSMA (AveXis)	Onasemnogene abeparvovec-xioi	SMA	AAV9	Episomal *SMN1* gene augmentation	Intravenous	2019
ZYNTEGLO (bluebird bio)	Betibeglogene autotemcel	Beta-thalassemia	LV (ex vivo)	$\beta^{A\text{-}T87Q}$-globin	Intravenous	2019
WAYLIVRA (Akcea therapeutics)[e]	Volanesorsen	Familial chylomicronaemia		apoCIII mRNA via ASO	Subcutaneous	2019

(continued)

Table 14.1 (continued)

Name	Active ingredient	Indication	Vector	Target	Route of Adm	Year of approval
LEQVIO (Novartis)	Inclisiran	LDL-C-hypercholesterolemia		*PCSK9* via siRNA	Subcutaneous (yearly twice)	2020
TECARTUS (kite pharma)	Brexucabtagene autoleucel	Mantle cell lymphoma	gRV (ex vivo)	CD19+ cancerous B-cells via CAR-T cells	Intravenous	2020
ABECMA (Bristol Myers)	Idecabtagene vicleucel	Multiple myeloma		BCMA-directed CAR T-cell	Intravenous	2021
BREYANZI (Bristol Myers)	Lisocabtagene maraleucel	Adult large B-cell lymphoma		CD19-directed CAR T-cell	Intravenous	2021
SKYSONA (bluebird bio)	Elivaldogene autotemcel	Cerebral adrenoleukodystrophy	LV (ex vivo)	CD34+ cells transduced with functional copy of *ABCD1* gene	Intravenous	2022
UPSTAZA (PTC Therapeutics)	Eladocagene exuparvovec	Aromatic L-amino acid decarboxylase (AADC) deficiency	AAV2	*AADC* gene augmentation	Intravenous	2022
ROCTAVIAN (BioMarin)[f]	Valoctocogene roxaparvovec	Adult severe hemophilia A	AAV5	*F8* gene augmentation	Intravenous	2022

[a]Withdraw of marketing authorization
[b]No longer authorized in EU
[c]In 2017 it has been withdrawn from the EU market due to its high price
[d] is no longer marketed in the US
[e]Only conditional marketing approval in EU
[f]Approval pending by FDA, approved by EMA

chromosome structure and thus will be transmitted during subsequent cell divisions to daughter cells [8].

The alteration in diseased cells is dependent on the type of genetic error: (1) if the disease is elicited by a loss-of-function mutation type (e.g., many metabolic disorders), the designed gene therapy usually acts through gene augmentation (by adding one or more functioning copies of the relevant gene), gene correction (alteration of the sequence by gene editing techniques) or by increasing the gene expression—resulting in biological active protein (e.g., by exon skipping with antisense oligonucleotides—ASO); (2) if the disease is caused by a gain-of-function mutation type, the gene therapy may act through gene ablation (by inhibiting the expression of the affected gene either by inhibiting the transcription or by applying a gene silencing approach via RNAs) or gene correction (by repairing the genetic lesion by genome editing).

Two major classes are distinguished: somatic gene therapy and germ-line gene therapy. In somatic gene therapy the consequences of the genetic modification cannot alter germ-line cells, the effect should be restricted to the treated patient. Germ-line gene therapy theoretically would imply the genetic modification of a gamete, zygote, or early embryo during in vitro fertilization [8]. Currently, due to ethical and safety issues only somatic gene therapy approach is permitted in humans. Somatic gene therapy can be achieved either as an ex vivo or in vivo approach. In most gene therapy trials in vivo gene therapy is the chosen form. The in vivo approach means that the genetic constructs are delivered via vectors in situ within the patient. Regardless of the in vivo/ex vivo approach, the **vector choice** is dictated and limited mostly by the proliferation rate of the target cells. If the targeted cells are mostly dividing cells, those vectors of the genetic construct should be chosen, which will allow the integration of the genetic construct into nuclear DNA molecules (e.g., lentiviral vectors). Otherwise the genetic construct delivered by non-integrating vectors will be diluted progressively after each cell division. In order to prevent this phenomenon, a repeated administration may also be an option [9]. If the targeted cells are mostly in a post-mitotic state, a non-integrating vector would be preferable [10]. The process of introduction of various genetic constructs into targeted human cells by viral vectors is known as **transduction**, whereas the delivery of genetic constructs by non-viral systems is known as **transfection**. A broader term, that encompasses both transduction and transfection, is **transgenesis**, which refers to the artificial transfer of genetic material into cells, regardless of the vector [8].

Genome editing enables the replacement, elimination, or modification of specific DNA sequences [11] and mostly relies on endonucleases, which cut the DNA and protein complexes that rejoin the broken DNA strands. In some scenarios, guide RNAs (gRNA) are used to specify the target DNA site and template oligonucleotides are also used to alter the original gene sequence (containing the pathogenic variant) for a desired sequence of choice. The result can be a homozygous gene inactivation (gene knockout), the introduction of a transgene (gene knockin), deletion of a target sequence or DNA sequence correction.

CRISPR-Cas system permits in situ gene sequence correction, gene ablation, or gene augmentation [12]. For gene replacement approach it is of key importance to achieve homogenous and physiologic gene expression, resembling the gene expression pattern of healthy tissues.

Two strategies can be applied for a gene augmentation approach: (1) successful delivery into a smaller number of cells and achieving a supraphysiologic expression of the transgene—resulting in restoration of tissue function or (2) reaching therapeutic levels by achieving a successful delivery in a greater proportion of the target cells in the affected tissue leading to a near-physiological gene expression level [10].

Of note, non-viral vectors have been successfully used in next-generation vaccines, to deliver viral-protein encoding nucleoside-modified mRNA in PEGylated nanoparticles intramuscularly to elicit cellular and humoral immune

response against SARS-CoV-2 [13], and more pathogens are on the list for development of this class of vaccines. Clinical data resulting from mRNA-based vaccines may also contribute in the near future to enhancement of optimal genetic constructs delivery strategies. Furthermore, chemically modification of nucleic acids, protecting against nuclease degradation may also contribute to the successfulness of gene therapy.

14.2.2 Current Pitfalls of Gene Therapy: Delivery, Durability, Off-Target Activity, Clinical-Scale Manufacturing

The two most commonly used viral vectors are the lentiviral and adeno-associated (AAV) viral vectors. Up-to-date worldwide more than 200 patients have been treated with lentiviral vector-based gene therapy for various diseases, including mucopolysaccharidosis, chronic heart failure, sickle cell disease, Fanconi anemia, and thalassemia [14, 15]. In order to achieve the therapeutic goal, a targeted and high delivery rate must be reached in the available timeframe-for-treating. This must be achieved without or minimal side effects along high safety. Delivery is also affected by the localization and accessibility of the targeted cells: some cells are easily targetable as blood, skin, muscle cells, and cells situated in the eye; some cells are harder to reach (e.g., brain cells). The engineering of novel vehicles capable of encapsulating genetic constructs and showing good targetability and penetrability is one of the bottleneck factors nowadays [16]. Another major hurdle is also the prediction of vector copy number per cell obtained after gene therapy [14].

Overall the viral vectors represent a more efficient delivery system; meanwhile, non-viral vectors represent the safer, more biocompatible delivery form. Another general pitfall is the development of humoral or cellular immunity against therapeutic product induced by gene therapy [7].

Vector-Associated Pitfalls

Overall, viral vectors have limited cloning capacity, tropism, and stability.

Lentiviral Vectors

Lentiviral vectors can reach the cell nucleus through the nuclear membrane pores easily and infect both dividing and non-dividing cells and integrate the genetic material into chromosomal DNA; however, their cloning capacity is around 8 kb. They also pose a low risk of oncogene activation; nevertheless, the long-term genotoxicity cannot be excluded, because of the quasi-random integration into the nuclear genome [14]. Currently, the manufacturing of lentiviral vectors at clinical scale is also very challenging [10].

AAV Vectors

AAV vectors are able to target both dividing and non-dividing cells but have a relatively low cloning capacity around 4.5–4.7 kb. The rep gene is accountable for the occasional integration of AAV-carried nucleic acid into chromosome 19 (mainly *AAVS1* site and surrounding regions). Even AAV vectors lacking the rep gene can integrate into nuclear genome, however at a low frequency of around 0.1% of total vector genomes [17]. Recently it has been reported that AAV vectors used for genome editing with CRISPR-Cas system may elicit a relatively high level of AAV integration at on-target CRISPR cut site in a preclinical study [17]. Many people have already preexistent wild-type AAV immunity, around 20–40% of adult population have both humoral and cellular anti-AAV capsid-specific immunity. The presence of such immunity leads to significant vector neutralization and consequently reduced therapeutic efficacy [10].

Delivery is hardened for in vivo gene therapy liver-targeting by at least three factors: (1) potential toxicity of gene therapy amount given in one-time shot into the bloodstream, (2) inactivation of gene constructs carrying vectors by pre-existing vector-specific neutralizing

antibodies, (3) clearance of gene therapy products before reaching target hepatocytes by phagocytes residing in the liver [7].

Adenovirus (Ad) Vectors

The main drawback is the immunogenicity that is evoked by the adenoviral vectors; however, newer designs result in weaker immune responses against these vectors. Ad vectors are used mostly for cardiovascular disease involving gene therapy clinical trials, where mostly a local delivery, e.g., intracoronary injection is desirable.

Genetic Construct-Associated Challenges

Genome Editing via CRISPR-Cas System

The downside of using the CRISPR-Cas system is the possible off-target activity, which refers to the unintended editing of DNA sequences which are similar to the target site. It has been reported that the binding of sgRNAs to target sequences can happen even up to 5 bp mismatches. Off-targeting may activate oncogenes or inactivate tumor-suppressing genes. The induction of DSBs may also increase the risk for genomic instability, which may lead to chromosomal breaks, resulting in translocation/inversion/deletion (on-target mutagenesis) [11]. Gene correction by homology directed repair (HDR) requires the delivery of a DNA template, which is not fully resolved yet [12]. Besides, before applying HDR-based genome editing, the *TP53* gene should be assessed for pathogenic variants. The rate of off-target is also influenced by the ratio of Cas: gRNA [18]. It is advised to analyze, both in vitro and preclinical studies, the frequency of off-targeting and its effects. However, extrapolation of these results must be taken with caution, as in clinical settings unforeseen, new off-targets or effects may arise. Another aspect to be kept in mind is that level of gene editing efficiency and heterogeneity in target cells should also be evaluated by a suitable method [11]. In vivo delivery in children and adults of reproductive age may also harbor the germ-line cells [11].

Genome Editing Without Genomic Cleavage

Base editing allows precise substitution of nucleotides [19]. It can also be used to engineer premature stop codons to abolish the expression of pathogenic variants [18]. The DNA modification is achieved by deaminase or dead Cas9 (with deactivated catalytic domain) that enables a direct and irreversible conversion of nucleotides [11]. However, base editing is limited to correct only transition mutations and most of the times requires dual-AAV for delivery, since the size of base editing complex is 6.1 kb, which is above the cloning capacity of AAV vectors [2]. Another shortcoming is the inaccessibility to certain genomic sites [18].

Overall, the transient expression of the components of genetic constructs is desirable, significantly decreasing the rate of unintended editing [18]. Still, the unwanted editing in other cells than target cells today remains a concern to be resolved in the near future [2].

14.2.3 Impact of Gene Therapy upon the Immune System

The immune system may recognize the delivery system or the genetic construct and develop long-term immunity against them. Gene therapy may trigger intracellular pathways, which in return may alter the proliferation, differentiation, or overall fitness potential of the treated cells [14]. Gene therapy itself may also induce antibody and cellular immune response against the transgene delivered by this therapeutic approach [20]. Addition of 4 mol% (of the total lipid) of a pro-drug of dexamethasone into lipid nanoparticles/synthetic extracellular vesicles containing nucleic acids may inhibit the therapy-induced immune response [21].

In a study of 200 human samples in 2.5% of the samples circulating anti-SpCas9 antibodies and in 10% of the samples circulating anti-SaCas9 antibodies were detected [11]. The wild-type AAV is recognized by the immune system and initiates the formation of long-term anti-AAV immunity, resulting in rapid clearance of

subsequent AAV-based gene therapies [22]. After 4–12 weeks of AAV vector-based gene therapy a delayed cellular immune response may arise, negatively impacting the efficacy of gene therapy [10].

An anti-vector immune response is rarely or not at all induced by non-viral delivery system, whereas viral vectors, depending on the vector may induce weak, medium, or even strong immune response. Because of the low immunogenicity of EVs even a pulsed delivery of genetics constructs could be feasible.

Anti-transgene immunity may exist even before gene therapy, developed, e.g., by recombinant enzyme replacement therapy. Nucleases and other enzymes used for genome editing are non-human proteins, they are heterologous antigens and immune response against these proteins may also be triggered [11].

Through in vivo induction of antigen specific regulatory T cells a persisted immune tolerance may be generated that has the ability to counterbalance the immune response against the genetic construct and/or vector [20].

14.2.4 Safety Concerns

The main safety concerns are genotoxicity, molecular, cellular, and tissue toxicity induced by various mechanisms [16]. The lack of universal delivery system is a main driver cause of safety concerns, as many gene therapy strategies rely on newly developed, altered, or unique vectors, which have not been fully mapped regarding their potential short- and long-term side effects.

Genotoxicity of viral vectors cannot be predicted due to the low numbers of included subjects in the different clinical trials [7]. It is also challenging to determine the long-term increase of oncogenic risk. As long as a viral vector can integrate into host nuclear DNA, the risk of gene inactivation (e.g., tumor suppressor genes) or gene activation (proto-oncogenes) cannot be excluded, potentially leading to insertional mutagenesis [7]. However, the chance for insertional mutagenesis for AAV vectors is very low [10]. Monitoring at molecular level is also quite hard and no established protocols are available, regarding timing, method of use, and tissue sampling.

Careful short-term follow-up is required to exclude the ongoing replication of viral vectors. This may happen as a consequence of reconstitution or mobilization of replication deficient lentiviral or adeno-associated viral vectors via (1) wild-type virus co-infecting the same tissue, (2) the vector carrying the genetic construct has been contaminated with replication-competent virus [10]. Long-term follow-up revealed that gammaretroviral vector-based (GV) gene therapy caused leukemia due to vector insertion around oncogenes [7]. Ex vivo lentiviral-based gene therapies have so far in 7 years of follow-up showed no life-threatening adverse effects [7]. In vivo systemic administration of AAV-based gene therapy-elicited most common side effect is the increased amino transferase levels, which may be addressed with glucocorticoid treatment [10]. Transiently, AAV vectors have been detected in the semen of men receiving AAV-based gene therapy [10].

Toxicity risk may arise if the transgene is too highly overexpressed [23]. There is also theoretical proof that phenotoxicity (ectopic/dysregulated expression of the transgene) may arise after gene therapy [10]. Furthermore, if the genome editing enzymes are designed to be expressed in a persistent fashion, new safety concerns may also need to be considered [11].

14.3 Role of Extracellular Vesicles in Gene Therapy

The ideal vectors are yet to be delivered for gene therapeutics. An ultimate vector candidate should possess high delivery efficiency of the genetic construct, to target cells of interest precisely,

have no toxicity, have very low/no immunogenicity, and be able to bypass intracellular sensors of genetic constructs. It is of uttermost importance for a successful gene therapy that the genetic construct is packaged into a suitable vehicle. EVs could be the most suitable vectors that are able to perfectly mask the exogenously delivered materials, since their membrane is a high fidelity derivative of the plasma membrane and is an endogenous carrier of nucleic acids [24].

The concept of hybrid EV vectors was introduced nearly a decade ago, and they are the result of EVs fusion with either liposomes, synthetic particles, or non-enveloped viruses [25]. EVs fused with viruses have been termed as vexosomes [26]. EV-associated AAVs outperformed the efficiency of gene therapy with only AAV in preclinical settings [27]. It has also been reported that EV-enveloped AAVs are also more resistant against neutralizing antibodies, when compared to standard AAV1 preparations [28]. Some EVs may become in the near future indispensable vector components, since theoretically EVs may possess and unite the desirable benefits of viral and non-viral vectors.

14.3.1 Benefits of EVs, as Vectors

Phase I and II clinical trials showed that in vivo administration of EVs is safe and feasible. Also repeated administration of EV preparations was safe and did not induce toxicity [16]. EVs have the ability to modify the phenotype of their target cells and they may also reeducate these cells to specific functions [29]. Genetic constructs carried in EV preparations are under protection of the vesicular structure. However, it seems that after systemic administration, the injected EVs are almost undetectable in the circulation after 4 h [16].

EVs are able to transfer between cells proteins, nucleic acids, lipids, and carbohydrates [25]. EVs have a rich surface and intraluminal molecular composition. The complex molecular structure of EVs is the result of numerous, heterogenous, and active biomolecules found in EVs. The most abundant nucleic acids found in EVs are miRNAs, vtRNA, tRNA, mRNA, Y RNA. A comprehensive EV-associated database has been published as an updated online database (http://www.exoRBase.org) [30]. As for proteins, tetraspanins, integrins, immunoglobulins, cytoskeletal proteins, heat shock proteins are the most prevalent. Last, but not least, phosphatidylserine, cholesterol, lysophosphatidylcholine, eicosanoids lipids, glycosphingolipids are also main components of EVs (mostly in membrane-associated form) [16, 25]. The functional effect of EVs is a result of the protein, lipid, and nucleic acid cargo altogether [31, 32].

The challenge for EV-based vectors is cellular uptake, endosome escape, and if nuclear localization is desired (in most cases) nuclear entry and release of genetic construct. As an ideal shelter, the lipid bilayer membrane protects the intraluminal cargo from enzymatic degradation [33, 34]. In in vivo mice and in vitro human cells studies successful delivery of red blood cell-derived EVs loaded with different RNA molecules (ASO, gRNA, mRNA) was achieved [35].

EVs possess homing properties, they are recognized and taken up mostly by targeted cells [34]. The source of EVs is a defining factor for preferential target cell interaction but is also dependent on the functional status of recipient cells [36]. The uptake of EVs is an active process. The exofacial surface of EVs is in general slightly negatively charged [37] and provides a complex docking platform for selecting and attaching to target cells. The rate and type of uptake are mainly dependent upon EV size, shape, surface charge, lipid components, and protein corona [16, 34]. The primary interaction between EVs and their target cells is considered that is initiating mainly upon EV carrying ligand and cell expressing receptor interaction. This specific docking makes room for a signal response that will either induce an intracellular pathway or permit the EV membrane protein transfer toward cell membrane and/or fusion of EV membrane with the cell membrane, proceeded by uptake of EV via (clathrin-mediated, dynamin-dependent, lipid-raft mediated or caveolin-mediated)

endocytosis/micropinocytosis/phagocytosis (actin-dependent, receptor-mediated)/complete direct fusion [25, 29, 36, 38]. Tetraspanin-enriched microdomains, thiol interactions, proteoglycans also contribute to the homing of EVs [34, 39]. The uptake of EVs can be further enhanced by altering their membrane with cloaking approach or surface display technology [16]. Modifying the target cells of EVs has been successfully done in 2005, using the surface display technology, by embedding moieties into EV surface involving fusion with C1C2 domain of lactadherin. Later on, in several studies by modifying the surface display pattern with different integrin subunits, EV tropism has been successfully manipulated, selectively targeting B-cells, Kupfer cells, endothelial or glial cells. By fusing cardiac targeting peptide to Lamp2 protein, EVs targeting the heart could be engineered and successfully delivered in vivo in mice [16]. Chemical methods, as click-chemistry (azide alkyne cycloaddition) or painting-EVs with targeting peptides are also viable methods to further define and refine the target cells of EVs. Overall, the manipulation of EV biodistribution and uptake with high efficiency and high specificity can be achieved via protein, lipid, and glycan EV surface corona alteration [34]. By applying synthetic lipids, the colloidal stability of EVs can be further improved [40].

So far, the following nucleic acids have been identified in EV cargo and trafficked by EVs: dsDNA, ssDNA, mtDNA, miRNA, rRNA, mRNA, Y RNA [25]. EVs are also able to transfer CRISPR-Cas system for genome editing in vitro and in vivo mice model [41].

14.3.2 Engineering EVs for Gene Therapy

It is possible that such EVs may be designed and delivered as synthetic vehicles, here the introduction of an ultimate synthetic EV concept for gene therapy will be presented. Synthesized EVs will have optimal biosafety (being manufactured under sterile conditions), present high homogeneity, confer enhanced target specificity, retain EV membrane protein and lipid topology, possess good circulation kinetics, with optimal loading capacity containing the right amount of genetic construct to restore the genome function and have a non-immunogenic nature. Bioinspired hybrid (EV membrane-camouflaged or fused vesicles) or fully artificial (bottom-up engineered vesicles via complex stepwise assembly of the building individual molecular blocks) EVs may provide the holy grail that satisfies all requirements and desired functions for a successful gene therapy [42]. The potential use of EVs in gene therapy is depicted in Fig. 14.1. One potential source for obtaining EVs are human red blood cells (RBC) from healthy donors with group O [43]. RBC-derived EVs naturally express CD47, which decreases immunorecognition of EVs, reduces the uptake by monocytes ("don't eat me signal"), and increases the circulation half-time [40]. These EVs may be harvested, engineered, and loaded with genetic constructs.

In in vitro settings and in vivo animal models engineered EVs have been successfully applied: EVs were loaded with siRNAs and induced gene silencing, resulting in reduced expression of (1) HER2, (2) MAPK1 (in monocytes and lymphocytes), or (3) Htt (bilateral silencing of huntingtin mRNA in mouse striatum). Also, EV-mimicking liposomes containing siRNA have been applied to inhibit expression of VEGF [25]. Using animal models, intracardiac administration of cardiosphere/cardiomyoblast/MSC-derived EVs loaded with miR-19, miR-156a, or miR-451 was assessed for cardiac regenerative and cardioprotective effects [25]. These studies proved that different RNA constructs delivered via EVs were therapeutically active. Currently, the encapsulation efficiency of siRNAs into EVs is around 10–50%, achieving up to 3000 siRNA copies loading per EV [33].

The surface patterning of EVs is of key importance for targeting [44]. To block non-specific interactions between EVs and cells, EVs and plasma components, respectively, a shielding with covalent coupling polyethylene glycol (PEG) may be performed [45]. Furthermore, the distal end of the PEG molecule can be functionalized with a targeting ligand to enhance

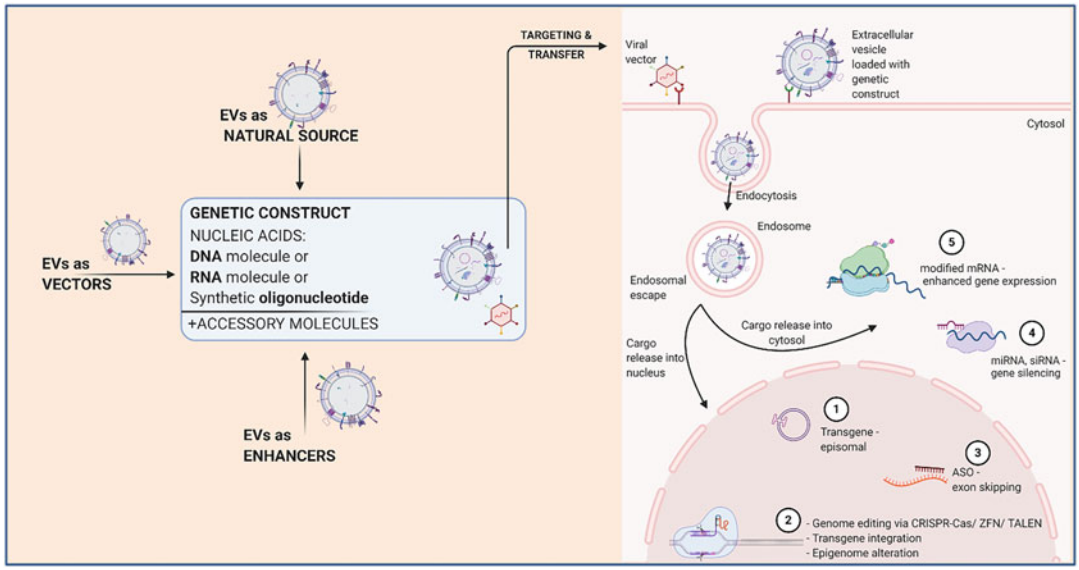

Fig. 14.1 Role of EVs in gene therapy, targeted molecules and effect of gene therapy. Possible role of EVs in gene therapy. EVs may act as a natural source, as vectors or as enhancers of gene therapy. EVs may be engineered or loaded with genetic constructs containing nucleic acids and accessory molecules. Successfully designed vector-containing gene therapeutics are targeted and transferred toward target cells. Through active interaction the therapeutic cargo is loaded either into the cell nuclei or cytosol. (*1*) Vector-transported episomal transgene expression is one of the most used options for cell nuclei targeted gene therapy. (*2*) At double-stranded DNA (dsDNA) level gene augmentation, gene disruption or gene correction are the main therapeutic goals. (*3*) At pre-mRNA level the correction of frameshift variants or key (multiple) trinucleotide pathogenic variations can be achieved by exon skipping effect. (*4*) Gene therapy targeting the cytoplasm (RNA therapeutics) may target protein translation by binding siRNAs, miRNAs, or ASOs to mRNA of choice. (*5*) Lastly, but not least, through delivery of synthetic modified mRNA molecules, a transient protein synthesis may be achieved, leading to an enhanced gene expression. (Created with BioRender.com)

targetability [29]. As a reprogramming of the natural EV surface, valency-controlled tetrahedral DNA nanostructures via cholesterol anchoring can be applied to enable specific EV targeting [46]. Enhancing the density of CD47 on the surface of EVs will enable the escape of phagocytosis by monocytes [29, 34]. The blocking of these non-specific interactions will also result in an increased circulation half-life [47]. It is also important that the annexin AI is reduced or absent in exofacial surface of EV membranes, because annexin AI induces the aggregation of EVs, thus reducing the efficiency of cargo delivery [48]. Also by achieving higher target specificity, the reduced uptake of non-target cells may also reduce the incidence of side effects [34]. It is desirable that the design of the vesicle to result in a near neutral surface exterior will prevent the adsorption of serum proteins [21]. However, it remains currently an open question that once an optimal EV corona is designed, what should be the density in the EV corona of the individual protein, lipid, and sugar components.

To escape the endolysosomal degradation and prevent degradation of active genetic construct, pH sensitive GALA peptides, basic peptide (e.g., melittin), pH sensitive polymers, or fusogenic lipids can be introduced into the membrane of EVs [16]. Once EVs escaped the endocytic degradation, the released EV cargo can exert its function in the cytosol, or if it contains NLS signaling motifs, it can reach the nucleus to engineer the genome function as it was designed (Fig. 14.2).

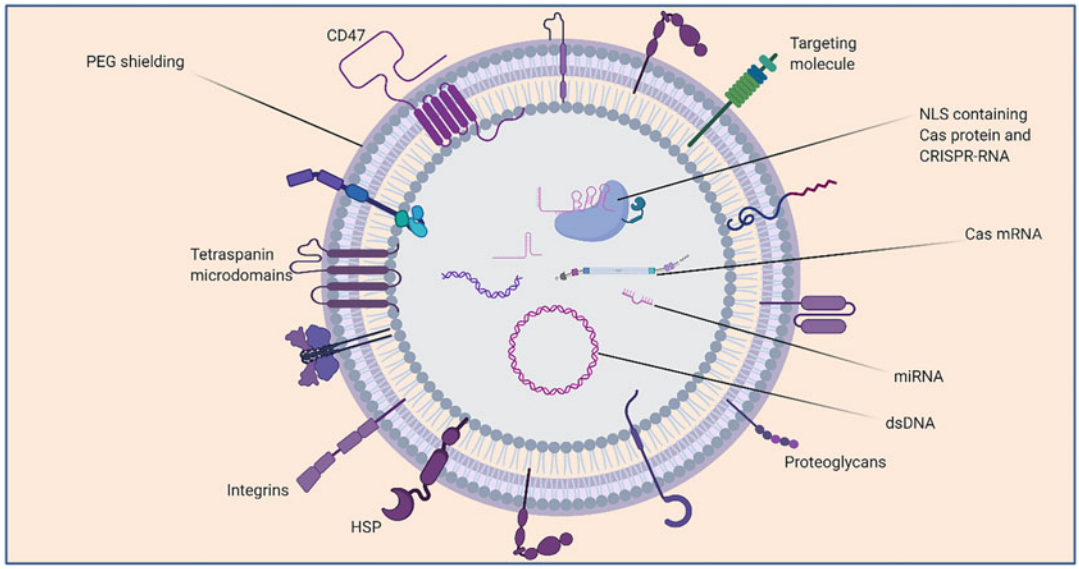

Fig. 14.2 Designing-EV based delivery system for efficient and safe gene therapy. The caterpillar of designing a vehicle suitable for gene therapy is resolving the targeting, stability, and efficient delivery (assembly of protein corona with lipid and carbohydrate moieties, shielding with PEG). Once the therapeutic agent is engulfed by the target cell, it needs to escape the endosome (which can be enhanced by fusogenic lipids, pH sensitive polymers, pH sensitive GALA peptides, or melittin). After endosomal escape, the cargo is loaded into the cytosol. Cargo containing NLS signal is further transported through nuclear entry into the nucleus, where it will exert its therapeutic effect. (Created with BioRender.com)

14.3.3 Active Loading of Genetic Constructs into EVs

Exogenous genetic constructs have been the most successfully loaded into EVs by electroporation (inducing transient pores). Different RNA and DNA molecules have been successfully loaded into EVs [35, 49]. The following factors seem to be critical for loading: size of the DNA/RNA molecule, nucleic acid:EV ratio, EV size distribution (larger EVs tend to take up more efficiently genetic constructs). Around 600 dsDNA copies/EV have been loaded. Furthermore, the electroporation process has a minimal effect on EV membrane components and does not damage the membrane due to heating (which is around 1°C/pulse), however may increase the risk of aggregation. To reduce the risk of aggregation, citric acid buffer, trehalose pulse media, or addition of other chelating agents such as ethylenediaminetetraacetic acid can be used [33, 42]. Other strategies involve sonification (through induction of mechanical shear force), saponin-assisted loading (pore formation through interaction of cholesterol), or hypotonic dialysis (EVs swell and form pores due to hypotonic solution induced osmosis) [25, 33]. Another, quite recently developed method is the targeted and modular EV loading (TAMEL) approach, which involves the use of a fusion protein (MS2 bacteriophage coat protein fused with Lamp2b or CD63) that has a transmembrane domain and an RNA-binding domain [33]. This approach provides a unique way of loading RNA molecules into EVs. For genetic constructs designed to engineer chromosomal DNA, instead of delivering the nuclease itself, in vitro transcribed nuclease-encoding mRNA could be included in the genetic construct, which could be an elegant option to limit off-target effects of these enzymes [50]. A

highly efficient loading method for miRNAs inclusion into EVs has also been described. Replacing the long pre-miRNA stem loop with a 51 nucleotide sized short stem loop induces many-fold enrichment of the desired miRNA (and theoretically even sgRNAs) into EVs [16]. Three miRNA binding proteins have been so far identified that may play significant role in miRNA packaging into EVs: synaptotagmin-binding cytoplasmic RNA-interacting protein (SYNCRIP, recognizing GGCU motif), hnRNPA2B1 (recognizing GGAG motif), and Y-box protein 1 (YBX1, the recognizing motif has not been identified yet) [51]. Moreover, it has been reported that inflammasome activation leads to inclusion of hsa-miR-24-3, hsa-miR-155p-5p, and hsa-miR-126-3p, miRNAs containing the AAUGC motif to sEVs [52, 53]. This mechanism once fully unlocked may be harbored for specific loading of certain miRNAs into EVs. Recently it has been reported that dual engineered EVs (CD47+ EVs-derived from MSC overexpressing CD47 and enriched in mmu-miR-21 via electroporation process) could reduce the size of infarction in mice [54]. The siRNAs can also be loaded into EVs with high efficiency in duplexes, reaching up to 0.14 pmol siRNA into 1 μg EVs [55]. The selective encapsulation of mRNA by a DNA aptamer approach recruits the AUG region of target mRNA and CD9 zinc-finger (ZF) region to the aptamer, resulting in sorting into CD9 ZF engineered EVs of DNA aptamer-mRNA complex [56]. The loading of specific mRNA molecules into small EVs via cellular nanoporation (via cells stimulated by biochip) could be also a viable alternative that may outperform bulk electroporation technique by up to 10^3-fold in mRNA loading. Moreover, this technique may also increase small EV secretion via HSP70/90-p53-TSAP6 signaling pathway (heat shock proteins 70 and 90-p53 protein-tumor suppressor-activated pathway 6 protein) [57]. Also, the half-life of ncRNAs can be expanded to weeks or months via certain chemical modifications (e.g., 2′-4′ ether bridge) [5].

For genome engineering purpose a new method has been described, called Genome Editing with Designed Extracellular Vesicles (GEDEX), which is an EV-based delivery of the CRISPR/Cas system. Biodistribution studies of GEDEX in mice revealed that GEDEX uptake was the highest in the liver, but it could be detectable also in the heart, kidney, and lungs [41]. This biodistribution study enlightens hope for GEDEX-based gene therapy approach for metabolic disorders as well as cardiovascular diseases. Also, a promising tool for EV-based genetic engineering is the extracellular nanovesicle-based ribonucleoprotein delivery system (NanoMEDIC), which involves a chemical induced dimerization for Cas9 recruiting and riboswitches for gRNA release into EVs. The NanoMEDIC system has been successfully used in preclinical settings for the treatment of *mdx* mice and in vitro enhancing of dystrophin in skeletal muscle cell-derived from Duchenne muscular dystrophy patient. The drawback of NanoMEDIC system is that HIV1 Tat is used for gRNA expression in loading cells, and the incorporation of Tat in a non-specific way could be a potential toxicity risk [58]. Other strategies have been also developed: (1) by giving a WW tag to Cas protein and overexpressing the Ndfip1 protein (that will ubiquitinate the WW tagged Cas) promote Cas loading into EVs (of note Ndfip1 is toxic to EV producing cells), (2) arrestin domain containing protein 1 (ARRDC1) mediated microvesicles technique involves the fusion of Cas9 protein with 2–4 ITCH domain, an advantage of this method is that provides a large spectrum of tropism [16]. It seems that exogenous loading of sgRNA post EV engineering seems the most promising approach. It is crucial to treat the in vitro transcribed sgRNAs with phosphatase, to remove cytotoxic 5′--triphosphate group, that could trigger the intracellular immune recognition (RIG-I RNA sensors). Chemical synthesis of sgRNAs already lacking the 5′-triphosphate group is also an alternative method, which also eliminates the need of phosphatase treatment. Significant increase in

on-target indel frequencies and increased specificity (reducing off-target effect) can be achieved via chemical modifications of 3′ and 5′ends of sgRNAs (2′-O-methyl, 2′-O-methyl 3-′-phosphorothioate, or 2′-O-methyl 3′-thioPACE) at three terminal nucleotides increased on-target [16].

14.4 Snapshot at the Current Development of/Available Gene Therapies for Cardiovascular Diseases

Cardiovascular diseases are the leading burden and cause of death worldwide, the outcome of cardiovascular diseases is often the progression of hypertrophy and heart failure [59]. Current therapies often slow down the progression of cardiovascular diseases but stopping or reversing the pathophysiological process cannot be achieved via traditional therapies. Gene therapy strategies may change the landscape and progression of cardiovascular diseases, but the inherited and acquired cardiovascular diseases demand a different therapeutical strategy. Hyperlipidemias, coronary artery disease (including refractory anginas), heart failure, arrhythmias, peripheral vascular diseases are among the most important pathologies that were addressed by a gene therapeutic approach [60]. Certain hematological diseases may also have a deep impact on the cardiovascular system; therefore, the inclusion of these pathologies is also of high relevance. Many pathogenic variants of several genes can lead to a specific cardiovascular phenotype. The most relevant viral vector-based gene therapies enlisted on clinicaltrials.gov for major cardiovascular and hematological (including only disorders of red blood cell and disorders of clotting factors) diseases are listed in Table 14.2.

Antisense nucleotide therapy targeting proprotein convertase subtilisin/kexin type 9 (PCSK9) was designed for individuals with **LDL-C-hypercholesterolemia**. However, in the first-in-human testing settings due to renal toxicity, the clinical trial was terminated [61]. Later on an siRNA-based drug targeting PCSK9 has successfully passed phase III clinical trial and has been approved by EMA and is under review by FDA [64], and its use has proved to be safe even for patients with renal impairment [65]. AAV-vector based episomal delivery of in vivo gene therapy for **lipoprotein lipase deficiency**, Glybera was retracted from EU market, due to its high cost of around 900,000 euros per treatment [66]. Subcutaneously administered ASO therapy for **familial chylomicronemia syndrome** that reduces apoCIII production has also been approved in 2019 [67].

Results from completed phase II clinical trial for treatment of **hemophilia B** with AAV vector mediated gene augmentation approach reported (NCT02484092 trial) no serious adverse effects, while mild or moderate adverse effects were seen in 93% of treated patients (14 out of 15). The most common adverse effects were upper respiratory tract infection (30%), followed by muscle strain (20%), nasopharyngitis (20%), and back pain (20%).

Peripheral artery disease is also a priority candidate for gene therapy, since no effective therapy is available to restore the circulation of an ischemic limb at molecular level. Most gene therapeutic studies aiming for vessel repair for peripheral artery disease target genes encoding for vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), or fibroblast growth factor (FGF). These studies mostly use the intramuscular administration route. *VEGF* gene augmentation showed the highest evidence for improvement, *HGF* gene augmentation led to complete ulcer healing [68].

At least 11 genes have been associated with **hypertrophic cardiomyopathy**, the two most common affected genes are the *MYBPC3* and *MYH7* and the phenotype is severed in homozygous or the presence of several pathogenic variants in gene sequence of an individual [3].

Angiogenesis promoting gene therapy strategy is applied for **coronary artery disease**. Catheter-mediated intracoronary administration

Table 14.2 Gene therapy clinical trials for cardiovascular and diseases affecting the cardiovascular system

Disease	Form of gene therapy	Vector	Clinical trial/ drug name	Ref
β-Thalassaemia major (*HBB* gene)	Ex vivo, for patients >12 years	SIN lentiviral vector, CD34+ target cells	NCT02151526 NCT01745120 Zynteglo	[7]
	Patients between 4 and 70 years		NCT03351829	
Hemophilia B (*F9* gene)	In vivo, for male patients >16 years iv. infusion	AAV8 vector, hepatocytes	NCT00979238 NCT01687608 NCT04272554 NCT02484092 NCT03861273	[7]
	Ex vivo, for male patients between 2 and 65 years	Lentiviral vector	NCT03961243	
Lipoprotein lipase deficiency (*LPL* gene)	In vivo, im adm.	AAV1 vector, muscle targeted	NCT00891306	
LDL-C-hypercholesterolemia (*PCSK9* gene)	In vivo, for patients between 18 and 65 years, subcutaneous injections	LNP, antisense oligonucleotide	NCT01350960 NCT02597127	[5, 61]
Familial chylomicronemia syndrome (*LPA* gene)	In vivo, for patients >18 years, subcutaneous injection	Antisense oligonucleotide	NCT03544060 WAYLIVRA	[5]
Sickle cell anemia (*HBB* gene)	Ex vivo, for patients between 2 and 53 years	Lentiviral vector	NCT04628585	[10]
	Ex vivo, for patients between 3 and 40 years	Lentiviral vector-containing short-hairpin RNA targeting *BCL11A*	NCT03282656	
	Ex vivo, for patients >18 years	Lentiviral-vector containing the βAS3 gene	NCT02247843	
	Ex vivo, for patients between 5 and 35 years	Lentiviral vector	NCT02151526	
	Ex vivo, for patients between 18 and 45 years	Lentiviral vector	NCT02186418	
Hemophilia A (*F8* gene)	Ex vivo, for patients >18 years	Lentiviral vector, CD34+ target cells	NCT03818763 NCT04418414	
	Ex vivo, for patients between 2 and 65 years		NCT03217032	
	In vivo, for male patients between 18 and 64 years, iv. infusion	rAAV2/6	NCT04370054	
	In vivo, for patients>18 years	rAAV2/8	NCT03001830	
		rAAV5	NCT03520712 NCT02576795	
Danon disease (*LAMP2* gene)	In vivo for male patients >8 years, iv. infusion	rAAV9	NCT03882437	
Homozygous familial hypercholesterolemia (*LDLR* gene)	In vivo for patients >18 years, iv. infusion	rAAV8	NCT02651675	
Severe coronary heart disease (*VEGFD* gene)	In vivo, endocardial injection system (NOGATM), patients between 30 and 80 years	Ad	NCT01002430	
Acute myocardial infarction (*HGF* gene)	In vivo, for patients between 19 and 75 years transendocardial injection	Naked plasmid DNA	NCT03404024	[62]

(continued)

Table 14.2 (continued)

Disease	Form of gene therapy	Vector	Clinical trial/ drug name	Ref
Refractory angina (*VEGF* gene)	In vivo, for patients >18 years, intramyocardial	Ad	NCT04125732	
Fanconi anemia subtype A (*FANCA* gene)	Ex vivo, for children and adult patients	Lentiviral vector, CD34+ target cells	NCT04437771	
Chronic heart failure (*AC6* gene)	In vivo, for patients between 18 and 80 years, intracoronary adm.	Ad5	NCT00787059	[63]
Chronic heart failure with left ventricular assisted device (*SERCA2A* gene)	In vivo, for patients between 18 and 74 years, percutaneous adm.	AAV1-CMV	NCT00534703 NCT01966887 NCT04703842	[63]
Stable heart failure (hsa-miR-132)	In vivo, for patients between 18 and 80 years iv. infusion	Antagomir-132	NCT04045405	[5]
Chronic lower limb ischemia (*HGF* gene)	In vivo, for patients between 20 and 80 years, im. Injection	pCK	NCT04274049	

of liposome/plasmid/adenovirus containing *VGEF* showed improved myocardial perfusion [60]. In preclinical studies with induced **heart infarction** AAV9 vector-based *SERCA2A* gene augmentation improved left ventricular function [60]. In a phase I/II clinical trial the incidence of cardiovascular events in a 3 year follow-up period was decreased in patients with **heart failure** treated with AAV1 vector-containing *SERCA2A* gene. However, in phase IIb clinical trial 123 patients were given AAV1-containing *SERCA2A*. This trial failed to deliver the expected results, after 12 months of follow-up no significant difference between the placebo and treated group could be observed [60, 63, 66]. Only around 2% of cardiomyocytes contained the genetic constructs, even the targeted local delivery via rAAV was not enough to reach high and effective transgenesis rate [66]. Ad-mediated delivery of adenylyl cyclase 6 (*AC6*) gene in phase II trial led to neutral results [66]. Phase Ib clinical trial with antagomir targeting has-miR-132 for heart failure may also be a viable option [NCT04045405], [5].

Heart failure can also develop, as a consequence of hereditary **transthyretin-mediated amyloidosis**, both intravenous and subcutaneous gene therapy have been already approved, the intravenously applied ASO is encapsulated into lipid nanoparticle.

In preclinical model, rAAV9 contains potassium voltage-gated channel subfamily.

A member 2 (*Kcna2*) targeting siRNA successfully inhibited the noradrenaline-induced **arrythmias** [69]. In preclinical model, the use of CMV enhancer upstream of a cardiac promoter induced high transgene expression in the heart [23].

Although many clinical trials are targeting main cardiovascular pathologies, many gene therapeutic candidates fell off during clinical trials [5]. Overall the need for efficient and safe delivery vehicles for genetics constructs seems to be the main limiting factor. In vitro and preclinical studies have shown that functional delivery of EVs containing certain miRNAs (e.g., miR-210, miR-132, miR-21, miR-451, miR-29b, miR-455) resulted in the inhibition of apoptosis, promotion of angiogenesis, and inhibition of myocardial fibrosis [70].

The uptake of cardiac tissue-derived EVs in preclinical model shows a different distribution uptake pattern, being mainly taken up by thymus, lung, and kidney [71]. Small EVs derived from mesenchymal stem cells and containing miR-19a have increased cardiomyocyte survival and promote myocardial repair in preclinical settings

[69]. Advances in preclinical studies push the limits and advancing of EV-based gene therapies that are still in infancy.

14.5 Snapshot at the Current Development of/Available Gene Therapies for Metabolic Disorders

The liver is the central metabolic hub and has been the main targeted organ for in vivo gene therapy for metabolic diseases [7]. Intravascular delivery of appropriate genetic constructs toward hepatocytes could be a main useful strategy for several inherited metabolic disorders. These approaches use the liver as a bioreactor to catalyze the therapeutic effect. In Table 14.2 the most relevant gene therapy clinical trials addressing metabolic disorders are depicted. Although for quite a few metabolic disorders enzyme replacement therapy, substrate reduction therapy, or specific dietary approach is available, some major complications or disease progression cannot be halted in every case. Therefore, there is a substantial need for gene therapeutic approach.

There are over 70 different lysosomal storage diseases (LSD), and in around 70% of LSDs there is a progressive central nervous system involvement. Intravenous administered AAV9-based gene therapy can successfully cross the blood–brain barrier, raising hope for many LSD prospective gene therapies [72]. In the most common LSD, **Fabry disease,** the preclinical models have shown that it can be successfully treated also via lipid nanoparticle containing mRNA-based genetic construct [73]. This approach can also be applied theoretically to other LSDs and metabolic disorders. **Gaucher disease** is the second most prevalent LSD, current disease-specific therapy cannot reach the central nervous system, thus neurological involvement is left untreated. In preclinical studies AAV1-based gene therapy successfully reduced substrate accumulation in the brain [74]. In mice studies the AAV-mediated episomal delivery of the *GBA1* gene has also been successfully carried out before birth, as prenatal gene therapy [75].

In preclinical settings both gene augmentation and mRNA therapeutics have been successfully applied to **methylmalonic acidemia** and **acute intermittent porphyria** animal models [73, 76].

Several clinical trials for glycogen storage disease (GSD) have been done and are ongoing with the aim to develop safe and efficient therapy options for patients affected by these disorders. Successful preclinical gene therapeutic approach has been achieved in GSD Type Ia and partially in GSD Type Ib. Clinical trials for GSD Type II, **Pompe disease**, are ongoing [77] (Table 14.3).

14.6 Outlook for the Near Future

Enriching knowledge with single-cell multiomics to decipher at highest resolution the genotype–phenotype correlations will pave the way to design better gene therapy strategies. The more than half century long promise of gene therapy is on its avenue of becoming a reality, a hope for a better humanity, for creating equal chances and fair play at individual level. The goal to heal in a personalized fashion now seems to become a light of hope for many affected ones. The Swiss knife of gene therapy, CRISPR-Cas system, shows a holy grail promise in human molecular genetics, emerging and facilitating both as a diagnostic and therapeutic tool. Emerging knowledge about EVs pave the road to deliver synthetic EV preparations, which will be fully compatible to carry different genetic constructs, offer stability until reaching target cells, be easily programable for desired cell targeting, and lead to high efficient delivery of genetic construct. Engineered EVs carrying optimal genetic constructs will change the fate of gene-corrected cells, offering

Table 14.3 Gene therapy clinical trials for the treatment of metabolic disorders

Disease	Form of gene therapy	Vector and target cells	Clinical trial/ drug name	Ref
Metachromatic leukodystrophy (*ARSA* gene)	Ex vivo, for patients <7 years	Lentiviral vector, CD34+ target cells	NCT01560182 NCT03725670 NCT03392987	[7]
	Ex vivo, for children and adult patients		NCT04283227	
	In vivo, for patients between 6 months and 5 years intracerebral adm	AAVrh10	NCT01801709	
Mucopolysaccharidosis I (Hurler syndrome) (*IDUA* gene)	Ex vivo, for patients <11 years, intracerebral adm	Lentiviral vector, CD34+ target cells	NCT02702115 NCT03488394	[12]
	In vivo, for patients >4 months	rAAV9	NCT03580083	
	In vivo, for patients >5 years, intravenous infusion	rAAV2/6 ZFN-mediated genome editing	NCT02702115	
Mucopolysaccharidosis II (Hunter syndrome) (*IDS* gene)	In vivo	rAAV2/6-IDA, mainly targeting cells in the liver	NCT03041324	[12]
	In vivo, for male patients between 4 months and 5 years and 5 and 17 years, intracisternal adm	rAAV9	NCT03566043 NCT04571970	
	In vivo, for patients >5 years, intravenous infusion	rAAV2/6 ZFN-mediated genome editing	NCT03041324	
Mucopolysaccharidosis III A (Sanfilippo syndrome) (*SGSH* gene)	Ex vivo, for patients between 3 and 24 months	Lentiviral vector, CD34+ target cells	NCT04201405	
	In vivo, for >6 months intracerebral adm	AAVrh10	NCT03612869	
	In vivo, for >6 months patients, iv. inj.	rAAV9	NCT04088734 NCT02716246	
Mucopolysaccharidosis III B (*NAGLU* gene)	In vivo for children and adults, iv. infusion	rAAV9.CMV	NCT03315182	
Adrenoleukodystrophy (*ABCD1* gene)	In vivo, for children and adult patients, intracerebral adm.	Lentiviral vector	NCT03727555	[12]
Krabbe disease (*GALC* gene)	In vivo, peripheral limb vein adm.	AAVrh10	NCT04693598	
Fabry disease (*GLA* gene)	In vivo for male patients >18 years, iv. infusion	rAAV2/6	NCT04519749 NCT04040049 NCT04046224	
	Ex vivo, for male patients between 18 and 50 years	Lentiviral vector	NCT02800070	
Type 1 Gaucher disease (*GBA* gene)	Ex vivo, for patients between 16 and 35 years	Lentiviral vector	NCT04145037	
Late infantile neuronal ceroid Lipofuscinosis (*TPP1* gene)	In vivo, for patients between 2 and 18 years, intracerebral adm.	AAVrh.10	NCT01161576 NCT01414985	
Late infantile neuronal ceroid Lipofuscinosis 6 (*CLN6* gene)	In vivo, for patients >1 year, intrathecal injection	scAAV9	NCT02725580	
Pyruvate kinase deficiency (*PKD* gene)	Ex vivo, for patients >8 years	Lentiviral vector, CD34+ target cells	NCT04105166	
Phenylketonuria (*PAH* gene)	In vivo, for patients >15 years, iv. infusion	rAAV	NCT04480567 NCT03952156	
Acute intermittent porphyria (*PBGD* gene)	In vivo, for patients between 16 and 64 years	rAAV2/5	NCT02082860	

(continued)

Table 14.3 (continued)

Disease	Form of gene therapy	Vector and target cells	Clinical trial/ drug name	Ref
GM1 gangliosidosis (*GLB1* gene)	In vivo, for patients between 6 months and 12 years	rAAV9	NCT03952637	
	In vivo, for patients between 4 and 24 months	AAVhu68	NCT04713475	
Methylmalonic Acidemia (*MMUT* gene)	In vivo, for patients between 6 months and 12 years	rAAV-LK03	NCT04581785	
Ornithine transcarbamylase deficiency (*OTC* gene)	In vivo, for patients >18 years, iv. infusion	rAAV8	NCT03636438	
Aromatic L-amino acid decarboxylase deficiency (*AADC* gene)	In vivo, for patients between 5 and 18 years, midbrain infusion	rAAV2	NCT02852213	
Glycogen storage disease type Ia—Von Gierke's disease (*G6PC* gene)	In vivo, for patients >18 years, iv. infusion	rAAV8	NCT03970278 NCT03517085	
Glycogen storage disease type II Pompe disease (*GAA* gene)	In vivo, for patients between 2 and 18 years	rAAV1-CMV	NCT03285126 NCT00976352	
Late onset Pompe disease (*GAA* gene)	In vivo, for patients >18 years	rAAV8	NCT04174105	
	In vivo, for patients between 18 and 50 years, im adm.	rAAV9	NCT02240407	
Wilson's disease (*ATP7B* gene)	In vivo, for patients between 18 and 60 years, iv. infusion	rAAV	NCT04537377	

sc self-complementary, *rh* rhesus, *r* recombinant, *adm* administration, *AAV* adeno-associated virus

the possibility to restore physiological states, resulting in improved quality-of-life for many affected humans. Finally, we should be able to closely monitor with advanced molecular tools (via single-cell whole genome amplification-based sequencing, single-molecule real-time sequencing, single-cell transcriptomics-based sequencing) the end-result of this complex therapeutic approach. A plethora of factors need to be considered and hurdles to overcome in order to pave the way for successful gene therapy application in wide clinical settings.

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Part V

Other Aspects and Future Prospects

Prospective Advances of Extracellular Vesicles Investigation in Cardiovascular and Metabolic Research

15

Shutong Shen

Abstract

Extracellular vesicles (EVs) play an important role in cardiovascular and metabolic diseases through intercellular communication. Although there has been extensive research on EVs, there are still some unsolved problems in the technologies of investigation of EVs. In this chapter, we reviewed the current knowledge of EVs functions in cardiovascular and metabolic pathophysiology and EVs as biomarkers and therapeutic agents in cardiovascular and metabolic diseases. We also addressed the challenges in isolation and identification of EVs as well as challenges in visualization and tracking of EVs. By addressing these challenges, we hope to have a more in-depth understanding of the biological functions of EVs.

Keywords

Extracellular vesicles · Cardiovascular and metabolic diseases · Challenges · Technologies of investigation

15.1 Background

In the late 1960s, it was first described that there are vesicles around the cells in mammalian tissue or fluid, such as cartilage [1], biological fluid, including blood [2] and semen [3], or mammalian cells, especially tumor cells [4] and platelets [5]. In 1983, Pan and Johnstone discovered a membrane vesicle from reticulocyte culture medium, which Johnstone named as exosome in 1987 [6]. In 2011, the general term "extracellular vesicle" was proposed to define all the extracellular structures surrounded by lipid bilayers.

Extracellular vesicles (EVs) can be divided into several subtypes according to their size and biogenesis. Exosomes (50–150 nm) originate from multivesicular bodies (MVBs). MVBs are formed by inward budding of the early endosomal membrane during endosome maturation process. Most of the MVBs fuse to lysosomes for degradation, but a subset of MVBs can merge with the plasma membrane, resulting in the release of exosomes in the extracellular space [7–9]. The direct blebbing of EVs from the plasma membrane produces microvesicles (150–1000 nm) and larger apoptotic bodies (>1000 nm) are outward blebbing of apoptotic cell membrane [10].

EVs secretion was initially thought to be a mechanism by which cells eliminate unwanted proteins. However, studies in the late 1990s proposed that EVs play a role in intercellular communication, especially in immune response and cancer [11, 12]. Strong support for this concept

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emerged in 2007, when Lötvall discovered that exosomes contain mRNA and microRNA [13]. Moreover, in vitro experiments show that mRNAs in EVs can be translated into protein in recipient cells [14, 15]. At present, it is recognized that EVs can carry lipids, proteins, nucleic acids (DNA, mRNA, microRNA, and long non-coding RNAs), and metabolites [16–20], mediating communication between cells locally within the same organ, or cross organ systems via liberation into peripheral blood [21, 22]. Binding of EVs to the recipient cell surface depends on specific receptors or ligands on both EVs and the plasma membrane of recipient cell [23–26]. Intracellular uptake of EVs occurs via endocytosis, membrane fusion, or receptor-mediated internalization [27]. On this basis, exosomes are involved in the occurrence and development of multiple diseases. In this chapter, we will give a comprehensive review on the prospective advances of EVs investigation in cardiovascular and metabolic disease.

15.2 Current Knowledge of EVs Functions in Cardiovascular and Metabolic Diseases

15.2.1 EVs Functions in Cardiovascular Pathophysiology

Almost all types of cells in the heart are known to be able to release EVs and communicate with other types of cells via EVs, such as cardiomyocytes, endothelial cells, smooth muscle cells, and fibroblasts. For example, Bang and Fredj reported a potential paracrine miRNA crosstalk between cardiac fibroblasts and cardiomyocytes [28, 29]. Cardiac fibroblasts secrete miRNA-enriched exosomes and when exposed to conditioned media from fibroblasts, fibroblast-derived miR-21* is transported to cardiomyocytes, leading to cellular hypertrophy [28]. In vitro experiments also validated that exosomes released by cardiomyocytes extracted from diabetic rats encapsulate higher levels of miR-320 and the exosomes are effectively taken up by mouse cardiac endothelial cells (MCEC), inhibiting MCEC migration and tube formation by targeting IGF-1, Hsp20, and Ets2 [30]. Exosomes also transfer between SMCs and ECs in atherosclerotic model. VSMCs derived exosomes loaded with miR-155 to ECs, destroying the integrity of tight junction between ECs, increasing endothelial permeability, and enhancing atherosclerotic progression [31].

In addition to the intercellular communication among cells in the cardiovascular tissue, circulating cells and cardiovascular cells also transfer EVs to each other. Gambim proposed that in sepsis, exosomes generated from platelet may contribute to endothelial dysfunction. In vitro data showed increased NO generation and LPS can trigger the release of platelet-derived exosomes, which induced caspase-3 activation and the apoptosis of endothelial cells through the generation of ROS and RNS [32]. In uremic mice, macrophages derived exosomes delivered miR-155 to cardiomyocytes, improving pyroptosis and uremic cardiomyopathy changes by directly targeting FoxO3a [33]. M2 macrophages can also carry miR-148a into cardiomyocytes and alleviate MI/R injury via downregulating TXNIP and inactivating the TLR4/NF-κB/NLRP3 inflammasome signaling pathway [34]. In the infarcted mouse heart, exosomes derived from macrophages are presented with miR-155 and it can be transferred into cardiac fibroblasts, inhibiting cardiac fibroblast proliferation and promoting inflammation [35]. Macrophages were also recipients of miR-155-enriched exosomes from endothelial cells. EC-EVs equipped with miR-155 shifted the macrophage balance from anti-inflammatory M2 macrophages toward proinflammatory M1 macrophages [36], playing critical roles in pathogenesis of atherosclerosis. Moreover, cells in the heart also communicate with immune cells via EVs. Necrotic HL-1 cells can drive bone marrow-derived dendritic cells to secret exosomes which can improve cardiac function via activation of CD4(+) T lymphocytes in the spleen [37]. And activated CD4+ T cells derive exosomal miR-142-3p to alleviate post-ischemic ventricular remodeling by activating myofibroblast [38].

15.2.2 EVs Functions in Metabolic Diseases

Since the content transduction mediated by EVs is similar to paracrine or endocrine processes, EVs are supposed to play a role in the metabolic diseases. It has been proved that EVs play as important mediators for metabolic organs to transmit metabolites and communicate with each other such as pancreatic β-cells, adipose tissue, skeletal muscles, and the liver [39].

Adipose tissue is supposed to be a major source of circulating exosomal miRNAs in both mice and humans. Circulating exosomes derived from adipose tissues might be transferred to other organs and tissues associated with metabolism. For example, exosomal miRNAs derived from adipocytes regulate FGF21 in the liver, thus influencing metabolism in multiple tissues [40]. The amount of adipocyte-derived EVs is influenced by weight and energy intake. Obese mice and humans have a higher level of circulating EVs originated from adipocyte, while energy restriction and bariatric surgery cause a decrease in the amount of EVs [16]. Besides the higher circulating levels, the level of contents in the EVs are also regulated in obese individuals [41].

As we all know, obesity is a high-risk factor of type 2 diabetes. Adipocyte hypertrophy may affect the function of islet cells [42]. Gesmundo proposes that obese adipocytes may affect the function of islet cells through EVs. Adipocyte-derived EVs may influence β-cell fate and function depending on the pathophysiological state of adipose tissue. EVs from healthy 3 T3-L1 adipocytes increase survival and proliferation of INS-1E β-cells and promote insulin secretion in human pancreatic islets both untreated or exposed to cytokines or glucolipotoxicity, while EVs from inflamed adipocytes caused β-cell death and dysfunction [43]. Besides, obesity is also associated with an increased risk of cardiovascular disease. EVs derived from adipocytes is also reported to be involved in atherosclerosis. Inflammatory adipocytes transfer EVs rich in VCAM-1 into vascular endothelial cells, enhancing leukocyte attachment [44]. In addition, nonalcoholic fatty liver disease (NAFLD) is another disease closely related to obesity. The pathogenesis of NAFLD is attributed to increased systemic inflammation and insulin resistance mediated by visceral adipose tissue (VAT). In vitro experiments have shown that adipocyte can convey exosomes into HepG2 and HHSteC cells. Obese adipocyte exosomes can induce TGF-β pathway dysregulation in both cell lines, while lean VAT exosomes do not exhibit the above effect, suggesting that adipocytes may act on hepatocytes via EVs communication to induce fatty liver disease [45]. And lipotoxic injury can induce the release of hepatocyte exosomes enriched with miR-192-5p, which play an important role in NAFLD by activation of M1 macrophages and hepatic inflammation [46].

Diabetes is one of the most common metabolic diseases and pancreatic β-cells play a central role in the regulation of glucose homeostasis by secreting insulin. It is well known that β-cell damage caused by autoimmune inflammation is the main cause of type I diabetes. Studies have proved that the composition of EVs cargo secreted by β-cells is regulated by pathophysiological conditions associated with diabetes [47, 48]. Proinflammatory cytokines have dual effects on β-cells. High concentration of cytokines induces apoptosis, whereas low concentrations exert protection on survival of β-cells. These dual effects were also observed in recipient β-cells incubated with exosomes released by β-cells expose to low or high doses of cytokines, which promoted survival or apoptosis of the recipient β-cells, implying that EVs are transferred between β-cells and mimic a positive feedback network in pathophysiological conditions [48, 49]. Conversely, EVs are found to participate in the communication between immune cells and β-cells during type 1 diabetes development [50]. As for type 2 diabetes, insulin resistance is the most characteristic pathological mechanisms. Skeletal muscles account for about 40% of the body mass and are the predominant site for insulin-dependent glucose uptake. Tracing of intravenously or intramuscularly injected labeled-exosomes from muscle cells

shows their accumulation in pancreas, liver, gastro-intestinal tract, and distal muscles, implying communication among these tissues via EVs. Muscle-released EVs isolated from mice fed on high palmitate diet transferred miR-16 to MIN6B1 cells in vitro and regulated Ptch1 expression in MIN6B1, which is related to pancreas development [51], implying that EVs mediate the communication between pancreas and peripheral glucose utilizing tissues, thus may play a role in the occurrence of type 2 diabetes.

15.2.3 EVs as Biomarkers for Diagnosis and Prognosis of Diseases

The cell-origin of EVs can be labeled by specific markers of host cells and the cargo in EVs is assembled in the host cells so the secreted EVs indirectly reflect the biological activities of host cells [52]. Secreted EVs are present in various available body fluids such as peripheral blood, urine, serous effusions, and so on. Analysis of EVs by non-invasive or minimally invasive liquid biopsy can not only help understand the pathology of specific cells or tissues but also avoid the trauma of tissue biopsy, such as heart and liver biopsy [53]. Furthermore, the contents of EVs, such as protein, mRNA, non-coding RNA, etc. are surrounded by lipid bilayers, avoiding the degradation by ribonuclease and proteases in body fluids [54, 55]. Therefore, the accessibility and stability of EVs make them ideal biomarkers for diagnosis and prognosis of disease.

Studies have shown that the dynamic changes in the number of EVs are correlated with diseases. A cross-sectional and longitudinal cohorts study shows that individuals with diabetes had significantly higher levels of EVs in their circulation than euglycemic control participants, especially erythrocyte-derived EVs. Furthermore, the levels of insulin signaling proteins were altered in EVs from individuals with high levels of insulin resistance and β-cell dysfunction [18]. Subgroup analysis of a randomized, controlled, multicenter, parallel-group clinical trial shows that increased levels of lymphocyte-/leukocyte- and SMC-derived circulating microparticles may predict an increased incidence of cardiovascular event in individuals at high cardiovascular risk [56]. While the increased level of circulating CD31+/Annexin V+ microparticles is an independent predictor of cardiovascular events in stable CAD patients [57]. Monocyte microparticles were significantly increased in peripartum cardiomyopathy compared with healthy pregnant women [58]. CD14 +, CD14 +/CD11b +, and CD14 +/CD142 + monocyte-derived circulating microparticles are related to long-term prognosis for cardiovascular mortality in STEMI patients [59]. High levels of endothelium-derived microparticles are an independent predictor of future cardiovascular events, but not for all-cause mortality in HF patients [60]. Increased CD62e(+) endothelial microparticle levels predict poor outcome in pulmonary hypertension patients [61].

Since contents in EVs are regulated under pathophysiological conditions, the contents concentration also has a potential to be biomarkers for diseases. Elevated circulating exosomal miR-92b-5p predicts hospitalization for acute heart failure of HFrEF patients [62]. Reduced exosomal miR-425 and miR-744 in the plasma predicts the progression of fibrosis and heart failure [63]. MiR-129-5p in plasma microvesicles inversely predicts heart failure in univentricular heart disease [64]. Serum exosomal miR-183, miR-21, miR-126, and PTEN are diagnostic biomarkers of acute coronary syndrome [65, 66]. Serum miR-192-5p levels in patients with NAFLD positively correlated with hepatic inflammatory activity score and disease progression [46]. Elevated exosomal miR-20b-5p is a diagnostic biomarker for type 2 diabetes [67]. Increased extracellular vesicle miR-21-5p serves as a biomarker of type 1 diabetes in children [68].

15.2.4 EVs as Therapeutic Agents for Diseases

Function experiments have shown that treatment of cells with EVs in vitro or intravenous administration of EVs in vivo can help improve the pathological state, implying that EVs are promising therapeutic agents for diseases. For example,

exercise induces endothelial cells to release exosomes rich in miR-342-5p into plasma. Intramyocardial delivery of these plasma exosomes from exercised rats shows cardiac protective effect by reducing infarct size and improving cardiac function after myocardial ischemia/reperfusion rats [69]. CPCs derived from atrial appendage explants from patients who underwent heart valve surgery secreted exosomes enriched with miR-210, miR-132, and miR-146a-3p compared with fibroblasts. When incubated with conditioned medium of CPCs, exosome miR-210 downregulates ephrin A3 and PTP1b, inhibiting apoptosis in cardiomyocytic cells and miR-132 downregulates RasGAP-p120, enhancing tube formation in endothelial cells in vitro [70]. Hypoxia/reoxygenation treatment promoted miR-133 expression in endothelial progenitor cells (EPCs) and EPC-derived exosomes, and exosomes with miR-133 transmitted to cardiac fibroblasts could increase the angiogenesis and mesenchymal-endothelial transition of cardiac fibroblasts [71].

Mesenchymal stem cell (MSC) therapy is a promising approach against myocardial infarction (MI). Bone marrow MSC-derived exosomes show cardioprotective effect in a mouse model of ischemia/reperfusion injury by deriving miR-25-3p-containing exosomes into cardiomyocytes, directly targeting the pro-apoptotic genes FASL and PTEN and decreasing the levels of EZH2 and H3K27me3, leading to derepression of the cardioprotective gene eNOS and the anti-inflammatory gene SOCS3 in vitro and in vivo [72]. Exosomes from adipose-derived mesenchymal stem cells ameliorate cardiac damage after myocardial infarction by activating S1P/SK1/S1PR1 signaling and promoting macrophage M2 polarization [73].

15.3 Challenges in EVs Study

15.3.1 Challenges in Isolation and Identification of EVs

To date, there are various isolation methods of EVs based on the biophysical and biochemical properties of EVs, including size, mass density, shape, charge, and antigen exposure. Each of the isolation methods available today has its own advantages and disadvantages. Ultracentrifugation is by far the most commonly used isolation method. Based on the size and density, dead cells and cell debris are removed at low-speed centrifugation and EVs are purified out at high-speed centrifugation [74]. On this basis, the combination of ultracentrifugation and density gradient centrifugation, such as sucrose or iodixanol density gradient tube, can make EVs in the sample enrich in a certain density range, with higher purity [75, 76]. Although this combination method can obtain highly purified exosomes, it also has some disadvantages, such as time-consuming and low recovery. The centrifugation force is likely to cause damage to EVs and reduce its activity [77]. Moreover, soluble protein and virus in the sample may agglomerate together with EVs and then cause sample pollution [78–80], limiting its clinical application. Size exclusion chromatography (SEC) or ultrafiltration enables size-based separation of EVs on a single column or filter with a cutoff pore size [81, 82]. Since EVs are larger than most soluble components, EVs larger than the cutoff pore size can be isolated and soluble contaminations can be ultimately removed. These two methods are simple and efficient [83] and does not affect the integrity and biological activity of EVs [84, 85], making them more clinically applicable. Conversely, ultrafiltration is effective to concentrate EVs up to 240-fold, which may benefit for the detection of contents in EVs. However, SEC and ultrafiltration cannot separate exosomes from other types of EVs which have a similar size to exosomes. Immunocapture assays use monoclonal antibodies to capture EVs that expose a specific ligand. Based on the presence of such ligands, subpopulations of EVs can be isolated. It is the preferred method to extract EVs secreted by specific cells from blood [86]. However, it is inefficient, and the biological activity of exosomes is easily affected by pH and salt concentration, which is not conducive to the downstream experiment. New methods are constantly being invented and it is important to choose the right method for different samples and research

purposes. For clinical biomarker detection, high purity, easy operation, and surface labeling are very important. For functional research, integrity and biological activity are most important. For disease treatment via EVs, high concentration of EVs is better than low concentration.

15.3.2 Challenges in Visualization and Tracking of EVs

At present, most of the research in EV field has stopped at an indirect level. By extracting EVs from tissues or fluid, incubating cells with EVs in vitro, and detecting the changes of EV cargo in recipient cells, we can indirectly confirm the communication between cells via EVs. However, compared with the indirect method, "lighting" EVs can help directly observe the EVs, which is helpful to further understand the biological behavior and function of EVs. Therefore, the establishment of a stable and reliable tracer imaging technology to realize the visualization of EVs is a hot and urgent demand. Fusion luciferase between a membrane-bound variant creates a sensitive EV reporter, thus facilitating fluorescence-mediated imaging of EVs in vivo, displaying a predominant localization of intravenously administered EVs in organs [14]. Similarly, the specific wavelength of fluorescent protein can be used to label exosomes, which can also be captured by fluorescence microscope [15]. In addition to the above optical imaging based label, magnetic resonance imaging or radionuclide based tracers are also used [87, 88]. Although the above technologies can help to show the distribution of EVs in cells, tissues, and organs, they are still limited to the static imaging level. In the future, the technology of dynamic tracking of EVs in vivo or in vitro will be a big challenge in the field of EV research, which can reflect the dynamic transmission process of EVs among organs, tissues, and cells. Meanwhile, more friendly tracers and more sensitive multimodal imaging techniques are key to the dynamic tracking of EVs.

15.4 Conclusions and Perspectives

EV has become a new hotspot in the field of life science in recent years. EVs are thought to play a role in pathophysiological processes by mediating intercellular communication. In the research of cardiovascular and metabolic disease, it is found that EVs play an important role in signal transduction and participate in the occurrence and development of many diseases. It has also been proved that the EVs can help to diagnose and predict the prognosis of cardiovascular and metabolic diseases. At the same time, the research technology of EVs has also made rapid progress. Researchers are continuing to explore more convenient and lower cost extraction technology to obtain more pure EVs from various complex components of fluid, in order to better study the function of EVs and improve the feasibility of it as clinical biomarkers and therapeutic agents. But at the same time, we can also see that there are still many questions to answer. At present, EVs are usually divided into three subtypes according to different biological origins, among which exosomes are the most widely studied. Now the three subgroups of EVs are roughly distinguished according to the size of vesicles. However, there is no consensus on the specific identification of the three subtypes although several specific markers have been found to expressed on the surface of exosomes. Since exosomes and microvesicles are both considered to have the function of mediating cell communication, the differences between these two subtypes have not been clarified. In addition, most of the researches concerning the function of EVs are still at an indirect level by measuring the changes of EVs contents in recipient cells. More and more researchers are trying to make EVs visible by label them with specific tracers, which are important techniques for the functional study of EVs, especially in vivo. However, in the future, dynamic tracking of EVs in living life will be a new challenge in the research field, which can help to fully understand the biological function of EVs.

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Effects of Exercise on Circulating Extracellular Vesicles in Cardiovascular Disease

16

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Abstract

The evidence that physical exercise has multiple beneficial effects and is essential to a healthy lifestyle is widely accepted for a long-time. The functional and psychological changes promoted by exercise improve clinical outcomes and prognosis in several diseases, by decreasing mortality, disease severity, and hospital admissions. Nonetheless, the mechanisms that regulate the release, uptake, and communication of several factors in response to exercise are still not well defined. In the last years, extracellular vesicles have attracted significant interest in the scientific community due to their ability to carry and deliver proteins, lipids, and miRNA to distant organs in the body, promoting a very exciting crosstalk machinery. Moreover, increasing evidence suggests that exercise can modulate the release of those factors within EVs into the circulation, mediating its systemic adaptations.

In this chapter, we summarize the effects of acute and chronic exercise on the extracellular vesicle dynamics in healthy subjects and patients with cardiovascular disease. The understanding of the changes in the cargo and kinetics of extracellular vesicles in response to exercise may open new possibilities of research and encourage the development of novel therapies that mimic the effects of exercise.

Keywords

Exosomes · Coronary heart disease · Heart failure · Cell-to-cell communication · Aerobic exercise

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16.1 Background

The terms physical activity, exercise, and physical fitness are often used interchangeably, but there are important differences worth mentioning. Physical activity is defined as any bodily movement produced by the contraction of skeletal muscles, which results in energy expenditure

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above the resting values [1]. Physical activity may include occupational, sports, conditioning, household, or leisure activities such as walking, bicycling, or swimming. Physical fitness is a construct of health-related and skill-related attributes that refers to the ability of our body systems to work together efficiently. The set of health-related attributes includes body composition, cardiorespiratory endurance, flexibility, muscular endurance, and power, while the skill-related components comprise balance, agility, coordination, speed, and reaction time [1, 2]. Physical exercise is a subset of physical activity aiming to improve and/or maintain one or more components of physical fitness; exercise is a planned, structured, and repetitive physical activity [1]. Exercise prescription and training are framed by the FITT-VP principle: Frequency (how many sessions per week), Intensity (how hard to exercise), Time (duration of the exercise session), Type (modalities of exercise training), Volume (the total amount or dose of exercise), and Progression (the rate of increasing the dose of exercise). The quantification of the intensity of physical activity is usually made using a percentage of maximal (or reserve) oxygen consumption, heart rate (reserve or maximal), or metabolic equivalents (1 MET = 3.5 mL/kg/min). Table 16.1 summarizes the physical activity intensity categories.

In the context of cardiovascular and metabolic diseases, exercise-based cardiac rehabilitation has received the highest class of recommendation and level of evidence in several clinical conditions such as stable angina/chronic coronary syndromes [4, 5], diabetes [6], after myocardial infarction [7–9], after myocardial revascularization [10], peripheral arterial disease [11], or in the setting of chronic heart failure [12, 13]. It is also recommended after cardiac transplantation or heart valve surgery [14, 15]. Figure 16.1 summarizes major chronic adaptations resulting from exercise training in patients with cardiovascular disease. In brief, an exercise training program or exercise-based cardiovascular rehabilitation program improves exercise capacity, lipid metabolism, resting blood pressure, endothelial function, inflammatory status, antioxidant defenses, parasympathetic tone, and quality of life [17–24]. These functional and physiological changes will consequently improve clinical outcomes and prognosis, by decreasing mortality, disease severity, and hospital admissions [21, 22].

The evidence that physical exercise yields many beneficial effects and is essential to a healthy lifestyle is recognized for a long-time. Nonetheless, little is known about the mechanisms that regulate the release, uptake, and communication of several factors (including proteins, miRNA, and myokines) in response to exercise. The link between extracellular vesicles (EVs) and exercise is in its primordial days; nonetheless, increasing evidence suggests that the release of peptides, mRNAs, and miRNA within EVs (including exosomes) into the circulation, in response to exercise, could mediate its systemic adaptations [25]. These EVs interact with and modify local and distant cellular targets, mediating organ crosstalk to promote systemic adaptations to exercise (Fig. 16.2). This chapter aims to explore the acute and chronic effects of exercise in EVs production and consequently the role of exercise-induced EVs in mediating the well-known benefits of exercise in cardiovascular diseases.

16.2 Acute Effects of a Single Bout of Exercise

The following sections provide an overview of the acute effects of a single bout of aerobic exercise, either submaximal or maximal/strenuous, on EVs among healthy individuals and patients with cardiovascular disease. The kinetics of the EVs released after a bout of exercise seems to be different depending on the population of EVs analyzed, and the type, intensity, and duration of the exercise bout. In general, an acute bout of exercise triggers the release of EVs into circulation by a vast array of cells and modifies the load that will be transported to different parts of the body [26, 27]. A summary of the studies assessing the circulating EVs profile in response

Table 16.1 Physical activity intensity categories for cardiorespiratory (aerobic) and resistance activities

	Aerobic (endurance) exercise				Resistance exercise
	Absolute intensity	Relative intensity			Relative intensity
Intensity	MET	%VO_2R or % HRR	% VO_{2max}	%HR_{max}	% One repetition maximum
Very light	<2.0	<30	<37	<57	<30
Light	2.0 to <3.0	30 to <40	37 to <45	57 to <64	30 to <50
Moderate	3.0–6.0	40 to <60	45 to <64	64 to <76	50 to <70
Vigorous	6.0 to <8.8	60 to <90	64 to <91	66 to <96	70 to <85
Near maximal to maximal	≥8.8	≥90	≥91	≥96	≥85

Adapted from [3]
HR_{max} maximal heart rate, *HRR* heart rate reserve, *MET* metabolic equivalent, VO_{2max} maximum oxygen uptake, VO_2R oxygen uptake reserve

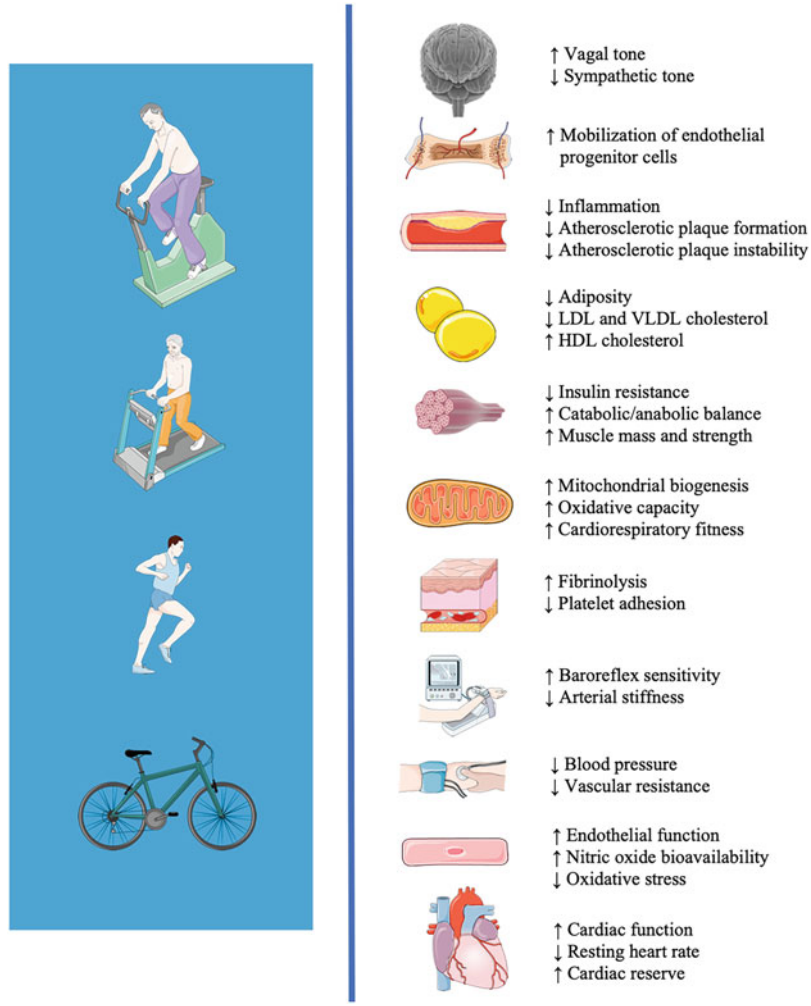

Fig. 16.1 Main cardioprotective benefits of physical activity and exercise. The elements of the figure were obtained from SERVIER Medical Art image bank (https://smart.servier.com). *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *VLDL* very low-density lipoprotein. ↑ Improved/increased; ↓ Decreased. (Adapted from [16])

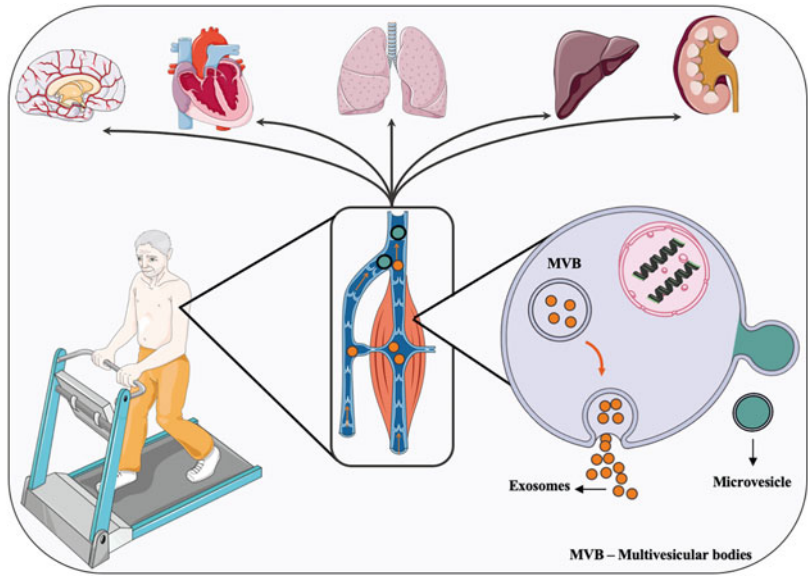

Fig. 16.2 Exercise training promotes the release of extracellular vesicles from several cell types, mainly from muscle cells, into circulation. These circulating extracellular vesicles induce the crosstalk between distant organs mediating systemic cardioprotective adaptations to exercise, such as improvement of endothelial function, tissue remodeling and repair. This figure was created using resources from SERVIER Medical Art (https://smart.servier.com)

to an acute bout of exercise is presented in Table 16.2.

16.2.1 Healthy Subjects

The study of the release of EVs in response to exercise is a recent topic of research, with most of the studies assessing the acute effect of aerobic exercise. Most trials tested the acute effects of a single bout of cycling or running, either maximal or submaximal, in circulating levels of EVs assessed at different time points (e.g., immediately after, one or 2 h after the exercise bout) and showed that one bout of aerobic exercise triggers an efflux of total EVs into circulation, starting immediately after the beginning of the session and returning to baseline after recovering a few hours (Table 16.2). Whitham et al. [27] performed a very comprehensive study characterizing the exercise-induced secretion of proteins contained in EVs following a 1-h bout of cycling exercise (30 min at 55%, 20 min at 70%, and approximately 10 min [until exhaustion] at 80% of VO_2max) in 11 healthy adults. The authors showed that a single exercise session increased the circulating levels of EVs; they further evaluated the proteome of the EVs by nano-ultra-high-performance liquid chromatography-tandem mass spectrometry analysis and found than 300 significantly different proteins between rest and after exercise, most of them associated with the biogenesis of exosomes. Additionally, 35 myokines (proteins that are synthesized and secreted by muscle cells) were found in plasma-derived EVs from exercised healthy men, suggesting that EVs might mediate the transport of those proteins by a process independent of the classic secretory pathway. Using a mice model, the authors also demonstrated that the exercise-derived EVs can communicate and transfer their protein cargo to the liver, indicating a crosstalking pathway between the tissues involved in the exercise and systemic tissues, which might include other organs such as lungs, kidneys, heart, and brain [40].

Exercise Characteristics

There are some characteristics of the exercise bout (e.g., type, intensity, duration) that seem to be moderator variables of the effect of exercise on EVs. It is important to mention that cycling or running protocols may induce different EVs response. For instance, Fruhbeis et al. [31] tested the effects of two incremental exercise tests until exhaustion (cycling on a bicycle ergometer versus running on a treadmill) and observed that both tests triggered a rapid release of EVs with the size

Table 16.2 Summary of the studies assessing the acute effects of exercise on EVs profile

Study	Participants/ model	Exercise protocol	Fluid	Isolation method	EVs characterization method	Exercise-induced effects/changes
Annibalini et al. [28]	Healthy men ($n = 8$)	Resistance exercise: 5 sets of 10 maximal squats On a flywheel	Plasma	UC	NTA	– Two-fold ↑ in EV yield 2 h after exercise – ↑ miR-206 and miR-146a in EVs (2 h after exercise)
Bei et al. [29]	Adults with risk factors or established cardiovascular disease ($n = 16$)	Exercise stress test	Plasma	N.A.	FC	↑ EVs after exercise
Chaar et al. [30]	Healthy men ($n = 7$)	High-intensity interval exercise (3 maximal ramp exercise interspaced with 10 min of recovery)	Plasma	Centrifugation	FC	– ↑ PMPs and PMNMPs levels – No changes for red-blood cells MP, MMPs, and EMPs
Frühbeis et al. [31]	Healthy men ($n = 12$)	Incremental aerobic exercise (cycling or running) until exhaustion	Plasma	dUC	NTA; WB	– ↑Small EVs after exercise – Slower clearance of running-derived EVs vs cycling EVs in recovery phase
Guescini et al. [26]	Healthy men ($n = 18$)	– Treadmill fitness test – 40-min of aerobic exercise at 80% of the VO_{2max}	Plasma	UC and DG or IP	WB, FC	Exercise-induced EVs subpopulation enrichment in α-sarcoglycan and muscle-specific miRNAs
Guiraud et al. [32]	Men with CHD ($n = 19$)	High-intensity interval (2×10: 15-s at 100% of PPO and 15-s recovery) or isocaloric continuous exercise (28.7 min at 70% of PPO)	Serum	N.A.	FC	No change in EMPs and PMPs after both exercise bouts
Lovett et al. [33]	Healthy men ($n = 9$)	Plyometric jumping and downhill running	Plasma	SEC	NTA, TEM	– No changes in mean EVs size or number

(continued)

Table 16.2 (continued)

Study	Participants/ model	Exercise protocol	Fluid	Isolation method	EVs characterization method	Exercise-induced effects/changes
Rakobowchuk et al. [34]	Healthy men ($n = 12$)	45 min of eccentric or concentric cycling below ventilatory threshold	Plasma	N.A.	FC	↑ PMVs but not EMVs levels
Rigamonti et al. [35]	Obese ($n = 15$) and normal-weight adults ($n = 8$)	30-min or until voluntary exhaustion exercise at 60% of VO_{2max}	Plasma	UC	NTA, FC	– ↓ Total EVs number – ↑ MV release in women vs men – ↑ Exosome release in men vs women
Serviente et al. [36]	Healthy women ($n = 11$)	30-min of aerobic exercise at 60–64% of VO_{2peak}	Plasma	Centrifugation	FC	↓ Concentration of total MPs, $CD62E^+$, and $CD31^+/42b^-$ EMPs
Schwarz et al. [37]	Marathon runners ($n = 99$)	Marathon	Plasma	N.A.	FC	– ↑ EMPs after the marathon – EMPs returned to baseline after 2 days
Shill et al. [38]	Healthy adults ($n = 20$)	Aerobic interval exercise (10×1 min intervals at ~95% of VO_{2max}) and continuous exercise (65% VO_{2max})	Plasma	N.A.	FC	– ↓ $CD62E^+$ MPs after continuous exercise (only in women) – CD34+ MPs did not change in response to both exercise protocols
Wilhelm et al. [44]	Healthy men ($n = 9$)	60-min of aerobic exercise at ~46% or ~ 67% of VO_{2max}	Plasma	N.A. or centrifugation	FC	– ↑PMVs, but not EMVs, after the higher exercise intensity – Exercise-derived MVs improved endothelial cells proliferation and migration and stimulated angiogenesis

(continued)

Table 16.2 (continued)

Study	Participants/ model	Exercise protocol	Fluid	Isolation method	EVs characterization method	Exercise-induced effects/changes
Whitham et al. [27]	Healthy men ($n = 11$)	60-min of aerobic exercise at increasing intensities (55%, 70%, and 80% of VO_{2max})	Plasma	UC	NTA; Cryoelectron microscopy; nano-UHPLC-MS/MS	– ↑ Number of small EVs and proteome changes – ↑ Proteins related to EVs biogenesis and GO terms GTPase signaling and glycolysis – EVs release of new myokine candidates
Yin et al. [39]	Male Sprague-Dawley rats ($n = 84$)	90-min of downhill or uphill (±15%) running (20 m/min)	Plasma	ExoQ	–	↑ Muscle-specific exosomal miRNAs levels following downhill run, which returned to baseline after 48 h

DG density gradient, *dUC* differential ultracentrifugation, *EMPs* endothelial cell-derived MPs, *EMVs* endothelial microvesicles, *ExoQ* ExoQuick, *EVs* extracellular vesicles, *FC* flow cytometry, *IP* immunoprecipitation, *MMPs* monocyte-derived MP, *miRNA* microRNA, *MPs* microparticles, *MVs* microvesicles. *N.A.* non-applicable, *nano-UHPLC-MS/MS* nanoscale liquid chromatography coupled to tandem mass spectrometry, *NTA* nanoparticle tracking analysis, *PMNMPs* polymorphonuclear neutrophils microparticles, *PMPs* platelet-derived MP, *PMVs* platelet-derived microvesicles, *PPO* peak power output, *SEC* size-exclusion chromatography, *TEM* transmission electron microscopy, *UC* ultracentrifugation, *WB* Western blot

and marker profile of exosomes into circulation; although the clearance of the exosomes from circulation was faster after cycling than after running exercise. A potential explanation for this difference in aerobic exercise modalities is the greater muscle mass recruited and the higher eccentric burden for the muscle during a running protocol per comparison to a cycling protocol, which may promote a more pronounced exosome generation after exercise cessation. Indeed, the increase in circulating EVs may be related to (1) the disposal of cellular waste that is generated by stress conditions caused by tissue and cell stimulation during exercise [31] and (2) the higher inflammatory, tissue repair, and angiogenesis response (EVs work as a signaling transporter of several players involved in this response) [41]. Indeed, it is not completely unexcepted to find a more pronounced EVs response to treadmill exercise per comparison to cycle ergometer exercise because treadmill exercise may recruit more muscle mass and induce a higher muscle damage stimuli (higher eccentric work).

Few studies assessed the effects of dynamic resistance exercise. In this regard, Annibalini et al. [28] recruited eight resistance-trained men to perform 5 sets of 10 maximal squats and showed a significant increase in circulating EVs and EV-encapsulated miRNA levels (miR-206 and miR-146a) 2 h after the exercise bout. The muscle damage caused by the maximal flywheel squat protocol was also associated with the increase in circulating EVs [28]. Although there was a marked increase in inflammatory and skeletal muscle damage markers after the resistance exercise bout, EVs were enriched in miR-206 and miR-146a. These miRNAs might influence the inflammatory profile after the exercise and the

muscle recovery since miR-206 plays an important role in muscle development and differentiation, [42] and miR-146a negatively regulates proinflammatory cytokines and chemokines expression [43].

Regarding exercise intensity and duration, there is evidence indicating that both factors influence the release of EVs [31, 38, 39]. Wilhelm et al. [44] tested the effect of exercise intensity (semi-recumbent cycling at two intensities: 46 ± 2% VO_{2max} versus 67 ± 2% VO_{2max}) and showed that endothelial ($CD62E^+$) and platelet ($CD41^+$) EVs concentration did not change with moderate exercise, but platelet EVs, in particular microvesicles (MV), had a two-fold increase after the more vigorous exercise. The effect of intensity is not a surprise, since a myriad of acute physiological responses to exercise are determined by exercise intensity; also, maximal and strenuous exercise protocols generally inflict severe muscle damage, inflammation, and prolonged recovery, [45] which may promote a higher efflux of EVs. There is limited information on the effects of the duration of the exercise bout in the release of EVs. As described above, even small bouts of exercise (such as few minutes of a maximal exercise test) promote a significant increase in circulation EVs, while some studies using long-duration exercise showed modest increases in EVs. For instance, Schwarz et al. [37] demonstrated that the changes in the EVs profile were transient and the EVs levels returned to baseline after 2 days of a marathon; they also observed that the increase in endothelial and platelet EVs was correlated with the number of marathons, but not with the time of running. Similarly to exercise protocols of shorter duration, long-duration exercise (e.g., marathon) is associated with the increase in endothelial EVs ($CD144^+$, $CD31^+$, and $CD62E^+$) and total platelet EVs ($CD62P^+$ and $CD42b^+$), suggesting acute endothelial damage due to the increase in apoptotic endothelial EVs and a high pro-thrombotic and proinflammatory profile [37]. Nonetheless, a study assessing the release of EVs during 1 h of continuous aerobic exercise observed that circulating EVs increased in the first half of the exercise (assessed at 30 min of exercise) and remained stable in the second half (assessed at 1 h of exercise) [44]. Taken together the available evidence suggests that exercise duration has less impact on EVs release than exercise intensity.

Sources of the EVs

Skeletal muscle is the major source of EVs in response to exercise, confirmed by the presence of several myokines in EVs during exercise or after recovery [25, 27]. Although there are other important sources of EVs in response to exercise such as endothelial cells, platelets, and red and white blood cells. Since exosome release is associated with the increase in intracellular calcium (Ca^{2+}) [46, 47] and that exercise stimulates the sarcoplasmic reticulum to release Ca^{2+} to proceed with muscle contraction, [48] muscle cells may trigger the release of exosomes faster than other organs upon stimulation [46].

Interestingly, the EVs response to acute exercise in healthy individuals seems to be population-dependent [40]. For instance, Chaar et al. [30] reported a higher total concentration of EVs, including the platelet-derived microparticles (MPs) ($CD41^+$), in plasma immediately after strenuous exercise, whereas erythrocyte-derived EVs ($CD235a^+$) were not significantly changed after exercise. Also, Rakobowchuk et al. [34] found an increase in platelet-derived MVs ($CD41^+$) after a bout of either eccentric or concentric cycling at a matched aerobic power output below the ventilatory threshold, while there were no significant differences in endothelial derived EVs ($CD62E^+$).

Despite most studies showed an increase in the circulating levels of EVs as a result of a single bout of exercise, there are also some contrasting data, indicating a reduction in the release of EVs upon acute exercise. Lovett et al. [33] did not found significant differences in the size and concentration of EVs after two consecutive bouts of strenuous exercise, while Serviente et al. [36] observed a decrease in the total count of MPs, following an acute bout of exercise on a treadmill. Moreover, some studies reported also gender-related differences in the postexercise release of EVs [35, 38]. Women showed higher levels of MVs after an exercise session and lower levels of

exosomes in comparison to men [35]. There is probably a hormonal interference in the release of EVs and the generally higher muscle mass in men could boost the release of exosomes.

It is also important to mention that there are some confounding variables among the studies assessing the effects of exercise on EVs release. The difference in the type and/or source of EVs that is evaluated, the methods of extraction and detection of EVs are factors that may influence the results [49]. The study of exosomes is particularly hard because the isolation methods do not guarantee that only the smallest EVs are extracted, and it is expected that MVs and apoptotic bodies may also appear in the evaluation. Further, contradictory results might also occur due to the evaluation of different subpopulations of EVs. For example, there are distinct phenotypes of endothelial-derived EVs, activated and apoptotic endothelial MPs, which might show different responses to exercise [36].

In general, a single bout of aerobic exercise stimulates the efflux of EVs into circulation and modifies the content to be delivered; the effects of exercise seem to be modulated by the characteristics of the exercise bout. Moreover, the kinetics of EVs in response to exercise is different depending on the source/population of EVs evaluated.

16.2.2 Cardiovascular Disease

Cardiometabolic risk factors and established cardiovascular disease interfere with EVs release and may also modulate the EVs response to exercise. Aging, inflammatory status, oxidative stress and antioxidant defenses, endothelial dysfunction, and even medication may affect the cargo and release of EVs. For instance, patients with diseases with a marked increase in inflammation will likely show higher cytokine-mediated EVs [50]. The cargo of EVs might be compromised in pathological conditions and exercise training may restore their properties [51]. Nonetheless, in contrast with healthy individuals, in patients with cardiovascular and metabolic disease the information is scarce on whether acute exercise changes the concentration and the cargo of EV [40]. Similarly to healthy adults, Bei et al. [29] also observed a rapid increase in plasma EVs in patients (mean age: 54 ± 11 years old) with cardiometabolic risk factors (including history of cardiovascular disease in some of them) undergoing an exercise stress testing. The authors reported a significant increase in the EVs at peak exercise in comparison to resting state (43.1 ± 24.6–54.7 ± 25.7%, $P < 0.05$); despite inter-individual variations in the resting levels of EVs, the response to maximal exercise was similar among all patients. Rigamonti et al. [35] also reported differences in the release of EVs in adults with obesity per comparison to adults with normal body mass index in response to an acute bout of exercise. A significant decrease in the total EVs after exercise was found; however, adults with normal body mass index had higher levels of EVs in comparison with the participants with obesity. This result might be related to the physical capacity of the normal body mass index participants, which allowed them to perform at higher intensities of aerobic exercise and for a longer time.

The impact of acute exercise on the dynamics of EVs in circulation is still not clear in patients with established cardiovascular disease. Giraud et al. [32] compared the effects of a single session of high-intensity interval exercise (two 10-min sets composed of repeated bouts of 15 s at 100% of peak power output interspersed by 15 s of passive recovery) with an isocaloric moderate-intensity continuous exercise (cycling during 28.7 min at 70% of peak power output) on endothelial ($CD42b^-/CD31^+/CD62E^+$) and platelet-derived MPs ($CD42b^+$) of patients with coronary heart disease. EVs levels were assessed before exercise and 20 min, 24 h, and 72 h after both exercise sessions; they observed that endothelial and platelet-derived EVs remained unchanged after both exercise sessions. However, the authors reported that those patients with higher levels of EVs at baseline experienced a bigger drop in the amount of EVs after exercise.

The contrasting results concerning the effects of acute aerobic exercise on circulating levels of EVs in patient populations may suggest that, per

opposition to healthy individuals, in patients with cardiovascular diseases the induction of EVs by acute exercise is blunted. Whether the acute response to exercise (maximal or submaximal) predicts future cardiovascular events or the chronic response to an exercise training intervention is a question that deserves an answer in future research.

16.3 Chronic Effects of an Exercise Intervention

Exercise stimulates the release of EVs after a single bout of exercise, however, this effect is transient; their levels return to baseline after a few hours, suggesting that EVs are taken away from circulation and captured by other cells and tissues [46]. Regular exercise participation promotes a constant articulation between the EVs from the cells that will be subjected to metabolic and mechanical stimuli by exercise (i.e., skeletal muscle, endothelial cells) and the systemic organs, guaranteeing a consistent acute stimulus that will likely have a chronic effect. Indeed, the uptake of the EVs in systemic target cells starts just after exercising, but long-term exercise seems to regulate, modify, and adapt the cargo inside the EVs. While acute exercise is generally associated with an increase in circulating EVs, chronic exercise has a distinct response. In general, exercise training will exert multiple alterations on EVs kinetics, namely reduction of endothelial and inflammatory cell-derived EVs, which are associated with many of the beneficial effects promoted by exercise. A summary of the studies assessing the effects of chronic exercise on EVs profile is presented in Table 16.3.

16.3.1 Healthy Subjects

Regular aerobic exercise induces positive changes in the release and uptake of EVs. Trained people seem to have the same concentration of total EVs at baseline compared to non-trained subjects, however, after completing a single session of aerobic exercise, the increase in EVs is more pronounced in trained subjects. For instance, Sossdorf et al. [62] demonstrated that trained individuals show different MPs release and uptake by target cells in response to exercise despite having levels of circulating MPs at baseline similar to untrained participants; a single session of moderate-intensity aerobic exercise induced a significant increase in MPs derived from platelets ($CD42a^+$), monocytes ($CD14^+$), and endothelial cells ($CD62E^+$) in the trained participants, although the number of these type of EVs returned to baseline levels after 2 h. In contrast, the untrained participants only had increased platelet-derived MPs, which remained elevated 2 h after exercise, suggesting that the trained participants have more efficient EVs uptake and trafficking systems.

The cargo of EVs is also modified by exercise training interventions. Although Hou et al. [63] did not show any difference between the size and concentration of plasma exosomes between healthy trained and untrained participants, changes were detected at the molecular level. Exosomes of trained participants reduced myocyte apoptosis and lactate dehydrogenase release and increased cardiomyocyte viability in cultured human cardiomyocyte subjected to hypoxia/reoxygenation, while the exosomes from untrained participants did not exert the same beneficial effects on cell culture. Furthermore, the authors also confirmed that exosomes from trained participants were enriched with a potent cardioprotective molecule (miR-342-5p), which support the idea that regular exercise has a protective role and that EVs may be responsible for carrying the molecular content (myokines, proteins, ncRNA) in charge of cell-to-cell communication that allows continuous assistance to cells and tissues and maintain homeostasis.

Contrasting with the effects of acute exercise, circulating levels of endothelial EVs seem to decrease after an aerobic exercise training intervention. For instance, Kim et al. [58] assessed the effects of supervised aerobic exercise training for 6 months on EVs and reported a decrease in total

Table 16.3 Summary of the studies assessing the chronic effects of exercise on EVs profile

Study	Participants/ model	Exercise protocol	Fluid	Isolation method	EVs characterization method	Exercise-induced effects/changes
Chaturvedi et al. [52]	Db/db mice as models of type 2 diabetes	Treadmill running for 8 weeks at 7 or 10 m/min for 300 m/day, 5 days/week	Heart tissue and serum	UC	Electron microscopy; FC; WB	↑ Exosome release
Chen et al. [53]	Healthy men ($n = 60$)	– 5-week AIT: 5 × 3-min intervals at 40% and 80% VO_{2max} – 5-week MCT: 5 days/week., 30 min at 60% VO_{2max}	Plasma	Filtration (0.2 μm)	FC	↓ Neutrophil-derived MPs after both AIT and MCT
Van Craenenbroeck et al. [54]	Patients with CAD ($n = 200$)	– AIT: 4 × 4-min intervals at 90–95% separated by 3-min at 50–70% HR peak – MCT: 37 min at 70–75% of HR peak – 3 sessions a week. for 12 weeks	Plasma	N.A.	FC	No changes in EMPs levels after both AIT and ACT
Eichner et al. [55]	Adults with obesity ($n = 18$)	– AIT: 12 sessions alternating 3 min intervals at 90% and 50% HR peak – MCT: 12 sessions at 70% HR peak	Plasma	Centrifugation	FC	– No changes on platelet and leukocyte EVs – ↓ Endothelial EVs after AIT vs MCT (effect lost after accounting for dietary sugar intake)
Feairheller et al. [56]	Adults ($n = 26$, 9 with prehypertension and 7 with hypertension)	6 months (3 days/week) of aerobic exercise (progressing from 20 min at 50% to 40-min at 65% VO_{2max})	Plasma	N.A.	FC	↓ EMPs levels after chronic exercise
Hsu et al. [57]	Patients with HFrEF ($n = 38$)	AIT: 3 sessions per week composed by 5 × 3-min intervals at 40% and 80% VO_{2peak}, for 12 weeks	Plasma	N.A.	FC	↓ EMPs and ↑PMPs, LMPs and RBMPs

(continued)

Table 16.3 (continued)

Study	Participants/ model	Exercise protocol	Fluid	Isolation method	EVs characterization method	Exercise-induced effects/changes
Kim et al. [58]	Prehypertensive adults ($n = 21$)	6 months (3 days/week) of aerobic exercise (40-min at 65% maximal HR)	Plasma	N.A.	FC	↓ Total and activated EMPs
Kränkel et al. [59]	Adults with chronic coronary syndrome ($n = 25$)	4 weeks of aerobic exercise (4 × day for 30 min, 5 × week) at 70% of the individual ischemia/ angina–free exercise capacity	Plasma	Filtration and UC	NTA, FC	↓ Leukocyte-derived EVs
Ma et al. [60]	C57BL/6 J mice	– Low exercise: Running at 5 m/ min, 5 days per week. for 4 weeks – Moderate exercise: Running at 10 m/min, 5 per week., for 4 weeks	Plasma	UC followed by microbead-based isolation	NTA, WB	↑ Endothelial progenitor cell-derived exosomes with moderate-intensity exercise
Wang et al. [61]	C57BL/6 mice, subjected to stroke after the exercise training	Moderate treadmill exercise (10 m/ min for 4 weeks, 5 days per week)	Plasma and brain tissue	UC	NTA	↑ Plasma and brain-derived exosomes ↑miR126 exosomal levels and ↓ infarct volume and cell apoptosis

ACT aerobic continuous training, *AIT* aerobic interval training, *CAD* coronary artery disease, *dUC* differential ultracentrifugation, *EMPs* endothelial microparticles, *EVs* extracellular vesicles, *ExoQ* ExoQuick, *FC* flow cytometry, *HFrEF* heart failure with reduced ejection fraction, *LMP* leukocyte derived-microparticles, *MCT* moderate continuous training, *miR* microRNA, *MMP* matrix metalloprotease, *MP* microparticles, *N.A.* non-applicable, *NTA* nanoparticle tracking analysis, *PMP* platelet microparticles, *RBMPs* red-blood cells microparticles, *TEM* transmission electron microscopy, *UC* ultracentrifugation, *WB* Western blot

($CD31^+/CD42a^-$) and active ($CD62E^+$) endothelial MPs after the training program. Moreover, the authors correlated the increase in laminar shear-stress with mitochondrial biogenesis and the decrease in endothelial-derived MPs, suggesting a protective role against endothelial dysfunction. The reduction of endothelial-derived MPs is most likely associated with the repeated exposure of endothelial cells to shear-stress, since laminar shear-stress suppresses apoptosis of endothelial cells [64]. Feairheller et al. [56] also observed a decrease in endothelial MPs ($CD31^+/CD42^-$ and $CD62^+$) after 6 months of aerobic exercise training in adults, accompanied by an increase in plasma nitric oxide levels and flow-mediated dilation and a decrease in carotid artery intima-media thickness. Another study also showed a significant decrease (~50%) in a subset of circulating endothelial MPs ($CD62^+$) that are derived from activated endothelial cells after 24 weeks of

aerobic exercise of moderate intensity (50–65% of VO_{2max}) together with a reduction in circulating inflammatory biomarkers and an increase in endothelial function assessed by flow-mediated dilation [65]. Interestingly, reducing daily physical activity levels of healthy recreationally active men for 5 days increased apoptotic endothelial MPs ($CD31^+/CD42b^-$) in circulation, [66] blunting the benefits of exercise and suggesting a rapid detraining effect.

The chronic effects of aerobic exercise are also dependent on the intensity of the exercise. Chen et al. [53] showed that 5 weeks of aerobic interval training (five exercise cycles, 3-min each at 80% of VO_{2max} alternated with a 3-min active recovery at 40% of VO_{2max}) and moderate continuous training (30 min at 60% of VO_{2max}) reduced thrombin generation, procoagulant activity, and the MPs derived from neutrophils ($CD16^+$) in young men at rest and after a hypoxic exercise test, but the changes were more noticeable in the interval training. Eichnner et al. [55] corroborated these results using a similar approach; they compared the effects of 12 supervised sessions of high-intensity interval training (alternating 3-min intervals at 90% and 50% of heart rate peak, respectively) versus moderate-intensity continuous training (at 70% of heart rate peak). High-intensity interval training significantly decreased endothelial EVs ($AV^-/CD105^+$) in comparison to continuous training, while leukocyte ($CD45^+$ and $CD45^+/CD41^-$) and platelet ($CD31^+/CD41^+$) derived EVs did not change. The high-intensity interval training also induced a greater increase in VO_2 peak in comparison to moderate continuous training, which was related to the decrease in the endothelial EVs. These results suggest that aerobic interval training has greater cardiopulmonary adaptations than moderate continuous exercise, which might be mediated by changes in EVs from endothelial and inflammatory cells.

16.3.2 Cardiovascular Disease

The capacity of EVs carrying bioactive markers to participate in the pathophysiology of several diseases suggests that EVs can be modulated by chronic changes, such as those imposed by regular exercise [40]. It is widely accepted that exercise training is beneficial for cardiovascular health, [67] and the exercise-induced modifications in the quantity and cargo of the EVs may reflect an improvement in cardiovascular homeostasis.

Despite the small number of studies assessing the effects of chronic exercise on the EVs dynamics in cardiovascular diseases, there is a clear tendency for the reduction of apoptotic endothelial EVs and the increase in EVs derived from endothelial progenitor cells (Table 16.3). For instance, coronary heart disease is characterized by increased levels of endothelial and leukocyte-derived EVs, which were significantly reduced after 4 weeks of high-intensity interval training [59]. This is particularly relevant because elevated circulating endothelial EVs have been associated with several cardiovascular diseases, and these particles may have pro-coagulation and inflammatory properties [41, 68]. The procoagulant activity of endothelial EVs is evidenced by elevated activation of the extrinsic coagulation pathway and by allowing the interaction with coagulation factors [69].

The decrease in the apoptotic endothelial EVs may be linked with the augment of EVs derived from endothelial progenitor cells with exercise training, since these vesicles were also reported to have antiapoptotic characteristics [61]. Wang et al. [61] demonstrated that moderate aerobic exercise training increases the levels of circulating endothelial progenitor cell-derived exosomes ($CD34^+/VEGFR2^+$) in plasma and brain tissue. Moreover, aerobic exercise increased the levels of miR-126 on the exosomes derived from endothelial progenitor cells and decreased infarct volume and cell apoptosis in mice subjected to ischemic stroke [60, 61]. miR-126 regulates angiogenic signaling and vascular integrity [70] and is often downregulated in cardiovascular diseases [71–73]. Hence, reverting the increase in apoptotic endothelial EVs may mitigate vascular damage and improve endothelial function in patients with cardiovascular disease.

In patients with coronary heart disease, high-intensity interval training also reduced the levels of EVs derived from the leukocytes ($CD45^+/$

$CD41^-$) and restored the capacity of vascular repair, despite not changing the total count of EVs [59]. These results are in line with the well-known anti-inflammatory effect of exercise training among patients with cardiovascular disease [19], which may halt the release of the EVs from lymphocytes. Although another study [54] failed to show an increase in circulating endothelial MPs ($CD31^+/CD42b^-$) after 12 weeks of aerobic interval training or moderate continuous training in patients with coronary heart disease. However, the baseline endothelial MPs were inversely correlated with the increase in VO_{2peak} and a better endothelial function following exercise training [54].

Increased circulation levels of platelet EVs have been described in several diseases, mostly associated with coagulation, immune response, and inflammation [74]. Although when properly stimulated, platelet EVs also have a positive influence on angiogenesis and hound healing [75], since these extracellular vesicles can carry several growth factors such as platelet-derived growth factor, vascular endothelial growth factor (VEGF), insulin-like growth factor 1, and transforming growth factor-β, which are essential to vascular growth. There are few studies assessing platelet-derived EVs in response to an exercise training intervention. Hsu et al. [57] showed that 12 weeks of high-intensity interval exercise reduce the levels of endothelial MPs in patients with heart failure with reduced ejection fraction. Moreover, the authors found an increase in procoagulant EVs and EVs derived from platelets, leukocytes, and erythrocytes. Considering that exercise is capable of modifying the cargo of EVs, it is plausible that exercise may also recover the profile of platelet-derived EVs in pathological conditions.

Exercise training may also impact the cargo of EVs and not just their circulating levels. The concentration of exosomes isolated from serum and heart of mice with diabetes (db/db mice) increased after 8 weeks of treadmill running [52]. Furthermore, exercise may mitigate cardiac dysfunction and remodeling by increasing the levels of miR29b and miR455 in the exercise-derived exosomes. These miRNAs bind and inhibit MMP9, a matrix metalloproteinase that is linked to endothelial dysfunction [76].

In summary, aerobic exercise training reduces the levels of endothelial EVs that are released into circulation and increases the EVs derived from endothelial progenitor cells, reflecting an improvement in endothelial and vascular function. It is still not clear how total EV concentration responds to chronic exercise; also, the available information about the effects of exercise training on several populations of EVs (e.g., erythrocytes, muscle cells, platelets) is still limited, hindering the understanding of the mechanisms of cell-to-cell communication in response to chronic exercise in patient populations.

16.4 Summary and Future Perspectives

Exercise has multiple health benefits in both healthy subjects and patients with cardiovascular diseases. A single bout of exercise triggers the efflux of EVs and modifies their molecular cargo. The transient increase in the levels of total EVs in circulation in healthy individuals in response to an exercise bout may reflect the normal response to the metabolic and mechanical stress triggered by exercise. The exercise characteristics seem to induce different effects on the concentration and cargo of circulating EVs, which is not surprising since the physiological stress and the acute cardiovascular and metabolic responses to exercise are dependent on the exercise intensity, duration, and type. There are few reports on the influence of acute and chronic exercise in the cargo of EVs and their dynamics in cardiovascular disease. However, the positive chronic effects of an exercise training intervention on EVs derived from endothelial cells seem to reinforce the beneficial role of exercise on vascular integrity and endothelial function, in both healthy adults and patients with cardiovascular disease.

Therapies based on EVs seem to have a lot of potential in several clinical conditions [77–80], although repeated EVs injections might be needed to achieve a sustainable effect

[81, 82]. In this context, exercise may be a handy option since an acute bout of exercise may boost EVs protective role. Further analysis of the cargo of EVs—regarding proteomic, lipidomic, transcriptomic, and metabolic profiles—in response to an exercise intervention could help understanding how exercise promotes systemic benefits in patients with cardiovascular disease. Ultimately, this understanding may open new possibilities of research and foster the development of novel therapies mimicking the effects of exercise.

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Platelet-Derived Extracellular Vesicles in Arterial Thrombosis

17

Matthew T. Harper

Abstract

Blood platelets are necessary for normal haemostasis but also form life-threatening arterial thrombi when atherosclerotic plaques rupture. Activated platelets release many extracellular vesicles during thrombosis. Phosphatidylserine-exposing microparticles promote coagulation. Small exosomes released during granule secretion deliver cargoes including microRNAs to cells throughout the cardiovascular system. Here, we discuss the mechanisms by which platelets release these extracellular vesicles, together with the possibility of inhibiting this release as an antithrombotic strategy.

Keywords

Platelets · Thrombosis · Extracellular vesicles · Microparticles · Exosomes · Phosphatidylserine · Exocytosis

17.1 Introduction

Blood platelets are necessary but sometimes deadly. Platelets aggregate to form a haemostatic plug at sites of vascular injury, preventing excessive bleeding and starting the process of wound healing. However, where an arterial atherosclerotic plaque has ruptured, platelets form large intraluminal clots (thrombi) that can occlude blood flow to downstream tissues and precipitate ischaemic stroke or acute coronary syndrome (ACS: unstable angina or myocardial infarction). Anti-platelet drugs are therefore the major pharmacological tool used to prevent ACS in at-risk patients with coronary artery disease (CAD) [1–3]. These agents, such as aspirin and clopidogrel, reduce the incidence of ACS but are associated with bleeding risk. The search continues for additional anti-platelet strategies that effectively prevent ACS but with lower bleeding risk.

Extracellular vesicles (EVs) are small membrane vesicles released by a wide range of cell types. Platelets are the greatest source of circulating EVs in healthy individuals [4, 5] and remain a major source of EVs in many pathologies including CAD. When activated, platelets release many EVs that not only promote thrombosis but also modify cardiovascular function far from the plaque rupture site. Activated platelets release at least two broad types of EV, either by shedding them from the plasma membrane or by storing EVs in intracellular granules and releasing them during granule secretion. In this chapter, the molecular mechanisms of EV release from activated platelets and their contributions to arterial thrombosis will be discussed. These contributions make them attractive pharmacological targets. However, the

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mechanisms and consequences of EV release are less clearly understood that other platelet functions in thrombosis, such as platelet aggregation and granule secretion.

17.2 Platelet Activation in Arterial Thrombosis

Arterial thrombi mainly form at rupture or erosion of atherosclerotic plaques [6, 7]. Plaque rupture exposes collagen fibres in the plaque cap and tissue factor (TF) and other prothrombotic and pro-inflammatory mediators deeper in the necrotic lipid core. Arterial thrombi must form under high shear, especially if the atherosclerotic plaque has resulted in extensive stenosis of the artery [8–10]. Platelets are essential to arterial thrombosis because they can adhere to exposed subendothelial collagens under high shear through plasma von Willebrand factor (vWF) and platelet glycoprotein (GP)Ib [7]. As platelets are captured by vWF, GPVI clusters on collagen fibres triggering intracellular signalling cascades, including increased cytosolic Ca^{2+} concentration ($[Ca^{2+}]$cyt) and activation of protein kinase C (PKC), small GTPases, and phosphatidylinositol 3-kinase (PI-3 K). Extensive rearrangement of platelet structure occurs as they extend filopodia and lamellipodia and secrete their granule contents by SNARE-mediated exocytosis [11, 12]. Platelet dense granules contain ADP, ATP, serotonin (5-HT), Ca^{2+}, and polyphosphates. The more numerous α-granules contain a wide range of proteins and small peptides with diverse functions, including adhesion molecules, coagulation factors, growth factors, cytokines, chemokines, and anti-microbial molecules [11]. α-granules also contain intraluminal vesicles (ILVs) that become exomes when the granule contents are secreted [13, 14]. In addition, integral proteins in the granule membrane become incorporated in the platelet plasma membrane. Surface expression of these proteins is often used as markers of platelet activation, including CD63 from dense granules and CD62P (P-selectin) from α-granules.

Increased $[Ca^{2+}]$cyt also triggers inside-out activation of platelet integrins, notably αIIbβ3 (GPIIb/IIIa; CD41/CD61) and α2β1 [15]. Both contribute to stable platelet adhesion, with α2β1 binding directly to collagen fibres and αIIbβ3 binding to vWF. αIIbβ3 is also crucial to platelet aggregation. Active αIIbβ3 binds plasma fibrinogen. Since fibrinogen can bind two αIIbβ3 molecules on different platelets, it acts as a bridge between activated platelets. Aggregated platelets form the body of the thrombosis adherent to the ruptured plaque wall. Platelets flowing past the growing thrombus are activated by ADP released from dense granules and by thromboxane A2 (TxA2) synthesised from membrane arachidonic acid. ADP and TxA2 activate Gαq-coupled receptors P2Y1 and TP, respectively, increasing $[Ca^{2+}]$cyt and activating αIIbβ3 in these platelets. ADP also binds to Gαi-coupled P2Y12 receptors. Gαi signalling sustains platelet aggregation by maintaining αIIbβ3 in its activated, fibrinogen-bound state [16]. Current anti-platelet drugs reduce arterial thrombosis by inhibiting platelet aggregation. Aspirin inhibits TxA2 synthesis by irreversibly inhibiting cyclo-oxygenase 1 (COX1). P2Y12 antagonists such as clopidogrel prevent the maintenance of αIIbβ3 activation by P2Y12-Gαi. αIIbβ3 antagonists (GPIIb/IIIa inhibitors), such as abciximab, block fibrinogen binding to this integrin, preventing aggregation. Although these drugs prevent arterial thrombosis in many patients, the remaining high level of myocardial infarctions indicates that further therapies may provide additional benefit.

Coagulation stabilises the growing thrombus. TF in the plaque core initiates coagulation by forming the extrinsic tenase complex with FVIIa, activating FX. FXa and FVa then form the prothrombinase complex, generating a small amount of thrombin. Thrombin amplifies coagulation by activating FV and XI and releasing FVIII from vWF [17]. Thrombin is also a potent platelet activator, triggering granule secretion and aggregation and promoting further growth of the thrombus. Crucially, a subpopulation of activated platelets become procoagulant. These platelets expose phosphatidylserine (PS) in the outer leaflet of their plasma membrane, forming a binding

site for the intrinsic tenase complex (FVIIIa/FIXa) and the prothrombinase complex (FXa/FVa), generating a burst of thrombin that cleaves fibrinogen to fibrin and stabilises the thrombus. In addition to PS exposure, procoagulant platelets lose mitochondrial membrane potential, downregulate αIIbβ3 activity, and swell to form 'balloons' [18–20]. The mechanisms underlying procoagulant platelet formation are not clearly established. Procoagulant platelets are more likely to form when platelets are coincidentally stimulated by thrombin and collagen [21, 22], although thrombin or collagen alone may be effective when platelets are adherent to a surface or platelets are subjected to high shear [23, 24]. In particular, it is not fully understood why some platelets undergo this procoagulant phenotype, while other platelets aggregate to form the body of the thrombus [25]. Different patterns of cytosolic Ca^{2+} signalling are likely to be crucial [25]. In all activated platelets there is an increase in $[Ca^{2+}]$cyt. Some of this Ca^{2+} is transported into mitochondria, increasing the mitochondrial matrix Ca^{2+} concentration ($[Ca^{2+}]$mito). In a subpopulation of platelets, $[Ca^{2+}]$mito is sufficiently high to trigger opening of the mitochondrial permeability transition pore (mPTP) [19], resulting in high sustained $[Ca^{2+}]$cyt [26, 27] that we have recently termed 'supramaximal Ca^{2+} signalling' [26]. This converts the platelet into a procoagulant platelet. Inhibition of mitochondrial Ca^{2+} uptake or of mPTP opening inhibits supramaximal Ca^{2+} signalling and reduces procoagulant platelet formation [19, 26, 28, 29]. Procoagulant platelet formation resembles regulated necrosis, with high, sustained $[Ca^{2+}]$cyt, mPTP opening, PS exposure, and plasma membrane blebbing [26, 29, 30]. PS exposure can also be induced by the intrinsic apoptosis cascade, which can be triggered by Bcl xL-inhibiting BH3 mimetics [31, 32], but this pathway does not appear to contribute to thrombosis [33].

PS exposure in the outer leaflet requires activation of TMEM16F, a phospholipid scramblase. PS is normally restricted to the inner leaflet of the plasma membrane by an ATP-dependent amino phospholipid translocase, or 'flippase'. Although ATP11C is the flippase in erythrocytes [34], this protein does not appear to be the flippase in platelets and its identity in platelets is currently unknown [35]. The flippase is inactivated in procoagulant platelets, presumably by the high sustained rise in $[Ca^{2+}]$cyt. At the same time, the scramblase function of TMEM16F is activated [36]. TMEM16F allows bidirectional movement of phospholipids between the membrane leaflets with the net result that membrane asymmetry is lost, and PS is exposed on the outer leaflet. PS is not the only asymmetrically distributed phospholipid in unstimulated cells, with phosphatidylethanolamine (PE) also enriched in the inner leaflet and sphingomyelin and phosphatidylcholine (PC) enriched in the outer leaflet. These asymmetric distributions are also lost with scramblase activation, which may be important in extracellular vesicle shedding from procoagulant platelets.

17.3 Mechanisms of Extracellular Vesicle Formation and Release during Arterial Thrombosis

17.3.1 Microparticles

EV Shedding from the Plasma Membrane (Microparticles)

Isolation and classification of different populations of EVs has been extensively reviewed elsewhere [37, 38]. EVs shed from the plasma membrane are often referred to as microparticles and sometimes as 'ectosomes'. Here, we will use microparticles to refer to these shed EVs, acknowledging that this a broad characterisation that may mask distinct sub-types of vesicles.

The characteristic features of platelet-derived microparticles are PS exposure in the outer leaflet of the microparticle, which can be detected by annexin V binding, and expression of platelet-specific markers such as CD41 (integrin αIIb). As microparticles are usually shed by activated platelets, they also generally show markers of platelet activation, such as CD62P (P-selectin). As with any broad characterisation, there are

reported exceptions, which will be discussed further below.

The primary trigger to PS-exposing microparticle release is a large, sustained increase in [Ca^{2+}] cyt. Extensive release of PS-exposing microparticles can be triggered by physiological activators that trigger procoagulant platelet formation, such as a combination of collagen and thrombin [39]. Although PS-exposing microparticles release is also triggered by activators that induce platelet aggregation but only a small subpopulation of procoagulant platelets, the microparticles may be only shed from the few procoagulant platelets. Whether PS-exposing microparticles are shed by activated but non-coagulant platelets is not clear. Intriguingly, however, PS-exposing microparticles are shed by unstimulated platelets during prolonged storage [40]. Microparticle release is also triggered by membrane pores that directly increase [Ca^{2+}]cyt such as the complement membrane attack complex [41], or bacterial pore-forming toxins such as streptolysin O [42, 43]. Histones, released during trauma, also trigger microparticle release by increasing cytosolic Ca^{2+} concentration [44]. Ca^{2+} ionophores are also often used as experimental tools to promote microparticle release, bypassing activation of cell surface receptors and proximal signalling pathways.

Microparticles are presumed to be formed by outwards blebbing of a region of plasma membrane, followed by scission of the bleb from the plasma membrane (Fig. 17.1) [45, 46]. Blebbing platelets have been observed in many studies (for example, Refs [23, 47–49]). How such blebs are formed from the platelet membrane is unclear but may involve local degradation of the cytoskeleton to detach a region of membrane and an increase in cytosolic hydrostatic pressure, inflating the detached membrane by intracellular fluid flow [50]. The increased hydrostatic pressure could be a result of an influx of ions and water, increasing platelet volume, or contraction of the cortical action cytoskeleton. In many cells, Rho kinase (ROCK) phosphorylates myosin light chain phosphatase to promote cytoskeleton contraction and blebbing in apoptotic cells [51]. ROCK similarly regulates blebbing in apoptotic platelets [33] and may also be required for platelet microparticle release in response to activation of PAR4 [52].

Ca^{2+}-dependent effectors are necessary to generate the outward-curving bleb. Calpain, a Ca2 $^{+}$-dependent protease, cleaves cytoskeletal proteins including talin, actin binding protein (ABP), and filamin [53], detaching membrane αIIbβ3 and GPIb from the underlying cytoskeleton [53]. Calpain inhibitors reduce microparticle release in response to Ca^{2+} ionophores [41, 43]. Calpain also cleaves phosphatidylinositol 5-phosphate 4-kinase IIβ (PIP4Kβ), inhibiting synthesis of phosphatidylinositol(4,5) bisphosphate (PI(4,5) P2) [42]. PI(4,5) P2 inhibits microparticle release, although the mechanism is not known.

TMEM16F, the Ca^{2+} dependent phospholipid scramblase described above, is also required for release of PS-exposing microparticles from stimulated platelets [48], though why it is required is not clear. PS is not the only phospholipid whose usual asymmetrical distribution is scrambled by TMEM16F. PE is also exposed in the outer leaflet, and sphingomyelin and PC move to the inner leaflet [54]. Scrambling of these phospholipids may alter membrane curvature or change the binding or distribution of membrane-associated proteins. Alternatively, sphingomyelin hydrolysis to ceramide in the intracellular leaflet could contribute to outward blebbing of the membrane [55].

Microparticles are preferentially released from sites of lipid rafts in platelets. Cholesterol depletion inhibited PS-exposing microparticle release from Ca^{2+} ionophore-stimulated platelets. These microparticles also bound Cholera Toxin B, which binds to GM1 ganglioside and is a widely used marker of lipid rafts. Stimulated platelets bound less Cholera Toxin B, suggesting that GM1 ganglioside is shed into microparticles. Platelet microparticles are also enriched in cholesterol, further supporting lipid rafts as the sites of microparticle release [56]. A similar role for lipid rafts in microparticle release is seen in erythrocytes and monocytes [57–59] Lipid rafts are specialised membrane domains that form foci for signalling microdomains and may localise the

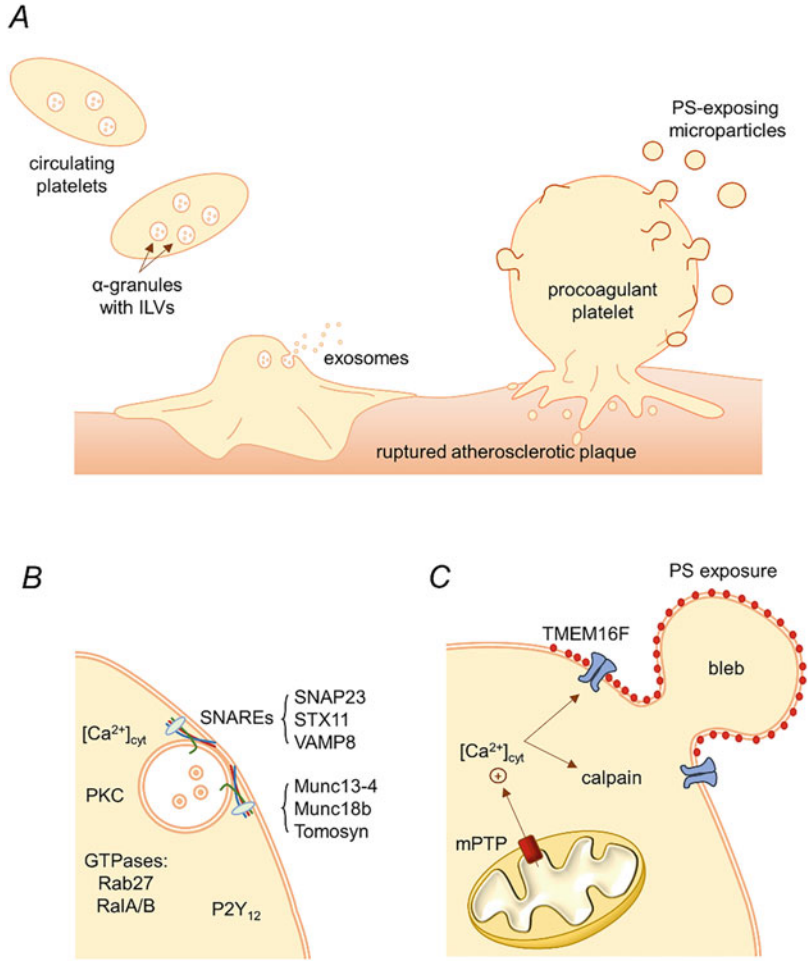

Fig. 17.1 (**a**) Circulating platelets contain α-granules with intraluminal vesicles (ILVs). In arterial thrombosis, platelets adhere to a ruptured atherosclerotic plaque and become activated (left). They extend filopodia and lamellipodia and secrete their granule contents. ILVs are released, becoming exosomes. Some platelets become procoagulant, forming swelling 'balloons' and releasing phosphatidylserine (PS)-exposing microparticles. (**b**) Granule secretion in activated platelets depends on SNAREs and SNARE regulators, as described in the main text. Granule secretion is regulated by intracellular signals, including $[Ca^{2+}]$cyt, protein kinase C (PKC), small GTPases, and P2Y12 signalling. (**c**) Mitochondrial permeability transition pore (mPTP) in procoagulant platelets increases $[Ca^{2+}]$cyt. TMEM16F activation leads to PS exposure. Calpain activation disrupts the cytoskeleton. The outward bleb is pinched off to form a PS-exposing microparticle

molecular machinery for bleb formation and scission.

Although blebbing is likely to underlie microparticle release from platelets in suspension, a distinct microvesiculation pattern in seen in studies with adherent platelets. For example, Kulkarni et al. showed that adherent platelets first extended filopodia and then lamellipodia, resulting in a flat, spread morphology. As these platelets developed into procoagulant platelets, the lamellipodia contracted and fragmented, leaving behind small membrane vesicles that remained attached to the substrate [60]. Membrane fragmentation was dependent on calpain and transglutaminase activity. Similar patterns of adherent, ballooning procoagulant platelets surrounded by the

fragmented remnants of lamellipodia are evident in other studies [20, 23, 40]. It is not clear whether the adherent membrane fragments are the same as microparticles formed from platelets stimulated in suspension, or whether they are released from the substrate once they are formed.

Platelet PS exposure can be triggered by the intrinsic apoptosis cascade. This apoptotic PS exposure depends on caspase activity, whereas caspases are not activated in procoagulant platelets [61]. In vivo, these PS-exposing platelets are rapidly cleared by phagocytosis leading to thrombo-cytopenia (low platelet count). In vitro, in the absence of clearance, apoptotic platelets progress to secondary necrosis and release PS-exposing microparticles. However, microparticle release from apoptotic platelets could be inhibited by a caspase inhibitor but not a calpain inhibitor, suggesting a different mechanism to microparticle release from procoagulant platelets [62]. In addition, caspase activation in apoptotic platelets prevented microparticle release in response to a Ca^{2+} ionophore. These data indicate crosstalk between two different mechanisms of PS-exposing microparticle release.

PS-exposing microparticles are also shed by megakaryocytes, the bone marrow resident cell that produces platelets. The microparticles form as beads along micropodia. Interestingly, and unlike platelet-derived microparticles, they contain full-length filamin, suggesting that calpain activation is not involved. Megakaryocyte released microparticles express platelet/megakaryocyte markers CD41 and GPIb (CD42b) but lack activation markers such as CD62P.

Although PS exposure is often seen as a defining feature of microparticles shed from the plasma membrane, several studies suggest the release of PS-negative microparticles that are distinct from exosomes. Using cryo-electron microscopy, Brisson et al. (Ref [63]) observed that activated platelets released CD41+ PS-negative tubular vesicles, larger than exosomes, and with internal fibres that resemble actin bundles. They proposed that these tubular vesicles are released from filopodia of activated but non-coagulant platelets.

Roles of PS-Exposing Microparticles in Arterial Thrombosis

The circulating level of platelet-derived microparticles is associated with risk of future atherothrombotic events [64–66]. PS-exposing microparticles are procoagulant. PS is a catalytic surface for formation of the tenase and prothrombinase coagulation complexes, increasing thrombin generation. Moreover, the coagulation capacity of platelet-derived microparticles is much greater than of platelets alone [4]. Although this may be partly due to increased surface area, platelet-derived microparticles are more procoagulant than activated platelets per unit surface area [67]. Microparticles have a greater degree of membrane curvature than platelets as their diameter is smaller. Computational studies predict that FX binding is increased by greater membrane curvature [68]. Microparticle release from procoagulant platelets will increase tenase activity and subsequent thrombin generation and fibrin formation.

Platelet-derived microparticles do not appear to express tissue factor (TF) activity [69–72]. TF initiates the extrinsic pathway of coagulation, forming the extrinsic tenase complex with FVIIa. Circulating TF-expressing microparticles, released by activated monocyte, endothelial cells, and some cancer cells, are procoagulant and can promote arterial thrombosis [73]. In contrast, platelet-derived microparticles initiate thrombin generation independently of TF in a FXII-dependent manner [71, 72]. Moreover, platelet-derived microparticles induce monocytes to release TF-expressing microparticles [74]. These data suggest that platelet-derived microparticles can initiate thrombin generation in addition to propagating thrombin generation through PS exposure.

In contrast to these prothrombotic roles of platelet-derived PS-exposing microparticles, they may have a protective effect. In healthy individuals, the microparticles may trigger a low level of thrombin generation in a FXII-dependent manner, activating protein C [75], which has antithrombotic, anti-inflammatory, and profibrinolytic properties [76].

17.3.2 Exosomes

Formation of Exosomes

Activated platelets release exosomes during granule secretion. Exosomes are small membrane vesicles (often <100 nm diameter), formed and stored as intraluminal vesicles (ILVs) in the endocytic pathway. Inward budding of an endosomal membrane generates ILVs within a multivesicular bodies (MVBs). The ILVs are released and become exosomes if the MVB fuses with the plasma membrane (Fig. 17.1). MVBs have been observed in megakaryocytes, the bone marrow resident factory-like cell that produces platelets. MVBs mature into platelet α-granules, some of which also contain small ILVs [13]. Dense granules also develop from MVBs [77], although how MVBs selectively mature into α- or dense granules is only partially understood [78, 79]. Whether dense granule also contains ILVs is unclear.

How α-granule ILVs are formed is also not resolved. In many cells, the ESCRT (endosomal sorting complex required for transport) complex controls cargo selection and ILV formation [80, 81]. The ESCRT machinery consists of five hetero-oligomeric sub-complexes (ESCRT-0, -I, -II, -III, and VPS4 [vacuolar sorting protein 4]) that are sequentially recruited to endosomal membranes by phosphatidylinositol 3-phosphate [PI(3)P] and phosphatidylinositol (3,5)-bisphosphate [PI (3,5)P2]. The main component of ESCRT-III, charged multivesicular body protein 4 (CHMP4), forms conical spirals (the ESCRT-III ring) that deform the membrane to induce budding away from the cytosol. Constriction of the ESCRT-III ring by VPS4 ATPase at the budding neck leads to membrane scission and release of the ILV into the lumen of the MVB [82, 83]. An alternative mechanism of activating ESCRT-III and ILV budding is by ALG-2 interacting protein (ALIX). This occurs independently of ESCRT-0, -I, and -II 80. Several tetraspanins, including CD63 and CD81, are enriched in exosomes. CD63 may form ILVs in an ESCRT-independent manner by forming membrane microdomains, although in some cells CD63 may be sorted to exosomes in an ALIX- and ESCRT-III dependent manner [84–86]. The ESCRT machinery could be involved in ILV formation in megakaryocytes. VPS4, CHMP4b, and ALIX have been detected in platelets in some proteomic [87, 88] and transcriptomic studies [89, 90], although involvement of these proteins is yet to be directly demonstrated. Platelet-derived exosomes are enriched in CD63, suggesting that this tetraspanin may also have a role in their biogenesis, but this is also not yet certain.

Platelet Exosome Release by Granule Secretion

Activated platelets release numerous small vesicles (40–100 nm) that are selectively enriched in CD63 but with little expression of platelet cell surface markers GPIb (CD42b) and PECAM-1 (CD31), indicating that they did not derive directly from the plasma membrane. These exosomes also did not bind annexin V, prothrombin, or factor X, distinguishing them from PS-exposing EVs shed from the plasma membrane [14]. Platelet exosome release is therefore likely to be due to exocytosis and secretion of granule contents.

During thrombosis, platelet activators rapidly trigger secretion of dense and α-granules. Platelet granule secretion is mediated by soluble NSF Attachment Protein Receptor (SNARE) proteins (Fig. 17.1). v-SNAREs are located in the granule membrane, whereas t-SNAREs are located on the target membrane. These SNARE proteins contain one or more SNARE domains that allow the SNAREs to interact in a coiled-coil structure, bringing the granule and target membranes together for fusion. Four SNARE domains are required for this coiled-coil structure, one from the v-SNARE, one from a Syntaxin-type t-SNARE, and two from a SNAP23/25/29-type t-SNARE. In contrast to currently limited molecular understanding of exosome biogenesis in megakaryocytes, the key components of the granule exocytosis machinery in platelets are more clearly described (reviewed in detail in Ref. [12]). The major v-SNARE in platelets is VAMP-8, with compensatory roles for

VAMP-2 and -3 and a distinct role for VAMP-7 in platelet spreading. Syntaxin-11 (STX11) is the major platelet syntaxin. Its loss in familial Haemophagocytic Lymphohistiocystosis type 4 (FLH-4) and in Stx11−/− mice prevents dene and α-granule exocytosis [91]. Syntaxin 8 also contributes to dense granule secretion [92]. SNAP23 is essential for secretion of both α- and dense granules, whereas SNAP29 appears to have no role, and SNAP25 is poorly expressed [93, 94]. Although the SNAP23-VAMP8-STX11 is the major SNARE complex in platelets, complexes containing other SNAREs may regulate secretion of specific cargoes [11]. The SNARE complex is tightly controlled. t-SNAREs are regulated by Munc18b (STXBP2) and tomosyn 1 (STXBP5) [95, 96]. Rab27a/b, a small GTPase, directs granule docking and tethering in many cells and regulates dense granule biogenesis and secretion in platelets [97–100]. Rab27 effectors, Munc13–4 and synaptotagmin-like protein 1 (SLP1), regulate dense granule secretion, although another Rab27 effector, myosin Va, does not [101–103].

The primary intracellular signals triggering granule secretion are $[Ca^{2+}]$cyt and PKC. Increased $[Ca^{2+}]$cyt activates Munc13–4 to promote SNARE-mediated membrane fusion. Munc13–4-deficient platelets have reduced dense and α-granule secretion, platelet aggregation, and thrombosis [102, 104, 105]. However, the α-granule secretion defect is overcome by extracellular ADP acting through P2Y12, indicating that dense granular secretion of ADP enhances α-granule secretion in an autocrine manner [106]. Exosome release from α-granules is likely to be similarly dependent on P2Y12 signalling, although this has not been directly tested. PKC activity is also required for granule secretion although the proteins that are phosphorylated by PKC isoforms are not clear [107, 108]. PKC may directly phosphorylate SNAP23 [109]. Alternatively, PKC may regulate IκB kinase (IKK)-β which itself directly phosphorylates SNAP23 [110]. Whatever the precise mechanism, Ca^{2+} and PKC dependent granule secretion usually results in exocytosis of granule contents and incorporation of granule membrane proteins such as CD62P into the plasma membrane. In contrast, in platelets from RalA/B deficient mice, α-granule contents such as platelet factor 4 (PF4) and TGFβ are released normally, but CD62P is not incorporated into the plasma membrane [111]. In the absence of RalA/B, granule fusion may be transient, leading to 'kiss-and-run' exocytosis of granule contents but not lasting incorporation of granule membrane proteins. Alternatively, CD62P and PF4/TGFβ may be localised in different α-granule subpopulations that are differentially regulated by RalA/B [79]. In either case, this raises intriguing questions about secretion of exosomes. Would exosomes be released during transient kiss-and-run secretion? Alternatively, are exosomes restricted to a subpopulation of α-granules? Although the molecular mechanisms of platelet granule secretion have been extensively studied, their relation to exosome release requires further investigation.

Role of Platelet-Derived Exosomes in Arterial Thrombosis

Exosomes have the potential to carry a wide range of cargoes from the cell of original, such as proteins, nucleic acids, and lipids. Exosomes are taken up by recipient cells by endocytosis, phagocytosis, or by direct fusion with the target plasma membrane [112]. After internalisation, exosome cargo may modify the function of the recipient cell. Platelet-derived exosomes carry a diverse range of cargoes, notably microRNAs (miRNAs), that can modify other cardiovascular cells. miR-21, miR-197, miR-223, and miR-339, for example, are enriched in platelet exosomes compared to exosomes from leukocytes and endothelial cells [113]. These miRNAs may regulate atherosclerosis progression and subsequent thrombosis. Plasma miRNA, likely to be from platelet exosomes, induces vascular smooth muscle cell (VMSC) apoptosis during atherosclerosis progression [114]. This may involve downregulation of platelet-derived growth factor receptor-beta (PDGFRβ) expression in VSMCs by platelet miRNAs [115]. PFGRβ inhibition induces apoptosis in VSMCs in vitro and in vivo [116] and is associated with

atherothrombosis [115]. miRNA in platelet-derived exosomes, particularly miR-223, may promote VSMC apoptosis and atherosclerosis progression. However, the effect on cardiovascular function and pathology is a complex one [117]. Some exosome miRNAs may reduce inflammation, which would reduce atherosclerosis [118]. miR-223 inhibits ICAM-1 expression in endothelial cells [119], although in another study, apoptosis was increased [120]. Platelet exosome miRNAs also regulate WNT signalling in endothelial cells [121]. Moreover, circulating levels of platelet-enriched miRNAs correlate with platelet reactivity in some studies but inversely correlated with platelet reactivity in others [122–125], making it difficult to directly link platelet-derived exosomes released during thrombosis to subsequent atherosclerotic and thrombotic risk. The emerging picture is that platelet-derived exosomes are complex regulators of vascular health, promoting angiogenesis and endothelial integrity after vascular injury [5], but also promoting counter-productive and damaging responses that promote atherosclerosis.

17.3.3 Release of Free Mitochondria

Activated platelets release functional mitochondria [126]. Extracellular mitochondria are also found in platelet concentrates [127]. Although some of these mitochondria are packaged in EVs, presumably shed as microparticles, others are released as intact but free organelles [128]. These mitochondria are able to undergo respiration [128, 129] and promote inflammation. Secretory phospholipase A2 (sPLA2)-IIA, which is secreted by platelets, liberates free fatty acids and lysophospholipids from extracellular mitochondrial membranes and release of mitochondrial DNA, a damage-associated molecular pattern (DAMP). The extracellular mitochondria bind to neutrophils where, combined with sPLA2-IIA, they promote LTB4 synthesis, EV release, oxygen consumption, and extrusion of neutrophil extracellular traps (NETs) [128, 130]. The contribution of free extracellular mitochondria to disease is still being explored. Activated platelets are not the only cell to release free mitochondria into the blood. Monocytes, for example, also release free mitochondria and mitochondria in EVs [131]. Mitochondria are also released by traumatic injury [132], including to the brain [133]. Since free mitochondria are bound by their own outer membrane and often submicron in size, they are likely to be detected as EVs in many experiments, suggesting that free mitochondria may contribute to some of the functions ascribed to platelet-derived EVs.

17.3.4 Clearance of EVs

EVs are rapidly cleared from the circulation. PS-exposing platelet microparticles injected into rabbits were cleared within 10 min [134]. Similarly, platelet microparticles infused into mice were cleared within 30 min (reported in Ref. [45]). In a separate study small EVs infused into mice had a circulating half-life of 7 min [135]. In human volunteers undergoing dobutamine stress echocardiogram (DSE), platelet-derived EVs levels increased immediately after DSE but had returned to normal within 1 h [136]. The mechanisms of this rapid clearance and not clearly defined and multiple mechanisms may be involved. PS is an ‘eat me’ signal on apoptotic cells and may have a similar role in PS-exposing EVs. Phagocytes recognise exposed PS through PS receptors on phagocytes, such as TIM4. Alternatively, soluble PS-binding proteins such as Gas6, lactadherin (MFG-E8), annexin I, and β2-GPI bridge PS on the apoptotic cell and receptors on the phagocyte [137], although whether some of these proteins accelerate clearance, or increase EV half-life by masking PS, is not clear [138]. Lactadherin, for example, delays clearance of PS-exposing platelets by the endothelium [139], whereas lactadherin-deficient mice have elevated levels of circulating platelet-derived EVs [140]. Although PS is likely to be important in the clearance of PS-exposing EVs, it is unlikely to contribute to the clearance of PS-negative EVs, such as exosomes. Complement activation and opsinisation of EVs by complement C3b may promote EV clearance. Opsinised EVs bind to

the CR1 receptor on erythrocytes, which are subsequently phagocytosed in the liver and spleen [45]. In addition, EVs may be degraded by sPLA2. Alternatively, sPLA2 may generate lipid mediators from the EV membrane, such as lysophosphatidic acid [141] and arachidonic acid. The latter is converted to 12(S)-hydroxyeicosatetraenoic acid [12(S)-HETE] by 12-lipoxygenase (12-LOX) in EVs, promoting EV phagocytosis by neutrophils [142]. sPLA2 is increased in inflammatory conditions, suggesting that the mechanisms of EV clearance depend on the disease context. Finally, platelet glycoprotein desialylation promotes their clearance by the liver at the end of a platelet's life span [143]. A similar mechanism may be involved in clearance of PS-negative EVs [144]. Whatever the mechanisms of EV clearance, elevated steady-state levels of EVs in coronary artery disease imply that there is either extensive and sustained release of EVs, that the clearance mechanisms are inhibited, or both. A similar increase in circulating EVs is seen in systemic lupus erythematosus (SLE), a systemic autoimmune disorder. Defective clearance of dead cell debris is a major causative factor in SLE. Platelet-, leukocyte-, and endothelial cell-derived EVs are more abundant in the blood of many SLE patients [145–148] and contribute to the progression of the disease [145, 149]. It is likely that both increased EV release and decreased EV clearance are involved in the higher steady-state levels of EVs in SLE.

17.4 Perspectives: Pharmacological Inhibition of Platelet EV Release

Platelet-derived EVs have a significant impact on arterial thrombosis and on cardiovascular disease more widely. PS-exposing microparticles enhance thrombin generation and thrombosis at the site of plaque rupture. miRNA carried by exosomes modifies cardiovascular cells. Although their effects are complex, there are associations with increased risk of atherosclerosis and subsequent thrombosis. Although EVs are cleared within minutes, this is sufficient time for EVs released during thrombosis to distribute throughout the vascular system, with widespread effects on cardiovascular function and health. Inhibition of EV release is therefore a tempting strategy. However, the current lack of clarity regarding their formation and release means that we lack an attractive molecular target.

Exosome formation in megakaryocytes and platelets may involve the ESCRT machinery or tetraspanin, as described in other cells. A high-throughput screen identified inhibitors of exosome biogenesis and release in several cancer cell lines, including the clinically approved drugs, tipifarnib and ketoconazole [150]. It would be interesting to discover whether these drugs also inhibit exosome formation in megakaryocytes, which could contribute to their clinical effects.

Granule secretion is increasingly well understood. Although important mechanistic questions remain, many of the major players have been identified [12]. Despite this, there are no selective inhibitors of platelet granule secretion available. This is, in part, due to the difficulty of targeting protein–protein interactions such as the SNARE complex. Cell-permeant peptides may be more effective than small molecules for this [151, 152]. However, inhibiting granule exocytosis would also inhibit the secretion of other granule cargoes. Patients lacking dense or α-granule secretion have increased bleeding, which is also seen in mice lacking these granules [11]. Inhibition of PKC, one of the primary signals leading to granule secretion, also results in many effects in platelets beyond granule secretion. For example, PKC inhibition increases $[Ca^{2+}]$cyt signalling and procoagulant platelet formation [108, 153–155]. Although there are no specific inhibitors of platelet exosome biogenesis or release, inhibition of exosome secretion from α-granules might contribute to the effects of some anti-platelet drugs. P2Y12 inhibitors, for example, inhibit α-granule secretion and release of CD61+/PS-negative EVs [156].

During thrombosis, PS-exposing microparticles are released by procoagulant platelets. mPTP opening and the subsequent sustained increase in $[Ca^{2+}]$i is likely to be a key step, resulting in PS

exposure and calpain activation. mPTP opening can be considered a tipping point in this process [27]. The receptors and intracellular signalling prior to this event are shared with other platelet responses, such as granule secretion and aggregation. Inhibiting these early signals results in general inhibition of platelet function. In this way, current anti-platelet agents such as P2Y12 inhibitors inhibit microparticle release, but also inhibit platelet α-granule secretion and aggregation [106, 156–158]. It is possible that reduced microparticle release may contribute to the therapeutic benefit of P2Y12 inhibitors, but they cannot be considered specific inhibitors of microparticle release. Moreover, P2Y12 inhibition did not inhibit microparticle release when platelets were stimulated with a Ca^{2+} ionophore to bypass early receptor signalling [43], demonstrating that P2Y12 signalling is not directly involved in the molecular mechanism of microparticle formation and release.

Preventing procoagulant platelet formation also inhibits microparticle release, in part because PS exposure is required. As noted above, platelets from Tmem16f−/− mice have reduced PS-exposing microparticle release [48]. However, pharmacological inhibition of TMEM16F would also inhibit platelet procoagulant activity. While this may be an attractive therapeutic strategy [159], TMEM16F inhibition cannot be a specific means of inhibiting microparticle release. Similarly, preventing procoagulant platelet formation by inhibiting mPTP opening also inhibits microparticle release, but also prevents the other consequences of mPTP opening in platelets, such as PS exposure and αIIbβ3 downregulation [18, 19]. Specific inhibition of microparticle release requires a much greater understanding of the underlying mechanisms to highlight a specific target. Compared to the detailed molecular picture of platelet granule exocytosis that has been uncovered, the mechanisms of platelet microparticle release are still largely hidden. Recently, we reported that 2-aminoethoxydiphenylborate (2-APB) and an analogue, 3-(diphenylphosphino)-1-propylamine (DP3A), inhibit platelet microparticle release without affecting PS exposure or calpain activity [160]. The target of these drugs is not yet known but they give us hope that there are additional mechanisms in platelet microparticle release that can be uncovered and inhibited for therapeutic benefit.

Platelet-derived microparticles and exosomes are released in abundance and contribute to the progression of arterial thrombosis. Currently, the mechanisms of their formation and release are not as well understood as other prothrombotic platelet functions. Advancing our understanding of these molecular mechanisms may provide new targets for antithrombotic therapy.

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