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Review Article

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Regulation of the Adaptive Response of Cardiac Cells to Ischemia: Role of Nanovesicles

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Abstract

Ischemic heart disease represents 1 in 4 of total global deaths worldwide. Myocardial intercellular cross-talk regulate simultaneously the tissue homeostasis between neighboring cardiac cells in response to acute or chronic ischemic insult. Different studies have suggested that the heterocellular communication of adult myocardium is mainly based on the release of several free soluble biofactors into the extracellular milieu and on the spreading of different ions through selective channels. In light of these findings, the past few decades have witnessed incessant research aimed at protecting the adult myocardium against ischemic injury, but the development of effective cardioprotection is still a desirable achievement. Nowadays, the rapidly evolving nanoscience is offering the opportunity to tailor new reliable therapeutic targets at ultrastructural level in order to prevent or attenuate the progressive myocardial trimodal response (cell death, matrix degradation and reactive cell hypertrophy) against ischemic injury occurring in infarcted heart. All cardiac cell types release nanovesicles termed "exosomes" (size 40-100 nm), which contain different cargo under normoxic and hypoxic microenvironment. In particular, we and other investigators have demostrated that cardiac fibroblasts and cardiac progenitor cells/stem cells interact with ischemic cardiomyocytes through the release of exosomes. Our review provides current insights into the role of nanovesicles in the modulation of injury and repair responses under ischemic microenvironment.

Keywords

Exosomes, Heart, Nanovesicles, Cardiac progenitor cells, Cardiac remodeling, Cardioprotection

Abbreviations

CDC: Cardiosphere Derived Cells; CPCs: Cardiac Progenitor Cells; CVD: Cardiovascular Diseases; ESCRT: Endosomal Sorting Complex Required for Transport; EVs: Extracellular Vesicles; FGF-2: Fibroblast Growth Factor Type 2; GTP: Guanosine Triphosphate; HIF1-alpha: Hypoxia-Inducible Factor 1-alpha; IL: Interleukin; ILVs: Intralumenal Vesicles; MI: Myocardial Infarction; MSCs: Mesenchymal Stem Cells; MVB: Multivesicular Bodies; MYBPC3: Cardiac-Type Myosin Binding Protein C; PDLIM5: PDZ and LIM domain 5; Rab: Ras-like monomeric GTPases; SORBS2: SH3 Domain Containing Protein 2; TEMs: Tetraspanin-Enriched Microdomains; Ub: Ubiquitin; VCP: Valosin-Containing Protein; VEGF: Vascular Endothelial Growth Factor

Nanofrontiers in the Management of Cardiovascular Diseases

Up to date cardiovascular diseases (CVD) constitute the main cause of

morbidity and mortality worldwide. In 2010 almost 52 million deaths have been recorded and 1 in 3 deaths is driven by CVD, which include coronary artery diseases, cerebrovascular diseases (e.g. stroke), diseases of aorta and peripheral arteries, but also congenital and reumatic heart diseases, cardiomyopathies and cardiac arrhythmias. The Food and Drug Administration declared that more than two thousand people die for CVD every day just in United States. Among all CVD, ischemic heart diseases represent 1 in 4 of total global deaths worldwide [1].

Despite the wide use of different drugs, such as beta-blockers, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, diuretics, or devices, such as implantable cardioverter-defibrillator and left ventricular assist devices [2], the 5-year survival is worse than cancer. The past few decades have witnessed incessant research aimed at protecting the adult myocardium against ischemic injury, but the development of effective cardioprotection remains a desirable achievement. The rapidly evolving sciences in nanotechnology is offering the opportunity to tailor new reliable therapeutic targets at ultrastructural level in order to hamper the progressive myocardial trimodal response (cell death, matrix degradation and reactive cell hypertrophy) against ischemic injury due to myocardial infarction [3].

The post-ischemic trimodal response

Myocardial infarction (MI) is the most common and clinically significant form of acute cardiac injury and is characterized by regional loss of a large number of cardiomyocytes, which triggers reactive hypertrophy of residual myocytes and large deposits of collagen. Other CVD, such as chronic pressure or volume overload and chronic idiopatic cardiomyopathy, cause more sporadic loss of cardiac cells and fibrotic response [4].

The progressive cell death activates native inflammatory cascade with large recruitment of circulating bone marrow-derived stem cells and immune cells in order to enhance the inflammatory cross-talk between the viable cells resident into the myocardium bordering the infarct area and to remove cell debris. Hence, the matrix degradation promotes the myocardial replacement with fibrotic tissue, which leads to a massive remodeling of the architecture of the beating ventricle [5, 6]. The matrix remodeling is an active process that affects both infarcted and non-infarcted myocardium leading to initial compensatory cardiac wall hypertrophy and stiffness up to chamber dilation and sphericity which is related to worse diastolic and systolic cardiac function [7] and the occurrence of sudden death [8-11]. Advancements in technology and knowledge have redefined the remodeling process as a group of ultrastructural, molecular and epigenetic modifications underlying the shape, size, metabolism and function of the myocardial cells exposed to reduced blood flow and increased oxygen demand [12-15]. The extent of the trimodal response depends on the tolerance of single cells to ischemic microenvironment and on the magnitude of the healing process mediated by the interplay between mature cardiac cells (i.e.: fibroblasts, cardiomyocytes, endothelial cells), progenitor and stem cells. Therefore, it is clinically relevant to address further investigations to dissect new pathophysiological features, such as cell-to-cell and cell-to-microenvironment cross-talk in the presence of acute or chronic ischemic hit [12].

Post-ischemic cardiac remodeling

To best of our knowledge, two types of cardiac remodeling have been described: physiological (adaptive) and pathological (maladaptive) remodeling.

The cardiac remodeling was initially defined as the recapitulation of different macroscopic morpho-functional changes affecting the cardiac chambers after myocardial infarction [5, 6]. The decay of myocardial blood flow is due to flow-limiting stenoses of the coronaries, but it may even occur in the presence of increased work load, such as during pressure (aortic valve stenosis or hypertension) or volume overload (valvular regurgitation), and inflammatory disease (myocarditis).

Cardiac hypertrophy is a common feature of the remodeled heart and it is difficult to predict as well as to counteract. Immediately after MI, the injured area progressively expands, promoting the thinning of the infarcted wall and the dilation of the ventricular chamber. In fact, the rise of end-diastolic volume due to progressive decay of systolic function leads to less elliptical and more spherical shape with initial eccentric hypertrophic response and final thinning of the walls [16]. In contrast, pressure overload stress leads to concentric hypertrophy, while ventricular radius is not modified [17].

Similarly, we have different features at the cellular level. The contractile-protein units are assembled in parallel resulting in width increase of cardiomyocytes during concentric hypertrophy; on the other hand, the contractile-protein units are organized in series in the cardiomyocytes of heart with eccentric hypertrophy, causing length increase of the cells [18]. However, it is still unknown whether the different architecture of contractile-protein units affects autocrine/paracrine activity and the ability of cardiomyocytes to communicate with neighbor cells [19].

Cell-to-cell communication into ischemic myocardium

Cardiac hypertrophy is usually considered as the first adaptive response to both extrinsic mechanical, biochemical and neurohumoral stressors. Despite changes of sarcomere structure, the hypertrophic microenvironment may affect cardiomyocytes gene expression, protein synthesis and metabolism [20-22]. Even if human cardiomyocytes has limited ability to proliferate during adulthood [23], the heart responds to pro-hypertrophic microenvironmental stimuli by cell growth or shrink [22], and cell mitophagy [24].

Nowadays, cardiac hypertrophy is classified as physiological or pathological [25]. Physiological hypertrophy occurs in response to a transient increase in cardiac work (i.e. exercise, pregnancy, overweight) [26] and it is distinguished from genetic familial hypertrophic cardiomyopathy mutations [27] in which the stimulus for hypertrophy is intrinsic to the cardiomyocyte. Conversely, reactive hypertrophy, a pathological form of hypertrophy, occurs after an injury that causes cell loss and matrix degradation and would provide compensatory

mechanical support to normalize ejection performance and systemic organ perfusion [28].

The physiological hypertrophy is related to normal cardiac function, while the pathological one is related to cardiac dysfunction due to MI or other cardiac pathologies [25].

The rise of cardiac mass, a hallmark of hypertrophy, is proportionally related to increased coronary capillary network, which is able to properly feed cardiac cells in physiological rather than pathological type. In fact, capillary density is reduced in pathological hypertrophy, suggesting that capillary number is controlled by myocardial signals, and thus rarefaction of capillary density significantly contributes to myocardial hypoxia and to the worsening of the contractile dysfunction towards heart failure [29].

Cardiac hypertrophy is a very complex process from molecular point of view and it may involve many different molecular pathways at the same time. In addition, it has been reported that different signals are simultaneously activated in the case of adaptive or maladaptive heart growth [30].

The intercellular cross-talk is a therapeutic target to attenuate the remodeling process and to prevent the onset of post-ischemic heart failure [19]. In such pathophysiological scenario, a balance between adaptive and maladaptive hypertrophy is provided by the communication among mature cardiomyocytes and fibroblasts or cardiac progenitor cells (CPCs) [31].

One cardiomyocyte borders several fibroblasts [32] and the communication between these two different types of cells is driven through the release of maladaptive (i.e. transforming growth factor-beta (TGF-beta), vascular endothelial growth factor (VEGF), endothelin-1 [19]) or adaptive (i.e. ST2, interleukin (IL)-33) humoral signals [33]. Long-term exposure of the cardiomyocytes to the abovementioned signals can be deleterious for the myocardium resulting in cell death and collagen deposition.

In particular, other free soluble factors released from activated fibroblasts (i.e. fibroblast growth factor type 2 (FGF-2)) may impair electrical properties of cardiomyocytes through both direct and paracrine interactions [34]. In addition, high levels of connexin-43 expressed on the surface of cardiac fibroblasts may alter the regular cross-talk with cardiomyocytes due to fibroblast-myocyte uncoupling that causes a chaotic electrical activity into beating heart [35].

Under normal physiological conditions, CPCs are thought to be quiescent in the cardiac stroma [36], yet they are activated after injury. We have demonstrated that the positive effects of CPCs in infarcted rodent heart is mediated through paracrine release of anti-apoptotic, immunomodulatory, proangiogenic host- and cell-derived factors [37]. In fact, the moderate positive effects of CPCs in infarcted myocardium is more likely due to increased neovascularization or favorable changes in the cardiac scar (which is not inert) rather than the formation of new cardiomyocytes [38]. Moreover, CPCs even communicate with cardiomyocytes through connexins, which play an important role in electrical coupling, and cadherins, which are essential for mechanical coupling [39]. Finally, progenitor/stem cells are able to communicate with cardiomyocytes through thin-membrane channels (tunneling nanotubes), which establish cytosolic connectivity and facilitate intercellular propagation of intracellular components (i.e. mitochondria) [40].

Recent reports have demonstrated that cardiac fibroblasts [41] and cardiac progenitor cells/stem cells [42-45] interact with ischemic cardiomyocytes through the release of nanovesicles termed exosomes.

Exosomes

Exosomes are smallest nanovesicles released by each type of cells [46] and were detected, for the first time, by Harding et al. [47]. However, the term 'exosomes' was coined by Johnstone et al. [48], which described exosomes as biologically active vesicles that could be isolated by ultracentrifugation.

Exosomes have diameter from 30 to 100 nm, have endosomal origin and are different from microvesicles (50-1000 nm), which derive from plasma membrane of donor cells, or from apoptosomes, which derive from apoptotic cells [46]. Recent study provided the first evidence of nanofilaments on the surface of exosomes using atomic-force microscopy peak force imaging [49]. Moreover, the exploration of nanofilaments may hold relevant significance for exosome biogenesis and their role in intercellular communication during critical illness [50].

Biogenesis of exosomes

Exosomes are released from donor cells and play their role after been uptaken by recipient cells. In particular, exosomes derive from intracellular multivesicular bodies (MVBs) which originate from plasma membrane invaginations and incorporate cargo proteins, processed by endoplasmatic reticulum and Golgi complex, RNAs, microRNAs, DNA and lipids. Plasma membrane of MVBs buds inside to form early endosomes, which have inverted membrane. During exosomes biogenesis, the potential cargo is in contact with outer endosomal membrane, which leads to the invagination of the membrane into the lumen and to the gripping vesicles into the endosomal lumen as well. Interestingly, the exosomes membrane has same orientation of plasma membrane after double membrane invagination [46]. So far, it is known that exosomes are released into the extracellular microenvironment following the fusion of MVBs with plasma membrane of the donor cell. This mechanism is driven by activity of guanosine triphosphate (GTP) hydrolases proteins, such as Ras-like monomeric GTPases (Rab) 27a and 27b [51]. In fact, the knockdown of Rab27a leads to enlargement of MVBs size and the knockdown of Rab27b induces the redistribution of MVBs in the perinuclear area [52]. Otherwise, the fusion of MVBs with lysosome determine degradation of the exosomal cargo.

It is known that the endosomal sorting complex required for transport (ESCRT) plays a key role in the exosomes biogenesis. Proteins that are both transported from Golgi complex and directly internalized into the cell, are ubiquitylated to be recognized by ESCRT0 complex. ESCRT0 binds

ESCRT2 complex through ESCRT1 complex. ESCRT1 and ESCRT2 complex triggers the intralumenal vesicles (ILVs) budding into the MVBs. Conversely, ESCRT3 complex is recruited to prevent the release of cargo proteins into the cytosol. In fact, the free ubiquitin (Ub) molecules and ESCRT complexes are released into the cytosol for recycling [53].

Finally, some proteins are sorted into ILVs by ESCTR independent pathways requiring the presence of raft-based microdomains, which are rich in sphingolipids and ceramide. Ceramide promotes microdomains union and leads to ILVs formation.

Thus, it is conceivable that differences in MVBs biogenesis determine the recruitment of selected proteins and confer the route of exosome traffic towards the extracellular milieu rather than the lysosomes.

Exosomes are highly enriched in tetraspanins, mainly CD63, CD9, CD81 and CD82 [54]. These proteins accumulate in specific sites of plasma membrane which are called tetraspanin-enriched microdomains (TEMs) and are involved in the exosomes biogenesis, release and protein sorting. In addition, exosomes may contain proteins involved in metabolism (such as enzymes) as well as in membrane trafficking, adhesion molecules, T cell stimulating molecules (such as Major Histocompatibility Complex (MHC)-class I and -class II) and cytoskeleton proteins.

Uptake of exosomes

Several *in vivo* studies have been performed to elucidate complex mechanisms underlying the exosomal uptake by different recipient cells. Fluorescent exosomes intravenously injected in mice displayed a plasma half-life of approximately 2 minutes and a spleen half-life of 2 hours [55]. On the other hand, exosomes have been detected in the brain and the gut of mice up to 3 hours after intranasal administration [56]. In both studies, exosomes have been detected within macrophages for at least 15 minutes. However, it is still unknown whether the dynamics of exosome internalization and trafficking is due to the clearance of exosomes by lysosomes or to the activation of intracellular pathways. It has been demonstrated that circulating exosomes tend to accumulate into the vessel wall, where the leukocytes and pericyte are usually detected [55]. This observation may also suggest how exosomes deliver signals into vascular monocytes/macrophages, probably by opsonization [57]. Moreover, the exposure of lysophatidylcholine on the exosomal membrane leads to binding immunoglobulin M and complement C3 that promote phagocytosis [58, 59].

However, little is known regarding the mechanisms that regulate exosome internalization.

So far, the main mechanisms regulating exosome uptake are related to soluble and juxtacrine signaling, membrane fusion, phagocytosis [60], macropinocytosis [61], receptor- and raft-mediated endocytosis [62].

Cardiac exosomes

All cardiac cells use different forms of communication to maintain cardiac homeostasis. It is already known that all cell types of the adult heart are able to release and to uptake exosomes delivering different cargo. The exosome-based intercellular communication may be more efficient than one based on factors released in free-soluble form. In fact, the exosomal content is stable and protected by a lipid bilayer membrane, which includes some proteins that are detectable using proteomics approach [63].

Adult cardiomyocytes release extracellular vesicles (EVs) with a size ranging from 40 to 300 nm [64], which include membrane caveolin-3 and flotillin-1, and even deliver sarcomeric and mitochondrial proteins, such as myomesin, tropomyosin, cardiac-type myosin binding protein C (MYBPC3) and valosin-containing protein (VCP) [65]. Moreover, external stimuli may affect the composition of the exosomal cargo. Indeed, the exposure to alcohol or oxidative stress alters the content of cardiomyocytes-derived exosomes. In addition, hypoxia increases the release of cardiomyocytes-derived exosomes [66].

Adult cardiomyocytes communicate with neighboring coronary endothelial cells via exosomes, but the effects of their cross-talk is still unclear. Vrijsen et al. [67] have shown that exosomes released by CPCs-derived cardiomyocytes trigger the migration of endothelial cells, which enhances the local angiogenic response and the cardiac repair. Conversely, other investigators have demonstrated that cardiomyocytes-derived exosomes deliver high levels of miRNA320, which inhibits the angiogenic ability of endothelial cells in rats with type 2 diabetes [68]. On the other hand, hypoxic endothelial cells release exosomes delivering miRNA126 and miRNA210, which display pro-angiogenic properties and increase the tolerance of CPCs to hypoxia [69, 70].

Interestingly, we have demonstrated that porcine aortic endothelial cells co-cultured with porcine adipose-derived mesenchymal stem cells (MSCs) under normoxic conditions release increasing amount of exosomes delivering hypoxia-inducible factor 1-alpha (HIF1-alpha), in a time dependent-manner [71]. The exosomal release was directly related to the accumulation of free radicals into the culture medium. The functional consequences of these modifications are not yet fully understood, yet HIF-1α activate the MSCs to release factors increasing the migratory potential of endothelial cells [72].

Cardiac fibroblasts are also able to communicate with different types of cardiac cells via exosomes. Fibroblasts-derived exosomes accumulate miRNA21*, which is a miRNA passenger strand usually degraded during miRNA biogenesis. Bang et al. [41] have demonstrated that exosomal miRNA21* induces hypertrophy of cardiomyocytes (recipient cells) by down regulating sorbin, SH3 domain containing protein 2 (SORBS2), PDZ and LIM domain 5 (PDLIM5). Conversely, the inhibition of miRNA21* in hypertrophic mice suppresses the hypertrophic response of cardiomyocytes. These findings highlight the role of fibroblast-derived exosomes in mediating the hypertrophic response of cardiomyocytes and may open new avenue in the treatment of hypertrophic cardiomyopathy.

It is noteworthy that exosomes released by cardiosphere derived cells (CDCs) [44] and cardiac progenitor cells (CPCs) [43] exert significant cardioprotection both *in vitro* and

in vivo. A first study has demonstrated *in vitro* that CDCs-derived exosomes inhibit cardiomyocytes apoptosis, increase cardiomyocytes proliferation and angiogenesis. These effects are not recapitulated by fibroblasts-derived exosomes. *In vivo*, the same authors have observed that the intramyocardial injection of CDCs-derived exosomes into the infarct border zone reduced the scar, increased the viable mass and improved the heart function compared to murine infarcted hearts receiving fibroblasts-derived exosomes [44].

We have observed that the intramyocardial delivery of CPCs-derived exosomes in the infarcted hearts limited the apoptosis of cardiomyocytes, reduced the infarct scar size and improved the cardiac function [43].

In both pre-clinical studies, the cardioprotective effects were related to the delivery of specific microRNAs, such as miRNA146a. Indeed, the upregulation of miRNA146a exerts anti-apoptotic effects by preventing the NF-κB activation and the inflammatory cytokine expression in infarcted animals [73].

In vitro, it was even reported that exosomes of donor cells may alter the exosomal cargo of the recipient cells. Tseliou et al. [74] have shown that the treatment of dermal fibroblasts with CDCs-derived exosomes increases the release of stromal cell derived factor 1 and VEGF and changes the miRNA profile in fibroblasts-derived exosomes. Fibroblasts primed with CDC-derived exosomes improved cardiac function, increased capillary density and reduced scar size in a mouse model of MI. Thus, CDC-derived exosomes converted inert fibroblasts-derived exosomes to therapeutically active cells.

Conclusions

The study of exosomes is an area that has recently become a topic of huge biomedical interest.

In the last five years, the scientific community is strongly interested in the use of exosomes derived from cardiac progenitor cells as an alternative therapeutic nanotool to treat infarcted hearts and to prevent the onset of post-ischemic heart failure. The exosomes-based therapy is a cell-free approach that would be expected to be well tolerated without overt immune rejection.

Exosomes are more manageable than the stem/progenitor cells. In particular, exosomes released in the culture medium as well as in the body fluids are more stable and resistant to environmental factors, such as temperature and mechanical force in the ultracentrifuge. For example, the exosomes isolated from urine have a more stable size and concentration profile than those from plasma. Moreover, 4 °C storage offers no additional benefit over maintaining at room temperature.

So far, exosomes-based therapy requires further studies to optimize critical methodological issues, such as the administration of exosomes by repeated injections in order to reach higher efficacy *in vivo*. Therefore, future investigations will be aimed to better address the methodological aspects of exosomes-based therapy in order to target specific recipient cells [75]. It should be considered that exosomes contain random cargo in terms of proteins, miRNAs, DNAs, lipids and this issue also requires further studies to drive the proper composition of endogenous exosomes under different microenvironment.

These findings will be helpful to improve the potential applications of exosomes as a smart nanoshuttle to prevent and/or to treat ischemic cardiac remodeling.

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