Advances in the Biological Functions of Animal Exosomes

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Abstract

Exosomes are vesicular structures secreted by diverse cell types, formed by a phospholipid bilayer membrane that encapsulates proteins, lipids, and nucleic acids. They can transport proteins, lipids, and nucleic acids between cells and serve as potent carriers mediating intercellular communication. Exosomes naturally exist in various body fluids, including blood, urine, saliva, cerebrospinal fluid, and milk, which provides the basis for their extensive regulation of physiological and pathological processes in the organism. Currently, research on exosomes in human malignant tumors and autoimmune diseases has been extensive, whereas studies on their role in regulating animal physiological and pathological processes remain limited. Recent studies have shown that exosomes are abundantly present in the milk of various mammals, providing immune protection to neonates through their contained miRNAs. Therefore, this review summarizes the biogenesis and origin, biological functions, and isolation and identification methods of exosomes, aiming to provide a reference for their research in animal husbandry.

Full Text

Advances in Research on the Biological Functions of Animal Exosomes

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Abstract

Exosomes are vesicular structures composed of a phospholipid bilayer membrane that encapsulates proteins, lipids, and nucleic acids, secreted by various cell types. They can transport proteins, lipids, and nucleic acids between cells and serve as powerful carriers mediating intercellular communication. Exosomes naturally exist in various body fluids such as blood, urine, saliva, cerebrospinal fluid, and milk, providing a foundation for their extensive regulation of physiological and pathological processes. Currently, exosomes have been extensively studied in human malignant tumors and autoimmune diseases, but research on their regulation of animal physiology and pathology remains limited. Recent studies have shown that exosomes are abundant in the milk of various mammals, providing immune protection to offspring through their contained miRNAs. Therefore, this review summarizes the biogenesis and origin, biological functions, and isolation and identification methods of exosomes, aiming to provide a reference for research in animal husbandry.

Keywords: exosomes; biogenesis; function; isolation and identification

Introduction

During biological evolution, both prokaryotes and eukaryotes have evolved concise and efficient strategies for intercellular communication. Classical cell biology holds that there are two ways of cell-cell communication: direct contact interactions or indirect effects through secreted soluble factors such as hormones, growth factors, and cytokines. Over the past decade, research has discovered that extracellular vesicles, particularly exosomes, represent a third newly identified important carrier for intercellular communication, capable of mediating the transport of biological macromolecules such as proteins, lipids, and nucleic acids between cells, and widely influencing physiological and pathological processes in the body [1]. Currently, the limited research on exosomes in animal husbandry has focused on milk. Lin et al. [2] reviewed the similarities and differences in exosomal miRNAs from porcine, human, and bovine milk. miRNAs are important bioactive components of exosomes, and after being encapsulated by exosomes, they can resist digestion in the gastrointestinal tract of young animals and degradation by RNases in blood, being transported via blood circulation to immune organs to regulate gene expression, providing an important immune protection mechanism for offspring [1].

Historical Background

In 1983, Pan et al. [3] first discovered that in vitro cultured immature sheep reticulocytes could release multivesicular aggregates produced through endocytosis during differentiation. These small vesicles contained the transferrin receptor from the cell membrane, and these multivesicular aggregates were subsequently

named exosomes. Initially, researchers believed that exosomes were cellular components for clearing membrane fragments and eliminating cell surface molecules, which would be completely degraded by lysosomes and not recycled back to the extracellular environment. Others considered exosomes to be products of dead cell membrane fragments isolated and purified from culture media. Ten years later, Raposo et al. [4] demonstrated that these vesicles could also be isolated from B lymphocytes infected with Epstein-Barr virus and possessed the functions of antigen presentation and induction of T cell responses. In 2007, RNAs and miRNAs were discovered in exosome contents, arousing great interest among researchers as a new medium for intercellular communication [1]. Along with these pioneering studies, researchers found that various cell types including endothelial cells, smooth muscle cells, and immune cells could secrete exosomes, and that exosomes naturally exist in various body fluids such as blood, urine, saliva, cerebrospinal fluid, and milk. At this point, people gradually recognized their important role in ensuring the healthy growth of animal organisms, and research on exosomes in cancer, autoimmune diseases, and physiological regulation has sprung up.

2. Exosome Biogenesis

Extracellular vesicles refer to membrane-bound vesicles secreted from cells in an evolutionarily conserved manner. Based on their size, biogenesis, and composition, they are mainly divided into three categories: 1) Exosomes, which are generated in endosomes through inward budding of the plasma membrane, forming multivesicular bodies that are released from the plasma membrane, with a diameter of 30-150 nm. 2) Microvesicles, also known as microparticles or ectosomes, which are produced by outward budding and fission of the plasma membrane, with a diameter of 100-350 nm. 3) Apoptotic bodies, which are generated by plasma membrane blebbing during apoptosis, with a diameter of 500-1000 nm [5].

Exosome biogenesis begins with early endosomes formed by inward budding (i.e., endocytosis) of the plasma membrane containing surface proteins. Early endosomes generate multivesicular bodies through the recognition, sorting, and selection of exosomal cargo proteins by vesicle sorting proteins such as the endosomal complex required for transport (ESCRT), or with the assistance of ceramide. This process is divided into ESCRT-dependent and ESCRT-independent pathways based on whether ESCRT participation is required. Currently, the sorting mechanism of miRNAs into exosomes remains unclear. The generated multivesicular bodies are partially degraded by lysosomes, while others fuse with the plasma membrane to release exosomes. [Figure 1: see original paper] shows a schematic diagram of exosome biogenesis and release [6-7].

When analyzing the biological composition of exosomes, it is surprising to find that their protein content is very limited in scope. They do not contain nuclear proteins, mitochondrial proteins, endoplasmic reticulum proteins, or Golgi apparatus proteins. All discovered exosomal proteins originate from the cytosol,

endocytic vesicles, or plasma membrane. Compared with their source cells, exosomes are not merely plasma membrane fragments because their proteins lack some proteins that are abundantly expressed on the cell surface, such as dendritic cell Fc receptors [8], T cell CD28 [9], CD40L, CD45, and B cell transferrin receptors [10]. Regardless of their secreting cells, most exosomes contain similar types of proteins, with only a small portion being cell-specific. These specific proteins can reflect the physiological and pathological state of their secreting cells [11]. Typical exosomal proteins include platelet-derived growth factor receptors, lactadherin, transmembrane proteins, lysosomal-associated membrane protein-2B, membrane transport and fusion proteins such as ubiquitin, GTPases, heat shock proteins, lipid-associated proteins, phospholipases, etc. [12]. Some proteins enriched in exosomes are commonly used as markers, including members of the tetraspanin family (CD9, CD63, CD81, and CD82), 14-3-3 proteins, MHC molecules, cytoplasmic proteins such as heat shock proteins (HSPs), Tsg101, and ESCRT-3 binding protein Alix. CD9, CD63, and CD81 were initially considered specific markers of exosomes, but they have now been found to also exist in apoptotic bodies and microvesicles [13].

Because exosomes carry a large number of variable and specific proteins, their carried proteins can determine their functions in different pathways; therefore, exosomes are regarded as vector signal vesicles. Receptors or ligands exposed on the exosome membrane surface are responsible for targeting them to specific cells or extracellular regions. Subsequently, exosomes can activate intracellular signaling pathways or alter cellular phenotypes by binding to or being internalized by receptors or ligands on the surface of target cell membranes [14]. Specific exosomal proteins such as MHC I molecules, MHC II molecules, and transferrin receptors are very active in some signaling pathways, such as integrin and Ca^{2} signaling pathways [15], mitogen-activated protein kinase (MAPK) signaling pathway, and natural killer cell group 2D (NKG2D) signaling pathway [16]. HSPs such as HSP60 and HSP70, as well as surface receptors primarily present on immune cell membranes like CD14, CD91, Toll-like receptor (TLR)-2, TLR-4, oxidized low-density lipoprotein receptor-1 (LOX-1), CD94/CD56, represent classic ligand-receptor binding models [17].

In addition to mediating intercellular communication through membrane surface proteins, exosomes also carry some important soluble protein mediators, such as cytokines. The most well-known example of exosome-associated cytokine transport is interleukin (IL)-1 , which can be released not only after the fusion of secretory lysosomes with the plasma membrane but also produced by exosomes [18]. Similar to IL-1 , IL-18 also lacks an N-terminal signal peptide and is secreted from macrophage surfaces by exosomes after being activated by inflammatory complexes [19]. Chemokines are a very important and unique class of cytokines, and studies have found that chemokines IL-8 (CXCL8) and CX3CL1 can be secreted by exosomes from apoptotic lymphocytes [20].

In 2007, it was first discovered that exosomes from mouse and human mast cells both contain mRNAs and miRNAs, which can be transferred to recipi-

ent cells and perform physiological functions there. The source of exosomes and different detection methods both affect the types and quantities of RNAs ultimately obtained. Sequencing analysis showed that in human plasma exosomal RNAs, miRNAs are the most abundant, with approximately 593 types. The five most common miRNAs—miR-99a-5p, miR-128, miR-124-3p, miR-22-3p, and miR-99b-5p—account for 48.99% of the total, ribosomal RNA accounts for 9.16%, long non-coding RNA accounts for 3.36%, piwi-interacting RNA accounts for 1.31%, transfer RNA accounts for 1.24%, small nuclear RNA accounts for 0.18%, and small nucleolar RNA accounts for 0.01% [21]. Exosomes from HeLa cell culture medium contain multiple types of RNA: miRNAs, mRNAs, rRNA, tRNA, piRNA, RefSeq, and ncRNA [22].

Exosomes provide protection for their contained lipids, proteins, and RNAs (especially mRNAs and miRNAs) against enzymatic degradation, and provide a pathway for their contents to enter cells through endocytosis. Human mesenchymal stem cell-derived exosomes contain approximately 239 types of mRNAs, most of which are closely related to cell proliferation, differentiation, and immune regulation. Two of these mRNAs can be translated into complete proteins in mouse renal epithelial cells both in vivo and in vitro, indicating that exosomes can transfer active mRNAs [23]. The content of mRNAs carried by mast cell exosomes differs significantly between oxidative stress and normal states, suggesting that the uptake of mRNAs by exosomes is regulated by the physiological state and stress of cells and may play an important role in maintaining tissue homeostasis [24]. Similarly, the mRNAs carried by cardiomyocyte exosomes are regulated by growth factors [25], and the mRNAs and proteins carried by glial cell exosomes are affected under hypoxic conditions [26], indicating that the function of exosome-mediated mRNA transport is closely related to the physiological state of animal cells.

miRNAs in exosomes can be transported through blood circulation without being degraded by RNases in blood, which forms the basis of exosome-mediated intercellular communication. miRNAs are 18-25 nt in length, with primary transcripts being hairpin-structured pri-miRNAs. Drosha releases the hairpin structure of pri-miRNAs to form pre-miRNAs, and Dicer is responsible for removing the loop structures on the 3'and 5'arms of pre-miRNAs to form miRNA duplexes. The miRNA duplex binds to Ago protein, the passenger strand is discarded, and the guide strand complements the miRNA-induced silencing complex (miRISC), ultimately forming mature miRNAs to regulate target mRNA expression [27]. Selective degradation of some miRNAs in exosomes is a potential way to rapidly regulate gene expression and a possible means to inhibit cancer cell spread.

Exosome-mediated miRNA transport is related to cell immune function. During the formation of immune cell synapses, miRNAs carried by T cell-derived exosomes, such as miR-335, are unidirectionally transferred into antigen-presenting cells to regulate their gene expression [28]. Milk-derived exosomes can inhibit cytokine production induced by Anti-CD3 and Anti-PHA and increase the number of regulatory T cell-specific populations [29]. Immune-related miRNAs

(miR181a and miR-17) are highly expressed in human milk exosomes during the first 6 months after delivery, indicating that exosomes can mediate the transfer of miRNAs between mother and infant and may have an important impact on the development of the infant' s immune system [30].

3.3 Lipids and Related Functions

Exosomal lipids play an important role in the physiological functions of vesicles. Although the lipid composition varies among exosomes from different sources, they are all rich in sphingomyelin, cholesterol, and glycosphingolipids compared with their source cells. Large amounts of sphingomyelin and cholesterol can consolidate exosome structure and enhance their resistance to adverse physicochemical environments [31]. Additionally, the bilayer structure of the exosome membrane also helps maintain stability in different extracellular environments. Research on exosome stability is of great significance for the development of exosome-based drug carriers.

4. Isolation and Identification of Exosomes

Although various characteristics of different types of extracellular vesicles have been identified, there is still a lack of widely accepted classification criteria. Typical isolation methods cannot accurately distinguish or purify various vesicles, only obtaining multivesicular complexes. Therefore, the combined use of multiple techniques such as differential centrifugation, density gradient centrifugation, filtration, and size-exclusion chromatography can obtain relatively pure vesicles [32]. Currently, there are several exosome isolation and purification techniques: ultracentrifugation, size-based separation, immunoaffinity capture, and exosome precipitation [33].

Ultracentrifugation is the standard method for isolating exosomes. This method is simple to operate, has low technical difficulty, does not take long, and requires no sample pretreatment. However, due to the heterogeneity of exosomes and the overlap in size with other extracellular vesicles, exosomes isolated by this method will contain other extracellular vesicles [34]. Théry et al. [35] detailed the steps for extracting exosomes from cell culture supernatants and various biological fluids such as urine, plasma, serum, and ascites, which is currently the most classic method for isolating exosomes. Ultrafiltration is a relatively common and mature technology for separating exosomes based on size, with the principle of using ultrafiltration membranes that restrict specific molecular weights and sizes for separation. Ultrafiltration saves time compared with ultracentrifugation and does not require special equipment, but the high pressure applied during ultrafiltration can cause deformation and rupture of larger vesicles, potentially affecting subsequent analysis results [36]. The exosome membrane surface contains a large number of proteins and receptors, and immunoaffinity separation techniques can be developed through the specific binding of these proteins to antibodies [34]. Specific protein molecules present on the exosome

surface, such as CD63, provide strategies for isolating exosomes from complex samples.

Exosome identification requires morphological observation by electron microscopy. For tissue cells, the standard electron microscopy sample preparation process includes fixation, dehydration, embedding, and sectioning, but this process is not suitable for exosomes because the dehydration and embedding process can damage the exosome membrane. Therefore, special methods are needed for exosome sample preparation [28]. When observed under an electron microscope, exosomes exhibit a typical cup-shaped morphology, with a diameter of 30-150 nm, appearing as flattened spheres [37]. Further identification of exosomes requires assistance from proteomic analysis, Western Blot, FACS, and other methods.

5. Conclusion

Exosomes were first discovered in 1983, and for more than a decade thereafter, they were considered cellular waste, leading to neglect of research in this area. In recent years, people have gradually recognized that exosomes can not only mediate intercellular communication but also have important effects on physiological and pathological processes in the body. Given the important role of exosomes in maintaining homeostasis in animals, their role in the occurrence and development of animal diseases is self-evident, and research in this area is currently needed.

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