Review

Possibility of Exosome-Based Therapeutics and Challenges in Production of Exosomes Eligible for Therapeutic Application

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Received February 16, 2018

Exosomes are cell-derived vesicles with a diameter 30–120 nm. Exosomes contain endogenous proteins and nucleic acids; delivery of these molecules to exosome-recipient cells causes biological effects. Exosomes derived from some types of cells such as mesenchymal stem cells and dendritic cells have therapeutic potential and may be biocompatible and efficient agents against various disorders such as organ injury. However, there are many challenges for the development of exosome-based therapeutics. In particular, producing exosomal formulations is the major barrier for therapeutic application because of their heterogeneity and low productivity. Development and optimization of producing methods, including methods for isolation and storage of exosome formulations, are required for realizing exosome-based therapeutics. In addition, improvement of therapeutic potential and delivery efficiency of exosomes are important for their therapeutic application. In this review, we summarize current knowledge about therapeutic application of exosomes and discuss some challenges in their successful use.

Key words exosome; therapeutic application; production

1. INTRODUCTION

Almost all cells secrete extracellular vesicles including exosomes, microvesicles, and apoptotic bodies. Exosomes are the smallest extracellular vesicles. Although the definition of exosomes is not clearly established, they are negatively charged lipid bilayer vesicles whose diameter is 30–100 nm and their density is 1.13–1.19 g/mL.¹⁾ Exosomes are secreted by fusion of multivesicular body (MVB) containing exosomes with plasma membrane. MVBs are formed by budding of intraluminal vesicles into late endosomes.²⁾ This process is regulated by a protein machinery known as endosomal sorting complex required for transport (ESCRT).^{3,4)} An ESCRT-independent MVB formation that requires ceramide has also been reported.⁵⁾ It has been shown that proteins other than ESCRT such as ARF6, PLD2, and sphingosine 1-phosphate receptor are related to biogenesis of exosomes.^{6,7)}

Several molecules including lipids, proteins, and nucleic acids of producing cells are sorted to exosomes (Fig. 1). Although mechanisms of cargo loading into exosomes are not clear, it has been reported that RNA-binding proteins^{8,9)} and modification of protein such as ubiquitination,¹⁰⁾ sumoylation,⁸⁾ and glycosylation¹¹⁾ are related to these processes. The composition of exosomal proteins is different among cell types. However, it is known that some proteins such as CD9, CD63, and Alix are enriched in exosomes and these proteins are currently used as exosome markers.¹²⁾ Exosomes contain mRNA and short RNAs including microRNAs (miRNAs).^{13,14)} Lipidomic studies showed that cholesterol, sphingomyelin, glycosphingolipids, and phosphatidylserine are enriched in exosomes.^{5,15,16)}

Exosomes induce many biological events by delivering bioactive molecules contained in exosomes. It has been reported that exosomes play important roles in intercellular transport in various pathological conditions such as cancer,^{17–20)} cardiovas-

cular diseases,²¹⁾ diabetes,²²⁾ and Alzheimer's disease.²³⁾ Hence exosomes may be expected to become therapeutic targets in these diseases. Additionally, exosomes may be useful as diagnostic markers. Because of their endogenous origin, exosomes are expected to be safe therapeutic agents. In this review, current knowledge about the therapeutic potential of exosomes is summarized and challenges in producing exosomes eligible for therapeutic application are discussed.

2. THERAPEUTIC APPLICATION OF EXOSOMES

2.1. Regenerative and Anti-inflammatory Therapies

Mesenchymal stem cells (MSCs) which are well known to possess anti-inflammatory and regenerative effects, are the most commonly used source of exosomes.²⁴⁾ It has been reported that MSC-derived exosomes are related to the therapeutic effects of MSCs.^{25,26)} Through the delivery of their components including miRNAs and proteins such as interleukin-10 (IL-10) and transforming growth factor- β , MSC-derived exosomes inhibit expression of pro-inflammatory cytokines to exert anti-inflammatory effect and promote tissue regeneration by enhancing extracellular matrix remodeling. Therefore MSC-derived exosomes have therapeutic potential against various diseases such as cardiovascular disease, liver

Fig. 1. Biogenesis and Composition of Exosomes

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Fig. 2. Therapeutic Application of MSC-Derived Exosomes

injury, renal injury, and neural injury^{27–34}) (Fig. 2). Although MSCs themselves can be used to treat these diseases, therapeutics using MSC-derived exosomes seem a safer option.³⁵ Exosome-based therapeutics have a lower risk for teratoma formation and embolization, which are major concerns for stem cell-based therapeutics. Exosomes secreted from induced pluripotent stem cell (iPS cell)-derived MSC also may exert therapeutic effects.³⁶ Therefore iPS cell-derived MSC can be a good source of exosomes since they can be abundantly prepared from patients. Moreover, exosomes secreted by iPS cell-derived MSC have superior therapeutic effect on osteoarthritis compared with the same number of exosomes secreted from synovial membrane-derived MSC.³⁷ It was shown that exosomes secreted from iPS cells, embryonic stem cells, and cardiac progenitor cells have therapeutic effects similar to MSC-derived exosomes.^{38–40}

Milk-derived exosomes have been shown to induce immunomodulatory effects.^{41–43} Arntz *et al.*⁴² showed that oral administration of bovine milk-derived exosomes attenuate arthritis. It was also demonstrated that plant-derived exosome-

like vesicles elicit immunomodulatory effects, although it is unclear whether these vesicles are strictly exosomes.^{44,45} Immunogenicity is a major concern of milk- and plant-derived exosomes because these are xenogeneic vesicles. Oral administration may be a safe route for the administration of these xenogeneic vesicles because they are derived from foods.

2.2. Cancer Vaccines Exosomes derived from dendritic cells (DCs) may be useful as anticancer vaccines because of the nature of DCs as antigen-presenting cells (APCs). Major histocompatibility complex (MHC)-I, MHC-II, and co-stimulating factor such as CD86 are expressed on the surface of DC-derived exosomes.^{46,47} Administration of exosomes derived from DCs incubated with cancer antigen induced cancer-specific T cell response.⁴⁸ Although direct activation of T cells by surface proteins of exosomes has been reported,⁴⁹ the T-cell response is considered mainly induced by APCs that captured the exosomes.⁵⁰ It has been shown that mature DC-derived exosomes induced strong T-cell response compared with immature DC-derived exosomes.⁵¹ In addition to antigen presentation, DC-derived exosomes activated natural

Biography

Takuma Yamashita was born in Gifu, Japan, in 1991. He obtained his B.S. degree at Faculty of Pharmaceutical Sciences, Kyoto University and pharmacist license in Japan in 2015. Now he is in the 4th year of the Ph.D. course at the Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University. He received the Nagai Memorial Research Scholarship from the Pharmaceutical Society of Japan for 2 consecutive years in 2015 and 2016. He has been a Research Fellow of the Japan Society for the Promotion of Science since 2017. His research focus is on the development of exosome-based therapeutics. He has investigated the biodistribution of injected exosomes, the major types of cells that are responsible for pharmacokinetics of exosomes, the effects of exosome isolation methods on the physicochemical and pharmacokinetic properties of exosomes, and an exosome-tropic RNA sequence for RNA loading into exosomes. Currently, his main research is focused on improving pharmacokinetic properties of exosomes.

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Fig. 3. Activation of T Cells and NK Cells by DC-Derived Exosomes

killer cells by their surface protein such as NKG2D ligand and IL-15R α ⁵²⁾ (Fig. 3).

Exosomes derived from cancer cells may also potentially be used as anticancer vaccines because they contain antigens. Our previous study showed that exosomes derived from B16BL6 murine melanoma containing melanoma antigens induced B16BL6-specific T-cell response and inhibited tumor growth.^{53,54)} On the other hand, it was reported that exosomes derived from cancer cells have roles on cancer progression, metastasis, and drug resistance.^{17–20)} Therefore safety evaluation is essential for using exosomes derived from cancer cells.

3. CHALLENGES IN THE DEVELOPMENT OF EXOSOME-BASED THERAPEUTICS

3.1. Large-Scale Production One of the major problems for realizing exosome-based therapeutics is low productivity of exosomes. Yield of exosomes is typically less than 1 μ g exosomal protein from 1 mL of culture medium,^{55,56)} whereas the useful dose of exosomes is approximately 10–100 μ g exosomal protein/mouse in most studies.^{29,57)} Thus effective large-scale exosome production methods are required.

Exosome-containing medium is normally prepared by culturing exosome-producing cells over a few days. Riches *et al.*⁵⁸⁾ and our preliminary data suggest that the amount of exosomes in culture medium reached upper limit after approximately 12-h incubation, although the time until saturation is probably dependent on the types of producing cells. Bioreactor may be useful to improve yield of exosomes. Watson *et al.*⁵⁹⁾ showed that yield of exosomes can be increased 5–10 fold using a hollow fiber bioreactor. However, whether yield of exosomes was actually increased by use of the bioreactor is not clear, since the obtained sample contained larger vesicles (200–800 nm in diameter).

Some reports demonstrated that exosome production was enhanced by applying stresses such as hypoxia, low pH, and anticancer drugs on exosome-producing cells.^{60–62)} However, the therapeutic effects and safety of exosomes secreted from stressed cells should be carefully evaluated because it was demonstrated that cellular stresses change the composition of exosomal contents.⁶⁰⁾ In addition, cellular stresses may increase contaminants derived from dead cells such as apoptotic

bodies, which may cause adverse effects and overestimation of exosome amount.

Efficient isolation of exosomes is another approach to increase yield of exosomes. Our previous research showed a small difference in the yields of exosomes between three conventional ultracentrifugation-based isolation methods (simple ultracentrifugation, density cushion, and density gradient ultracentrifugation).⁵⁶⁾ In addition to the ultracentrifugation-based methods, other exosome-isolation methods have been developed.^{63–70)} Some of these methods such as size exclusion chromatography, ultrafiltration, aqueous two-phase system, and polymer-based precipitation can be used for large-scale production of exosomes. However, there is little information about difference in efficiency of exosome isolation among these newly developed methods.

3.2. Collection of High-Quality and Uniform Exosomes

In addition to the yield of exosomes, many other properties are affected by choice of isolation methods. Table 1 summarizes the advantages and disadvantages of each isolation method. Purity and physicochemical properties of exosomes are the major factors affected by different isolation methods. Our previous research showed that simple ultracentrifugation causes more contamination of proteins and aggregation of exosomes compared with density cushion and density gradient methods.⁵⁶⁾ Therefore simple ultracentrifugation, which is the most commonly used method for exosome isolation, may not be suitable for therapeutic application in scenarios wherein these parameters affect efficacy. Additionally, polymer-based commercial reagents for exosome isolation are not suitable for therapeutic application because they are known to cause severe aggregation and contamination.^{56,71)} Recently, it was noted that there exist subpopulations in exosomes, which are different in their composition and size.^{72–74)} Subpopulations in the collected exosomes should be different among the isolation methods. Several studies indicated that composition of proteins and RNAs in exosomes are different among exosomes collected by different methods,^{71,75)} which may be caused by differences in subpopulations or contaminants. It is likely that therapeutic potentials are different among the subpopulations, although differences have not been reported yet. Other properties such as protein corona (that is, proteins adsorbed on the surface of nanoparticles) may also be affected by isolation methods. Although it is not clear whether protein corona is formed on exosomes, it was reported that the protein corona changes the properties of nanoparticles such as mechanisms of cellular uptake and clearance from blood circulation.^{76–78)}

Overall, exosomes collected from different isolation methods are different in many properties. Thus for therapeutic application of exosomes, optimization of the isolation method is important to preserve properties of exosomes and to reduce risk for side effects induced by contaminants or specific subpopulations.

3.3. Optimization of Storage Conditions The International Society of Extracellular Vesicles recommends⁷⁹⁾ that exosomes be suspended in phosphate buffered saline and stored at -80°C . It was previously shown that storage at higher temperatures decreased quantity of exosomes and their contents, whereas storage at -80°C was associated with fewer changes.^{80,81)} It was shown that addition of trehalose protected exosomes from cryodamage.⁸²⁾ For therapeutic application, storage at higher temperatures is desirable because it does

Table 1. Characteristics of Exosome Isolation Methods

	Advantages	Disadvantages	References
Ultracentrifugation-based methods			
Simple pelleting	Scalable, low running cost	High cost for equipment, aggregation, protein contamination	56)
Density cushion method	High purity compared to simple pelleting	High cost for equipment, aggregation	56)
Density gradient method	High purity, gentle	High cost for equipment, time-consuming, low sample capacity	56)
Size-based separation			
Ultrafiltration	Very scalable, fast	Low purity, damages by shear stress	63)
Size exclusion chromatography	High purity, gentle	Low sample capacity, dilution of samples	64)
Polymer-based methods			
Polymer-based precipitation	Easy, scalable, requires no special equipment	Low purity, severe aggregation	56,65)
Aqueous two-phase system	Easy, scalable, requires no special equipment	Little information	66)
Affinity-based separation			
Surface protein affinity method	High purity, can collect specific subpopulation	High cost for reagent, damages on surface proteins during elution	67)
Phosphatidylserine affinity method	Elution in a mild condition	High cost for reagent	68)
Others			
Microfluidic systems	Low cost, high purity	Less scalable	69,70)

not require special equipment. Lyophilization of exosomes may improve their stability at higher temperatures. In a patent of Kreke *et al.*⁸³⁾ it was shown that exosomes obtained from cardiosphere-derived cells by ultrafiltration can be lyophilized without significant loss of biological activity. However, it is not clear whether different types of exosomes can be lyophilized by the same methodology. Additionally, shelf-life of lyophilized exosomes remains unclear. Further research should be performed to reveal these points.

3.4. Improvement of Therapeutic Potential of Exosomes For overcoming low productivity of exosomes, improving their therapeutic potential can be one possible solution. Overexpression of molecules related to therapeutic effects of exosomes is a simple method to improve therapeutic potential. Several groups^{84,85)} demonstrated that overexpression of miRNAs enhanced therapeutic effects of exosomes. It has been reported that overexpression of proteins that change expression profile of miRNAs and proteins also may improve therapeutic potential of exosomes.^{86,87)} Modification of exosomes with functional molecules is also effective in increasing their therapeutic potential. In our previous study we demonstrated that modification of B16BL6-derived exosomes with CpG DNA, an immune adjuvant, was effective in decreasing the dose of exosomes required to induce cancer antigen-specific immune response by 10–100 fold compared with preceding studies in which unmodified exosomes were administered.⁵³⁾ Hypoxia treatment also changed the composition of exosomes, and resulted in enhancement of their biological effects.^{88,89)} However, because these modifications may cause unwanted changes in exosomes, the effects and safety of modified exosomes should be evaluated independently.

3.5. Delivery of Exosomes Because the biological effect of exosomes is exerted by their uptake by target cells, elucidation and control of biodistribution of exosomes are required for the therapeutic application of exosomes. Our research showed that intravenously injected exosomes rapidly disappeared from blood circulation and accumulated in the liver, spleen, and lung.^{90,91)} In addition, we have demonstrated that exosomes are taken up by macrophages in the liver and spleen and that the

negative charges of exosomes derived from phosphatidylserine contained in exosomal membrane play some roles in the recognition of the exosomes by macrophages.^{92,93)} In accordance with our study, other researchers have shown that intravenously injected exosomes mainly accumulated to the liver and spleen.^{94,95)} On the other hand, Grange *et al.*⁹⁶⁾ showed that exosomes accumulated to the kidney when intravenously injected in acute kidney injury model mice. This change in the distribution of exosomes may be due to the enhanced vascular permeability in the kidney caused by injury and inflammation.⁹⁷⁾ Wiklander *et al.*⁹⁸⁾ investigated the biodistribution of exosomes following injection *via* different routes: intraperitoneal injection resulted in higher accumulation in pancreas and gastrointestinal tract compared with intravenous injection, whereas subcutaneous injection resulted in much lower accumulation of exosomes in all measured organs. In most cases, the majority of an injected exosomes dose is rapidly taken up by macrophages in the reticuloendothelial system irrespective of cell source and route of administration.^{55,98)} This rapid clearance of exosomes by macrophages limits systemic administration of exosomes. Although there are several reports showing that ligand modification of exosomes enhanced their accumulation in target organs, the amount of exosomes in the target organ was limited.⁹⁹⁾

4. CONCLUSION

Exosomes are expected to become effective therapeutic reagents for various diseases. However, there remain several challenges to their optimal use. In particular, methods for large-scale production must be developed. Methods for isolation and storage should be carefully optimized since they affect various factors of collected exosomes. Additionally, improvement of therapeutic potential and delivery efficiency of exosomes is needed. In this review, we summarized current knowledge about these challenges in the process of exosome production. The information in this review will be helpful to develop superior methods for production of exosome formulation.

Acknowledgments This work was supported by Grant-in-Aid for Challenging Exploratory Research and Grant-in-Aid for Japan Society for the Promotion of Science (JSPS) Fellows from JSPS KAKENHI (Grant number 17K19390 and 17J09513).

Conflict of Interest The authors declare no conflict of interest.

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