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Review

Extracellular vesicles: Specialized bone messengers

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ABSTRACT

Mammalian cells actively secrete factors that contribute to shape their microenvironment. These factors either travel freely or they are enclosed within the lipid bilayer of extracellular vesicles (EVs), and regulate the function of neighboring and distant cells. EVs are secreted by a wide spectrum of cell types and are found in various biological fluids. They convey their message by mediating the horizontal transfer of bioactive molecules, such as proteins, mRNAs and miRNAs, between cells. Recent studies showed the vital roles of EVs in a wide range of physiological and pathophysiological processes. In this review, we highlight the recent developments in the newly emerging EV field, including their biogenesis, molecular content and function. Moreover, we discuss the role of EVs in bone biology and their promising applications in diagnosis, drug development and regenerative therapy.

Introduction

Multicellular organisms developed complex communication networks to regulate biological activities, and consequently maintain physiological homeostasis. Interruptions in intercellular communication lead to pathological complications and disease progression. Cells exchange information with each other via immobilized molecules as well as secreted factors. Traditionally, secreted factors include small soluble molecules such as neurotransmitters, chemokines, cytokines and hormones that can either act over short distances and affect the neighboring cells in a paracrine manner or travel long distances in an endocrine manner. The last couple of decades have witnessed the unprecedented dedication of scientists to the study of extracellular vesicles (EVs)[1] as novel mediators of intercellular communication.

EVs had long been regarded as cellular debris until Wolf and colleagues described their biological significance in coagulation in 1967 [1]. Later same year, Anderson and Bonucci reported the discovery of matrix vesicles as specialized EVs involved in biomineralization of the bone matrix [2,3]. Since then, EVs have been described to have a broad range of functions in development, immunology, angiogenesis and stem cell biology, as well as disease progression. EVs are considered to be bioactive organelles that carry genetic information in forms of lipids, proteins and nucleic acids between cells, and have effects on diverse molecular functions, such as signaling and regulation of gene expression of the target cells [4–8]. EVs embrace a heterogeneous group of vesicles, including microvesicles, microparticles, ectosomes, exosomes, shedding vesicles, apoptotic bodies and many others released under different biological circumstances. Even though the different names reflect their diversity in terms of biogenesis, structure, content and function, there are still conflicts over the definition and characterization of different vesicular structures. For simplicity, EVs are often categorized in three classes based on the well-defined processes for EV biogenesis: small vesicles (10–100 nm) released by exocytosis (exosomes, exosome-like vesicles), bigger vesicles (100–1000 nm) formed by budding from the plasma membrane (microvesicles, shed vesicles, matrix vesicles) and big vesicles (0.8–5 μm) released from dying cells (apoptotic bodies) (Fig. 1) [8–10].

Cells release EVs either constitutively during their growth or upon activation by biological stimuli. Apoptotic and diseased cells also release EVs providing valuable information about the health state of the cell [10,11]. EVs were detected in all studied cell types so far and most biological fluids, including blood, urine and breast milk [12–14]. Osteoblasts, the bone forming cells, also secrete EVs that have well characterized roles in mineralization [15]. There is increasing knowledge about the biological functions of EVs in other bone-related processes. Particularly, the regenerative role of mesenchymal stem cell (MSC) EVs makes them promising therapeutic agents for bone regenerative medicine [16–18]. In this chapter, we will review the various roles of EVs in diverse aspects of intercellular communication and highlight their function in bone biology.

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[1] *Abbreviations used:* EVs, extracellular vesicles; MSCs, mesenchymal stem cells; MVBs, multivesicular bodies; ESCRT, endosomal sorting complex required for receptor transport.

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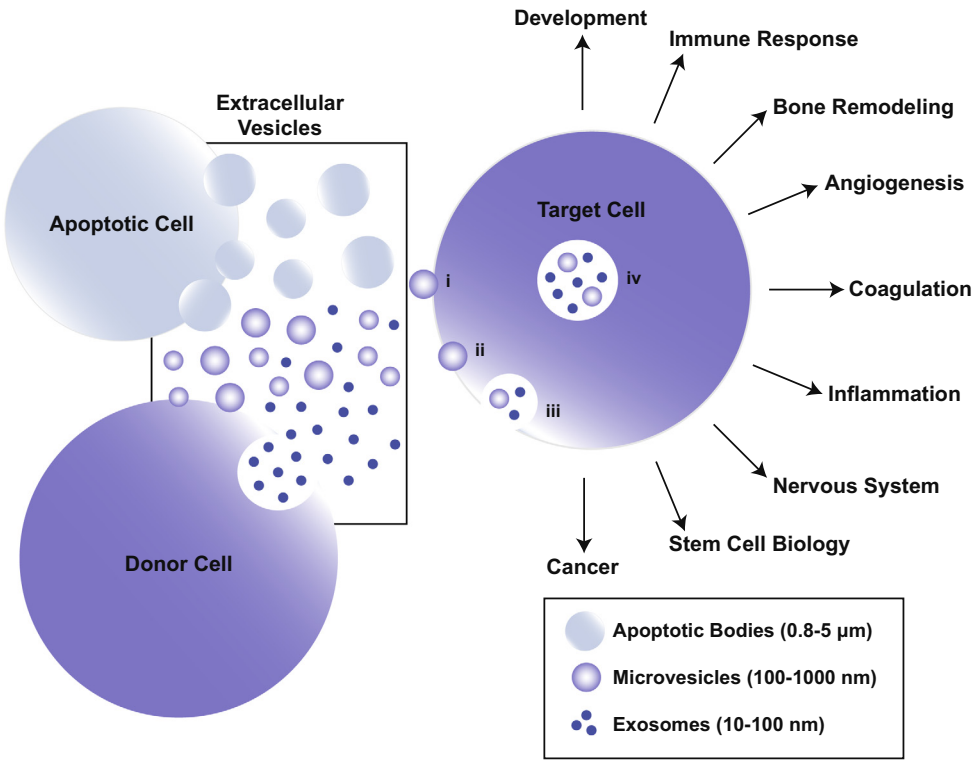

Fig. 1. Schematic representation of EV release from donor cells and the interaction with the target cells. EVs are released either by exocytosis of the multivesicular bodies or direct budding from the plasma membrane. Apoptotic bodies are released from breakdown of the apoptotic cells. EVs interact with their targets via (i) signal transduction mediated by docking at the plasma membrane of the target cell and/ or via releasing the bioactive cargo upon (ii) fusion or (iii–iv) endocytosis followed by fusing with the delimiting membrane of the endosomal compartment. Interaction with target cells leads to a broad range of biological functions. Interaction with the target cell is only shown for microvesicles but exosomes and apoptotic bodies utilize similar mechanisms.

Isolation and characterization

With the rapid development of EV research, the optimization and standardization of isolation techniques have become of utmost importance to improve the understanding of EV biology. Currently, the most commonly applied EV isolation method relies on differential ultracentrifugation protocol developed by Thery and colleagues in 2006 [19]. According to this protocol, biological fluids or the conditioned media from cultured cells are subjected to a series of centrifugation and ultracentrifugation steps to pellet the EVs. Cells break open at high speeds, and as a consequence cellular organelles and big protein complexes are co-isolated with EVs. Therefore, it is very important to start with a low centrifugation speed to remove floating and/or dead cells, and perform serial centrifugation steps in increasing speeds to remove the contaminants in a step-wise fashion. Furthermore, it is crucial that the purified EV pellet is free of bovine serum contaminants, such as serum proteins and vesicles. For cell cultures it is common to use EV-depleted serum and/or serum-free medium treatment before EV collection. This becomes, indeed, more challenging with biological fluids. The duration and speed of each centrifugation step varies between different protocols based on the EV source. Big microvesicles and apoptotic bodies are commonly pelleted using 10,000g centrifugation, while smaller microvesicles and exosomes require high speed centrifugation at about 100,000g [6,19]. The crude EV pellet can be subjected to further purification by sucrose density gradient or high performance liquid chromatography to remove possible contaminants. There have been attempts to separate EVs with different sizes based on their sediment density by sucrose density gradient. Recent studies have reported the sediment densities as the following: 1.11–1.21 g/ml for exosomes [20–22], 1.25–1.30 g/ml for microvesicles [23], and 1.18–1.28 g/ml for apoptotic bodies [24]. However, some small microvesicles can have even lighter densities than exosomes making the separation of the different EV populations very difficult using the conventional techniques [6].

Purified EVs are commonly characterized by microscopic, biochemical and fluidic analyses. EVs can be visualized by electron microscopy, which gives clues about their size and morphology (Fig. 2A). In the past, researchers used electron microscopy to verify the presence of exosomes based on their cup shape morphology; however, it was recently discovered that this was due to dehydration during sample processing [25,26]. Atomic force microscopy, on the other hand, allows samples to be in their native state, and hence it proves to be a better alternative to study the morphology of different EVs (Fig. 2B)[27]. Other common methods to study EVs include western blot and flow cytometry analyses using known vesicle markers. On-going studies are focusing on identifying markers specific to unique EV classes. A major challenge of EV characterization is the accurate quantification. ELISA and immunoadsorption are possible methods to determine the abundance of EVs using known markers; however, they do not give comprehensive information about the total EV concentration [28]. Conventional flow cytometry is a useful technique to quantify big EVs, while accurate quantification of EVs smaller than 250 nm is difficult. Van der Vlist and colleagues developed a high-resolution flow cytometry-based technique that can be used to detect smaller EVs based on their fluorescence intensity [29]. Recently, several companies developed techniques that allow multi-parameter analysis of EVs of certain size ranges providing greater accuracy than fluidic-based techniques. Nanoparticle tracking analysis measures