Extracellular vesicles: small bricks for tissue repair/regeneration

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Provenance: This is a Guest Perspective commissioned by Section Editor Dr. Mingzhu Gao (Department of Laboratory Medicine, Wuxi Second Hospital, Nanjing Medical University, Wuxi, China).

Comment on: Silva AM, Teixeira JH, Almeida MI, *et al.* Extracellular Vesicles: Immunomodulatory messengers in the context of tissue repair/regeneration. Eur J Pharm Sci 2017;98:86-95.

Abstract: Extracellular vesicles (EVs) are nano-sized membrane vesicles involved in intercellular communication. EVs have pleiotropic actions in physiological and pathological conditions. The ability of EVs to transports proteins, drugs and nucleic acid, to target specific cells and to increase the stability of therapeutic cargo, make EVs interesting as new devices for the treatment of human disease. In a recently published issue of European journal of pharmaceutical sciences, Silva and colleagues reviewed the ability of EVs to modulate tissue repair and regeneration, focusing on their roles and therapeutic potential as immunomodulatory messengers. In this perspective, we discussed the open questions regarding the dual role of EVs in immune system, as well as the technical limitation of the procedure for EVs isolation and administration in clinical practices. EV-based therapies require further studies to consider EVs as promising candidate for a novel cell-free therapy in the context of regeneration medicine.

Keywords: Extracellular vesicles (EVs); immune system; tissue repair/regeneration

Submitted Dec 01, 2016. Accepted for publication Dec 06, 2016.
doi: 10.21037/atm.2017.01.53
View this article at: http://dx.doi.org/10.21037/atm.2017.01.53

In the past decade, the scientific interest in the role played by extracellular vesicles (EVs) in human physiology and in pathogenesis of major human diseases has rapidly increased. Huang-Doran and colleagues considered EVs an "umbrella term" that includes exosomes and different microvesicles, distinguished by their size and biogenesis (1).

EVs are commonly heterogeneous in size, ranging from 20 to 1,000 nm in diameter depending on their origin and mechanism of release, direct shedding or budding from the plasma membrane. Exosomes are vesicles with a diameter of 20–100 nm formed by the inward budding of endosomal membranes to form large multivesicular bodies and released extracellularly when MVBs fuse with the plasma membrane. Larger microvesicles with diameter of 100 nm–1 mm result from the direct outward budding and fission of the plasma membrane (2).

In the 1980s, exosomes were thought to act as 'garbage bags' with the main function to discard the cellular waste, now they are considered as important intercellular communication devices (3).

Evs have been detected in several biological fluids such as blood, saliva, urine, bronchoalveolar lavage fluid, synovial fluid, breast milk, amniotic fluid and malignant ascites (4).

EVs carry a plethora of molecules including proteins, lipids, small and long, coding and non-coding RNAs that interact with local and distant cellular targets, thus modifying cell phenotypes. EVs add an alternative to the paracrine and endocrine cellular communication (5). EVs can interact with recipient cells with different endocytic mechanisms including clathrin-mediated endocytosis, clathrin-independent endocytosis, phagocytosis and macropinocytosis. The selected pathway depends on the expression of surface proteins on the recipient cells and on proteins present in EVs (6). After internalization by target cells, the mechanism by which EV cargos escape the degradative pathway of the recipient cells is still unclear (7).

EVs have a key role in several biological processes, they are considered as a new paradigm in cell signaling. EVs

carry molecules that can activate downstream canonical pathways in the target cells (8).

The widespread research in the field of the vesicle role in cancer highlights the effects of EVs in modulation of the microenvironment. Increasing evidence shows that tumor-derived exosomes have multiple roles in tumorigenesis; they can contribute to cancer growth inducing anti-apoptotic and oncogenic pathways such as invasion, metastasis and angiogenesis. Exosomes can promote tumor immune evasion with T-cell apoptosis induction (9-11).

Recently, exosomes attracted the interest of the scientific community for their potential use in therapy as a targeted and non-immunogenic delivery system for drugs or biological molecules (12).

EVs are involved in the maintenance of tissue homeostasis and they contribute to tissue repair and regeneration (13) as extensively showed in the review of Silva and colleagues (14).

Because Evs are involved in pathological processes and their presence in biological fluids allows their use for easy liquid biopsies (10), EVs have also attracted great interest for the potential use as disease biomarkers (15).

The authors of the review "Extracellular vesicles: Immunomodulatory messengers in the context of tissue repair/regeneration" preferred to indicate the nanovesicles discussed in the paper as EVs, in order to review studies that investigate a broad range of different population of vesicles (exosomes, microvesicles, ectosomes) according to the nomenclature guidelines proposed by ISEV.

In this review, the authors discussed the role of EVs in tissue repair and regeneration and the potential therapeutic effects of natural, modified and artificial EVs in control of inflammation and regenerative medicine. The authors described effects of EVs in tissue repair as mediators of cell proliferation and differentiation and they reported the effects of EVs in the extracellular matrix remodeling. Moreover, they focused the attention on the role of EVs in therapy as vehicles of new drugs and as a novel drug-delivery system.

After isolation, EVs could be utilized in regenerative medicine through several methods, in combination with cells and/or other therapeutics or separately. It was described that EVs can be directly injected into tissue or circulation. EVs can be introduced in regenerative therapies, mixed with hydrogels, or it is possible to coat scaffolds using fibrin gels.

Since EVs are complex devices with a pleiotropic role, it is necessary to understand their advantages and disadvantages before to candidate them as therapeutic agents, considering the limitations that have emerged in the clinical trials for their use.

In this perspective, we analyzed the dual role of EVs in immune system, the technical limitations of a non-standardized procedure for the purification and quantification, necessary to utilize the vesicles in therapy.

The authors in this review focus on the use of EVs, as a promising candidate for a "novel cell-free" therapy, in the resolution of inflammation during the repair of tissue damage. Resolution of inflammation is one-step of tissue repair and regeneration.

The role of EVs in regenerative medicine is supported by several experimental data. It was demonstrated that exosomes released by mesenchymal stem cells (MSCs) have been tested in preclinical settings for the treatment of cardiovascular diseases (15), kidney injury (16), osteochondral regeneration (17), skeletal muscle regeneration (18) and neurological disease (19). EVs can modulate the immune-activity (20), through the regulation of cytokine expression. EVs can also affect the infiltration of immune cells into the damaged tissue, inhibiting the pro-inflammatory processes and immune cells activity.

It has been also demonstrated the role of EVs in immune suppression and/or immune stimulation. Several papers reported preliminary application of EVs as immunotherapeutic agents for the treatment of inflammation triggered by the injury. Blazquez *et al*. demonstrated the immunomodulatory and anti-inflammatory effects of human adipose MSCs derived exosomes (exo-hASCs) in *in vitro* stimulated T cells. hASCs exosomes inhibit the activation and differentiation of cytotoxic and helper T cells, as well as reduced T cell proliferation and IFN-γ release on *in vitro* stimulated cells (21).

Zhang *et al*. also demonstrated an immunosuppressive role for exosomes isolated from embryonic stem cells (ESC). Exosomes were shown to induce high levels of anti-inflammatory IL-10 and tumor growth factor-β1 (TGF-β1) in human monocytes, and they reduced the levels of pro-inflammatory IL-1B, IL-6, tumor necrotic factor α (TNFα), and IL-12P40 (22).

Wen *et al*. demonstrated that exosomes released by human bone marrow mesenchymal stem cells (hBMSCs) suppress immune reaction during Langerhans islet transplantation, promoting islet function and inhibiting immune rejection (23).

On the other side, data in the literature report the role of exosomes in immune system stimulation. Rahman *et al*.

demonstrated the role of Islet-derived MSCs exosomes as autoantigen carrier in the promotion of local autoimmune response. The cells of Langerhans islet of NOD mice (MSC-like cells) release immunostimulatory exosomes that could activate autoreactive B and T cells endogenously. The immunization with exosomes accelerated the effector T cell-mediated destruction of islets (24).

The exosomal features and their potential role in immune regulation suggest a possible clinical use of EVs as adjuvant or therapeutic agents, but several problems limit the clinical use of EVs.

Clinical Good Manufacturing Practice (cGMP)-grade standards for EV clinical use require standardized criteria for EV isolation and storage methods. It is necessary to standardize a long-term storage method, in order to preserve the EV functions and minimize the variability of EVs stability and composition.

Nowadays, a reliable quality control assay to test EV membrane integrity after their preservation is lacking (25). This limit does not ensure the reproducibility of the effects obtained after the EV administration.

Another problem to use the EVs as therapeutic agents or drug delivery vehicle is the lack of an optimal method for their isolation. The techniques most commonly applied for EV isolation, ultracentrifugation and polymeric precipitation, are not ideal due to several reasons: (I) the technique is cumbersome; (II) it does not avoid the co-precipitation of culture medium components (serum or bovine albumin) with EV pellets; this could affect the EV biological effects; (III) recovery is low and the scale up process not easy to standardize. The culture medium components could induce modulation in target cells that can be confused with the effects assigned to EVs. Therefore, it is necessary the standardization of a unique EV isolation technique that could prevent the presence of other components in EV pellets.

The clinical application of EVs also requires the standardization of the administered dose, but actually does not exist a unique technique of quantification, the achievement of comparable results is therefore difficult to obtain. To date, the amount of EVs is calculated in two different ways: in several studies, EVs released by a defined cell number are used, others scientists utilize the count of the EV number (11).

Furthermore, the characterization of the active molecules contained in EVs is a fundamental step for EV approval in clinical practice by the National Agencies for Drug Regulation. It is also important to consider the oncogenic potential of EVs that could limit the clinical use of vesicles as therapeutic agent. EVs contain oncogenic proteins, mRNAs, miRNAs and transcription factor that can potentially alter the genome and the proteome of recipient cells, thus favoring angiogenesis, proliferation and metastasis (16-20).

In the review, the authors discussed about the effects of EVs in regenerative medicine, but it is important to consider the dual effect of EVs on the immune system. There are benefits and disadvantages about the use of EVs as therapeutic immune-modulators in the context of tissue repair/regeneration.

Although, MSC derived EVs have immune-modulating activity (20), the effects of long-term EV administration are unknown. Since EVs display MHC (26) and carry several allogeneic proteins, they could stimulate an auto immune response in the patient in which they are injected.

Few data support the use of allogenic and xenogeneic EVs as therapeutic application to enhance the amount of EVs injectable in therapy. It was demonstrated that allogenic EVs are tolerated in immune–competent animals (5,7).

Nowadays, one clinical trial reported the use of allogenic EVs in human therapy. EVs released by MSCs have been first administered for compassionate use in a patient with steroid refractory graft versus host disease (27). Multiple injections (every 2–3 days) of EVs released by MSCs of unrelated bone marrow donors showed anti-inflammatory effects and ameliorated the clinical symptoms of the patient without adverse effects, indicating the safeness of EV administration. The first trial on MSC-derived microvesicles and exosomes on β-cell mass in type I diabetes mellitus is now enrolling (NCT02128331).

Arslan *et al*. also demonstrated that xenogeneic EVs might be tolerated in mice, *in vivo* (28), but nowadays there are not data about the potential time-dependent effect on the immune system modulation, following repeated injection of EVs in human.

In order to use EVs for therapeutic applications might be optimal to derive therapeutic EVs from an autologous source, to reduce the side effects and cost of EVs therapies.

Escudier *et al*., demonstrated the role of exosomes isolated from conditioned medium of autologous dendritic cell (DC) of patients with advanced metastatic melanoma, incubated with melanoma peptide antigens to induce presentation of the antigen on the cell surface, in association with MHC. EVs reintroduced into patients promoted an immune response against melanoma. Some patients reported minor inflammatory responses at the

site of exosome injection (mild swelling, redness, and DTH responses) and low-grade fever were observed after exosome administration. The patients tolerated administration of exosomes for up to 21 months (29), but it is necessary to evaluate the exosome effects in a long-term treatment.

Jefferson *et al*., in the pilot immunotherapy trial for recurrent malignant gliomas, suggested the use of autolog exosomes isolated from tumor cells removed during surgical craniotomy. The isolated cells, treated with an investigational new drug (an antisense molecule), that target surface receptor protein, are re-implanted (encapsulated in a small diffusion chamber) in the abdomen of patient, within 24 hours after the surgery. The lack of the surface receptor led tumor cells, treated with the antisense molecules, to apoptosis. The authors supposed that released exosomes are full of tumor antigens that together with the antisense molecules could stimulate the immune system against tumor recurrence. The patient recruitment of this trial is currently ongoing. (NCT02507583) (www.clinicaltrial.gov).

One of the most important advantage in the use of a cell-free therapy is the possibility to inject EVs locally thus minimizing the side effects of cells administration. Moreover, EVs can be engineered and addressed to specific organs. The use of EVs in therapy is more attractive than the injection of the cells, in fact, injected cells may die or not fully home into the site of damaged tissue whereas EVs can be locally administered with a controlled dosage (30).

The authors in the review also discuss about the use of EVs as drug delivery vehicles in tissue repair and regeneration, taking advantage of their natural biocompatibility and cell targeting.

Recent studies suggest the production of engineering artificial lipid vesicles that incorporate EV components such as specific lipids or proteins in order to increase stability, targeting, and uptake. Engineered EVs with high levels of phosphatidylserine result in vesicles with a rigid membrane, resistant to lipolytic and proteolytic degradation in the circulation (31). The engineered structure of the EV membrane could enhance the stability of the vesicles *in vivo* and increase the probability to target the specific organ before to be cleared or degraded. The authors indicate that EVs may be regarded as a promising candidate for a novel cell-free therapy for tissue repair damage. Unfortunately, the data present in the literature does not suggest clear advantages of the EVs for the therapeutic applications. Several problems could be related to the use of EVs as immunomodulatory messengers in the context of tissue repair/regeneration. In the next future, the goal of the researchers should be to standardize the technique to manipulate the EVs for their use in therapy and demonstrate the long-term EVs effectiveness and safety for the treatment of human tissue damage.

Acknowledgements

This work was supported by Italian Association of Cancer (AIRC) to R Alessandro.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Cite this article as: Taverna S, Pucci M, Alessandro R. Extracellular vesicles: small bricks for tissue repair/regeneration. Ann Transl Med 2017;5(4):83. doi: 10.21037/atm.2017.01.53

