Role of stem-cell-derived microvesicles in the paracrine action of stem cells

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Abstract
The paracrine theory has recently changed the view of the biological action of stem cells and of the subsequent potential application of stem cells in regenerative medicine. Indeed, most of the beneficial effects of stem-cell-based therapy have been attributed to soluble factors released from stem cells. In this context, MVs (microvesicles) released as exosomes from the endosomal compartment, or as shedding vesicles from the cell surface, may play a relevant role in the intercellular communication between stem and injured cells. By transferring proteins, bioactive lipids, mRNA and microRNA, MVs act as vehicles of information that may lead to alteration of the phenotype of recipient cells. The exchange of information between stem cells and tissue-injured cells is reciprocal. The MV-mediated transfer of tissue-specific information from the injured cells to stem cells may reprogramme the latter to gain phenotypic and functional characteristics of the cell of origin. On the other hand, MVs released from stem cells may confer a stem-cell-like phenotype to injured cells, with the consequent activation of self-regenerative programmes. In fact, MVs released from stem cells retain several biological activities that are able to reproduce the beneficial effects of stem cells in a variety of experimental models.

Introduction
Experiments based on bone marrow transplantation in mice have suggested that bone-marrow-derived stem cells can contribute to the repair of several tissues [1]. In some studies, it is the haemopoietic stem cells rather than the MSCs (mesenchymal stem cells) that are implicated in the physiological repair [2]. However, the permanent engraftment of bone-marrow-derived stem cells and their differentiation into tissue-specific cells has been challenged by several studies [3–5]. In experiments of exogenous stem cell administration, despite a beneficial effect being observed in different pathological conditions, only a few of the injected stem cells undergo transdifferentiation or fusion [6], and certainly not in sufficient numbers to account for parenchyma reconstitution of injured organs [3–5,7]. Growing evidence indicates that many of the biological effects of stem cells can be attributed to paracrine mechanisms, related to the production of bioactive factors acting on neighbouring cells. This conclusion is drawn from the observation that stem-cell-derived bioproducts mimic the cell-regenerative effect. For example, in the heart, the extracardiac administration of MSCs favours cardiac repair through trophic mechanisms, independent of direct localization of stem cells in the myocardium [8]. In the kidney, Bi et al. [4] demonstrated that MSC-derived conditioned medium diminishes tubular cell apoptosis, increases tubular cell survival and limits renal injury in a model of cisplatin-induced acute kidney injury. Similarly, administration of MSC-conditioned medium induces significant survival in fulminant hepatic failure [9,10]. In general, experiments of administration of in vitro-expanded adult stem cells demonstrate a functional and morphological improvement in various pathological conditions with just a transient recruitment of stem cells at the site of injury. The immunomodulatory action of MSCs is also largely due to the secretion of soluble factors that can inhibit several T-lymphocyte functions and modify the cytokine production by DCs (dendritic cells), naïve and effector T-cells, and natural killer cells, leading to an anti-inflammatory phenotype [11]. In addition, MSCs can down-regulate immune responses by expanding regulatory T-cells. Several soluble factors produced by MSCs have been identified as potential immune modulators, including COX-2 (cyclo-oxygenase 2), prostaglandin E2, indoleamine 2,3-deoxygenase and transforming growth factor β1 [11].

The paracrine/endocrine hypothesis of stem cell action is thus based on these observations and suggests that factors produced and secreted by stem cells limit injury, modulate immune responses and information and promote self-repair from cells surviving injury [4,5]. In this context, MVs (microvesicles) released from stem cells may account for a reciprocal communication between stem and injured tissue cells as they may induce phenotypic changes in the recipient cells [12].
**MVs as paracrine mediators**

The release of extracellular vesicles is an evolutionarily well-conserved mechanism that cells exploit for exchange of bioactive proteins, lipids and nucleic acids. Vesicles released from cells are heterogeneous in origin and size, and include those derived from the endosomal membrane cell compartment, released by exocytosis after fusion of multivesicular bodies with the plasma membrane, as well as those formed by direct budding of the plasma membrane [13–15].

The first type of vesicles, known as exosomes, range from 30 to 120 nm in size, and their release is dependent on cytoskeleton activation, but not on Ca\(^{2+}\) influx. Exosomes can be distinguished from other vesicles by the expression of CD9, CD81 and CD63 tetraspanins, low amounts of phosphatidylserine, the presence of HSP (heat-shock protein) 60, HSP70 and HSP90, as well as expression of Alix (ALG-2 (apoptosis-linked gene 2)-interacting protein X), clathrin and annexin. Vesicles generated by direct budding of the plasma membrane, also known as shedding vesicles, are more heterogeneous in size and include vesicles ranging from 100 nm to 1 \(\mu\)m in size. Budding of small cytoplasmic protrusions that precedes vesicle shedding from the cell surface is a phenomenon dependent on cytoskeleton activation and an increase of intracellular calcium concentration that alters the balance of several transmembrane enzymes such as calpain, flippase, floppase, scramblase and gelsolin [16].

Consequent changes in the symmetry of phospholipid distribution within the plasma membrane lead to the formation of membrane nanodomains enriched in molecules commonly present in lipid rafts such as cholesterol, phosphatidylserine and flotillin-1 [17,18]. Ca\(^{2+}\)-dependent proteolysis is then responsible for the release of these nanodomains from the cell surface [18].

Both types of extracellular vesicle contain surface receptors and cytoplasmic components that are characteristic of the cell of origin. These include several biologically active molecules such as proteins and lipids, as well as nucleic acids [19–22]. The surface molecules of MVs facilitate the interaction with neighbouring cells bearing specific receptors. This may lead to direct cell stimulation, as well as transfer of receptors and biologically active molecules [23].

The exact physiological role of MVs, however, needs further clarification, but it is emerging that transfer of transcriptional regulators from one cell to another may induce epigenetic and functional changes in the recipient cells, revealing an unpredicted high plasticity of the cellular system. Functional modulation of recipient cells may derive from the delivery of intracellular proteins, as in the case of growth factors (vascular endothelial growth factor, basic fibroblast growth factor, platelet-derived growth factor, leptin, acidic fibroblast growth factor, tumour necrosis factor \(\alpha\), transforming growth factor \(\beta\) and others) [24], proteases [e.g. MMP (matrix metalloproteinase) 9, MMP2 and MT1-MMP (membrane-type 1MMP)] [25], and their activator EMMPRIN (extracellular MMP inducer) [26] that can stimulate angiogenesis. In addition, the exchange of genetic information may radically modify the behaviour of the recipient cell.

Ratajczak et al. [19] were the first to demonstrate that stem-cell-derived MVs may reprogramme neighbouring cells, in their study on murine embryonic stem-cell-derived MVs. These MVs were able to reprogramme haemopoietic progenitors by delivering both proteins and mRNA coding for several pluripotent transcription factors. Once transferred to the cells, mRNA was translated into proteins. Since RNase treatment inhibited the biological effects of MVs, it was inferred that the delivery of mRNA was critical [19]. In addition, we found that MVs derived from human EPCs (endothelial progenitor cells) activate an angiogenic programme in quiescent endothelial cells by transferring selected functional pro-angiogenic mRNA to them [20]. The transferred mRNA is then translated into the corresponding proteins; RNase pre-treatment of MVs reduced their pro-angiogenic effect, suggesting further a crucial role for RNA transfer [20]. Moreover, EPC-derived MVs induced changes in the recipient endothelial cells by triggering transcription of critical components of pro-angiogenic pathways. More recently, we found that MVs released from EPCs may deliver miR-126 and miR-296 pro-angiogenic miRNA (microRNA) that may explain the activation of pro-angiogenic pathways in the recipient cells [27]. These studies suggest that stem cells may exert their biological action by conveying genetic information and altering the gene expression of target cells by means of MVs.

Indeed, MVs derived from embryonic stem cells contain a considerable amount of mRNA that can then be transferred to mouse embryonic fibroblasts in vitro [21]. Since only certain miRNAs are efficiently transferred, the involvement of mechanisms of miRNA compartmentalization and delivery has been suggested.

We found that MVs produced by human MSCs and human liver stem cells contain selected patterns of miRNAs associated with ribonucleoproteins known to be involved in the intracellular trafficking of RNA, such as T-cell internal antigen-1, T-cell internal antigen-1-related and AU-rich-element-binding protein, which are multifunctional proteins expressed in nuclei and stress granules, along with proteins involved in the transport and stability of mRNA such as Staufen1, Staufen2 and Argonaute2, a protein of the Argonaute family involved in miRNA transport and processing. These observations suggest a dynamic regulation and compartmentalization of RNA in stem-cell-derived MVs [22].

The RNA species present in MVs are specific to the cell of origin. This is of particular relevance for stem-cell-derived MVs. In fact, mRNA, and in particular miRNA shuttled by MSC-derived MVs are linked to the mesenchymal phenotype of these cells, and are involved in the control of transcription, cell survival, multi-organ development, differentiation and immune system regulation [22,28]. In addition, MVs derived from embryonic stem cells contain mRNA for Oct-4, Rex-1, Nanog, SCL (stem cell leukaemia) and GATA-2 transcription factors, able to enhance haemopoietic progenitor cell pluripotency [19].

Quesenberry et al. [29,30] challenged the hierarchical vision of the stem cell niche requiring asymmetric division
of stem cells to ensure self-renewal and generation of a differentiated progeny [31], instead proposing a “continuum model” of stem cell biology. According to this model, the phenotype of stem cells is reversibly changing during the cell cycle, pending a terminal-differentiating stimulus provided by environmental factors. The stem cell phenotype is therefore continually adjusting to individual conditions [32]. In this context, MVs may play a critical role in modulating the plasticity of stem cells, accounting for exchange of genetic information in a defined microenvironment. The bidirectional exchange of genetic information between stem- and tissue-resident cells may explain both the functional and phenotypic changes occurring in stem cells and the activation of regenerative reprogramming in tissue-injured cells.

**MVs from injured tissue may modulate the plasticity of bone marrow stem cells**

Whether bone marrow cells can generate non-marrow cells in injured tissues remains a matter of debate [1,2,33,34]. Transdifferentiation and fusion are debated as mechanisms for marrow plasticity in tissue repair [35–37].

Dooner et al. [38] demonstrated that bone marrow cells that were co-cultured with injured lung cells express genes for lung-specific proteins such as surfactant B and C and Clara cell-specific protein. This was interpreted as being due to the transfer of lung-specific mRNAs to bone marrow cells via MVs released from the injured lung cells. However, the long-term persistence of a lung-like phenotype in marrow cells and in cross-species experiments, along with the observation that rat MVs induced expression of mouse surfactant mRNA in mouse marrow cells, also suggest transfer of a transcriptional activator to bone marrow cells [39]. Therefore MVs released from injured cells may deliver specific signals to stem cells that are able to trigger their differentiation, and may represent a mechanism involved in physiological tissue repair [32] (Figure 1).

On the other hand, stem-cell-derived MVs may incite changes in the phenotype of tissue cells that regulate regeneration and cell differentiation.

**MVs from stem cells may reprogramme injured cells**

Tissue-resident cells rather than marrow-derived cells seem to provide a major contribution to the repair of many organs, including liver [40], heart [41] and kidney [42]. In the kidney, for instance, tubule repopulation following acute kidney injury originates from tubular cells that survived injury that acquire a mesenchymal phenotype, proliferate, migrate to substitute lost cells and finally differentiate again into mature epithelial cells [3]. This phenomenon, named ‘epithelial–mesenchymal–epithelial cycling’, is enhanced by the administration of MSCs [42–45] as well as by MSC-conditioned medium [4]. MVs released from stem cells are possible modulators of tissue repair by reprogramming injured cells (Figure 1). Indeed, MVs released from human MSCs induce dedifferentiation to a stem-cell-like phenotype of renal tubular epithelial cells with subsequent activation of regenerative programmes, leading to proliferation and apoptosis resistance [28]. When injected *in vivo* in murine models of acute kidney injury induced by glycerol or ischaemia/reperfusion [27], MVs accumulate at the site of tissue injury and become incorporated into endothelial and tubular cells. Once incorporated, MVs act by limiting cell injury and inducing tubular epithelial cell proliferation, thus accelerating kidney recovery. In a lethal model of cisplatin-induced acute kidney injury, human MSC-derived MVs also enhanced survival [46]. Transfer of human mRNA via MVs, and its translation into protein has been observed both *in vitro* and *in vivo* in diverse experimental models. RNA inactivation in MVs resulted in significant loss of biological activities, suggesting that transfer of nucleic acids has a relevant role. However, MVs must also transfer regulators of transcription as they induce changes in gene expression of the recipient cells, such as up-regulation of *BCL2L1* (Bcl-xL), *BCL2* and *BIRC8* (baculoviral inhibitor of apoptosis repeat-containing 8) anti-apoptotic genes, and down-regulation of *CASP1*.
(caspase 1), CASP8 (caspase 8) and LTA (lymphotoxin α) genes involved in the execution phase of cell apoptosis [46]. Moreover, the miRNAs present in MVs are candidates for post-transcriptional regulation and induction of epigenetic changes in the recipient cells. We found that miRNAs shuttled by EPC-derived MVs are the main effectors of the proangiogenic and renoprotective activities of MVs [27]. In fact, the non-specific as well as the specific depletion of miRNAs in MVs obtained from Dicer- or miR-126- and miR-296-knockdown EPCs abate their biological effects [27]. EPC-derived MVs were also shown to protect the kidney from acute ischemic injury by reprogramming hypoxic resident renal cells, and, in a model of hindlimb ischemia, improve neovascularization and favour muscle regeneration [47]. Moreover, they promote angiogenesis in a model of pancreatic islet transplantation by an miRNA-dependent mechanism [48].

The activation of regenerative programmes has been also observed in hepatocytes after treatment with MVs released by adult human liver stem cells in a model of 70% hepatectomy in rats [49]. Reduction of infant size in pig and mouse models of ischaemia/reperfusion injury has been also observed after treatment with MSC-derived exosomes [50].

**Conclusions**

By delivering their biologically active contents, MVs may induce epigenetic changes in recipient cells that modify their behaviour. In particular, the horizontal transfer of genetic information between stem cells and tissue-injured cells may provide an explanation for the paracrine hypothesis of stem cell action. On the basis of the ability of MVs to mimic cell action. On the basis of the ability of MVs to mimic

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**References**


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