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Potential Immune Modulatory Action of Mesenchymal Stem Cell-Derived Extracellular Vesicles in Type 1 Diabetes

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Abstract

Several preclinical studies have shown potential immune-modulatory properties of mesenchymal stem cells (MSC) in type 1 diabetes leading to phase I/II clinical trials. Immune-modulatory properties of MSC have been mainly ascribed to their secretome. The extracellular vesicles (EV) have emerged as paracrine mediators of MSC actions. In fact, MSC-derived EV have been shown to carry proteins and nucleic acids capable to mimic the effect of originating cells. In the present short review we discuss evidences for contribution of EV to the immune-modulatory properties of MSC and mechanisms involved. In particular, EV were shown to inhibit T cell response to the glutamic acid decarboxylase (GAD) islet auto-antigen by inducing a shift of lymphocytes from Th1 to Th2 phenotype.

Keywords

Type 1 diabetes, Immunotherapy, Pancreatic β cells, T lymphocytes

Abbreviations

ALIX: Apoptosis-Linked Gene 2-interacting Protein x, DC: Dendritic Cell, ESCRT: Endosomal Sorting Complex Required For Transport, EV: Extracellular Vesicles, HGF: Hepatocyte Growth Factor, IDO: Indoleamine 2,3-Dioxygenase, MSC: Mesenchymal Stem Cell, PD1: Programmed Cell Death Protein 1, PD-L1: Programmed Cell Death 1 Ligand 1, TGF: Transforming Growth Factor, Th17: T Helper 17, Treg: Regulatory T Cell

Introduction

Type 1 diabetes and immune intervention therapies

Type 1 diabetes is an autoimmune disease characterized by chronic damage of pancreatic β cells, leading to lifelong insulin replacement therapy and to risk for development of chronic diabetic complications. The aetiology is multifactorial, but the role for susceptibility genes, environmental factors and immune system, remains to be elucidated [1].

Although disease incidence differs importantly between different countries and populations, incidence is increasing worldwide [2,3]. Several circulating auto-antibodies against pancreatic islet antigens, such as insulin, proinsulin GAD65, and islet antigen-2 and zinc transporter 8 can frequently be detected before clinical onset of the disease and the level of circulating autoantibodies is predictive for the disease development in subjects with increased genetic risk. However, the timing of diagnosis remains a challenge, and familiar aggregation accounts for approximately only 10% of cases [1-3].

It is widely assumed that T lymphocytes play the major role in the destruction of islet β cells. Several abnormalities of the function and phenotype of T cells in subjects with or at risk for type 1 diabetes have been detected. These include imbalances between type 1 pro-inflammatory T cells and type 2 regulatory T cells, abnormal distribution of cellular subsets and functional deficiency of naturally arising regulatory T cell populations that limit autoimmune response [4]. The emerging evidence is that CD8 T cells, the main lymphocyte population infiltrating islets, have a key role in β cell death [5].

Thymic and peripheral immune tolerance failure involves also dendritic cells (DC). Activated DC are required for priming naive T cells. It is likely that DC presentation of islet auto-antigens occurs during the development of islet auto-reactivity in type 1 diabetes and, thereafter DC can play a role in all stages of the disease [5].

The ability to detect islet auto-reactive T cells *ex vivo* and their effector function are key goals in type 1 diabetes research. This may allow to decipher the disease process, to provide markers for detection of at risk patients and to develop intervention strategies to preserve insulin-producing cells. Over the past 3 decades in fact, a variety of immunotherapy approaches for prevention or reversal of diabetes have been proposed, showing that in some cases immune intervention can attenuate and temporarily halt autoimmune diabetes [4]. Intervention strategies include auto-antigen-specific and non-specific approaches, such as immunosuppression, lymphocyte targeting, costimulation inhibitors, regulatory T cell enhancement and anti-inflammatory cell therapies. Lessons learned from phase II and III clinical trials indicate that it is critical for immune-modulatory therapies to induce β cell antigen tolerance, without causing long term immunosuppression, and combination immunotherapies are advocated [4].

Mesenchymal Stem Cells for Treatment of Type 1 Diabetes and of Diabetic Chronic Complications

Mesenchymal stem cells (MSC) represent an attractive therapeutic

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Table 1: EV- exosomes and microvesicles/ectosomes.

EV Source	Exosomes	Microvesicles
Size	30-120 nm	50-1000 nm (usually larger than 100 nm)
Origin	endosomal compartment of the cells after intraluminal budding of multivesicular bodies and subsequent fusion with cell membrane	direct budding from the plasma membrane and release as shedding vesicles
Markers	ESCRT components, Tetraspanins, TSG101, flotillin, ALIX, CD63, CD 81, CD9	Integrins, selectins, CD40 ligand, ARF6, VCAMP3
Content	Membrane protein and receptor that is reflective of the cell type of origin, mRNA, miRNA, non-coding RNAs, lipids and metabolites.	cytoplasmic proteins and membrane proteins, including receptors mRNA, miRNA, non-coding RNAs,

opportunity, satisfying several requirements to curb the autoimmune destruction of insulin-producing cells. MSC are multipotent, undifferentiated cells of mesodermal origin, characterized by their ability to differentiate into different mesenchymal cell lineages. Several lines of research have now established that MSC can exert an immune modulatory effect towards T cell functions [6]. MSC are endowed of poor immunogenic properties, since they express low levels of MHC class I molecules and lack of MHC class II molecules. Moreover, MSC do not express costimulatory molecules such as CD40, CD40 ligand, B7-1 and B7-2. The interaction between MSC and lymphocytes results in the suppression of lymphocyte activation markers [7]. The immune modulatory effects have been related to the interaction of MSC with several cell populations including dendritic cells, NK cells and activated T cells, thus resulting in inhibition of proliferation of T cells and induction of a regulatory T phenotype [8]. MSC-mediated immune suppressive effect has been attributed to several soluble factors such as prostaglandin E_2 (PGE_2), transforming growth factor-β1 (TGF-β1) and hepatocyte growth factor (HGF) [8]. MSC can promote a dendritic cell type 2 (DC2) anti-inflammatory signalling by the induction of mature DC2 and secretion of IL-10. MSC have been successfully used in the acute graft-versus-host disease (aGVHD), providing an effective alternative treatment for patients with steroid-resistant aGVHD [9].

Ongoing phase I clinical trials using *ex vivo* expanded MSC have shown to improve the outcome of allogenic transplantation of aGVHD [3,4]. It has been also suggested that MSC may be employed in autoimmune diseases. In the murine model of multiple sclerosis and in experimental autoimmune encephalomyelitis, MSC were shown to home lymphoid organs. Mice treated with MSC developed a milder disease compared with controls. MSC clustering around T cells was associated with a significant reduction of disease activity and of relapses. In the encephalomyelitis model MSC interfered with the pathogenic autoimmune response, showing a decreased production of interferon-gamma and tumor necrosis factor-alpha, without trans-differentiating into neurons [10]. A pilot clinical study on 15 patients with persistently active systemic lupus erythematous demonstrated the efficacy of human allogenic MSC transplantation, since after 1 year of follow up, a decreased disease activity was observed in 11 patients out of 13 [11].

In the context of diabetes research, a regenerative potential of MSC has been shown in NOD/SCID mice with diabetes [12]. The intra-cardiac infusion of human MSC in mice promoted an expansion of pancreatic β cells producing mouse insulin and favoured the recovery of diabetic glomerular injury. It has been suggested that MSC may act through paracrine factors, by promoting neovascularization and scavenging cytotoxic molecules [12]. Amelioration of streptozotocin-induced diabetes was shown after injection of genetically modified MSC with recombinant *PDX-1*. An increase of insulin and a reduction of blood glucose levels were observed in MSC-treated mice [13]. Allogenic MSC obtained from mice resistant to diabetes were injected into NOD mice, showing the reversal of hyperglycaemia and the deferral of diabetes onset [14]. The mechanism of action has been related to MSC homing to pancreatic lymphonodes with autoreactive T cells suppression [14].

A shift towards an anti-inflammatory profile and a tolerogenic signalling of T cells may be induced by MSC treatment [14], possibly as consequence of negative costimulatory PD1/PD-L1 pathway and regulatory T cell activation and of TGF-β and IL-10 production [15]. Moreover, MSC seem to have a role in conversion of fully differentiated pro-inflammatory Th17 in T cells with immunosuppressive activity [6].

Human allogenic bone marrow-derived MSC were shown to inhibit the *in vitro* activation of Th1 lymphocytes of patients with new-onset type 1 diabetes in response to the glutamic acid decarboxylase (GAD) islet antigen. MSC reduced the production of IFN-γ and stimulated the secretion of anti-inflammatory IL-10 and IL-4 cytokines [16]. This is suggestive of a T cell switch to a Th2 anti-inflammatory phenotype.

A report from the first cellular intervention clinical trial using MSC treatment in newly diagnosed type 1 diabetes showed no side effects and suggests that MSC treatment could represent a future application to block disease progression and to preserve β-cell function [17].

Taken together these results suggest that the beneficial effect of MSC is mainly related to their immune-modulatory and anti-inflammatory properties. The mechanism of action has been attributed to the secretome of MSC rather than to their trans-differentiation. Among the paracrine mediators, extracellular vesicles (EV) emerged as potential effectors of MSC immune-modulatory and regenerative activities [18].

MSC-derived EV biogenesis

Back in 1981 Trams et al. [19] described for the first time the presence of EV; however, at that time their functions were largely unknown and for a long time they were considered cellular waste [19].

The pivotal studies of Raposo et al. [20] showed that EV released from B lymphocytes may induce an MHC restricted T cell activation suggesting that vesicles may vehicle the MHC-2 peptide complex [20]. Since then several studies supported from technological advances helped to uncover the EV biogenesis and functions [21,22]. In 2014 the International Society for Extracellular Vesicles provided a minimal set of biochemical, biophysical and functional standards that should be used to attribute any specific biological cargo or function to EV [23]. Based on their biogenesis EV can be classified into two main categories: exosomes and microvesicles/ectosomes [22,24,25]. Exosomes derive from the endosomal compartment whereas microvesicles arise from budding of plasma membrane. The content of both EV types reflects that of the cell of origin and may contain receptors, cytoplasmic and surface proteins, proteins interacting with lipid rafts and nucleic acids (Table 1).

The inward budding of the multivesicular body (MVB) membrane gives formation of exosomes a homogeneous population of vesicles [22,24,25]. The cargo sorting of exosomes is mediated by the endosomal sorting complex required for transport (ESCRT) [26]. Programmed cell death 6 (also known as ALIX) in association with syntenin and tumour susceptibility gene 101 protein (TSG101) play a key role in exosome sorting [27]. Other cells require the neutral sphingomyelinase and lipid ceramide for the exosome production. The MVB fusion with cell plasma membrane is required for the exosome release from cells. In some cells this process involves small GTPases such as RAB11, RAB31 and RAB27A [28]. The fusion process to plasma membrane of MVBs is favoured by SNAP receptors (SNAREs), which belong to a protein superfamily involved in intracellular trafficking of vesicles [29].

Proteomic analysis provided evidence that exosomes released from different cell types have unique shared proteins and cell-type-specific proteins. Specific exosome markers include the tetraspanin family members, CD63, CD9, CD81, and other molecules such as CD82, Tsg101, and ALIX [30].

It is difficult to discriminate between exosomes and small microvesicles because of the overlapping characteristics. However, microvesicles represent a more heterogeneous population of EV formed through outward budding of plasma membrane consequent to a dynamic interplay between cytoskeletal proteins and phospholipid redistribution [22,30]. The translocation from the inner to the outer side of membrane leaflet of phosphatidylserine induces the membrane budding/vesicle formation through the activity of aminophospholipid translocases. In order to induce the microvesicle budding, ADP-ribosylation factor 6 (ARF6) initiates a signalling cascade that begins after the activation of phospholipase D (PLD), which allows recruitment to the plasma membrane of the extracellular signal-regulated kinase (ERK). ERK induces the activation of myosin light-chain kinase (MLCK), which finally triggers microvesicles release [25].

Pleiotropic Functions of EV

EV can be considered as paracrine mediators in cell-to-cell communication [21]. By transferring their content EV may deliver from the originator cells to the recipient cells membrane receptors, cytoplasmic proteins and nucleic acids inducing a functional reprogramming and phenotypic changes [31-33]. Several studies have shown that EV may modify the behavior of recipient cells in many ways. The identification of EV pleiotropic functions has driven the interest towards their use in regenerative medicine and immune therapies [34].

In particular MSC-derived EV have been widely studied since it has been shown that they may mimic the regenerative potential of MSC [18]. This led to the idea to use EV instead of MSC in cell therapy. MSC-derived EV contain proteins involved in self-renewal and differentiation of MSC [35] and mRNAs and miRNAs implicated in many different cell activity including regulation of immune response [18]. They also contain mRNAs involved in the angiogenic and adipogenic pathways and miRNA regulating cellular transport, apoptosis and proteolysis [36].

Several studies demonstrated that MSC-derived EV are able to induce phenotypic changes in target cells. Bruno et al. [37] showed that EV from bone marrow MSC are able to induce proliferation and to reduce apoptosis through the transfer and the consequent translation of MSC specific mRNAs in a mouse model of acute kidney injury. Human tubular epithelial cells injured by cisplatin and then treated with EV up-regulated several genes with anti-apoptotic activity (i.e. Bcl-xL, Bcl2, and BIRC8) and downregulated genes involved in apoptosis (i.e. Casp1, Casp8, and LTA) [37]. *In vivo* a single EV administration improved survival and kidney morphology and function in a mouse model of cisplatin-induced acute kidney injury. Whereas the single injection was not capable to prevent the onset of chronic damages, multiple injections exerted an anti-apoptotic effect leading to a prolonged mice survival and preservation of renal function and morphology [37]. Aliotta et al. [38] observed that MSC-derived EV were able to revert pulmonary hypertension induced by monocrotaline administration in mice [38]. Moreover, both murine and human EV were shown to revert toxic bone marrow damage caused by radiations [39]. Recent studies have also shown an immune-modulatory potential of EV derived from MSC.

MSC Derived Ev as Novel Immune-Modulator in Type 1 Diabetes

Depending on the cellular sources, EV have been shown to display different immune-modulatory functions as they may induce immune cell activation or inhibition [22].

In particular, vesicles secreted from B lymphocytes or antigen presenting cells were shown to activate T cells by direct presentation of the peptide-MHC complex, to activate dendritic cells (DCs), macrophages, natural killer (NK) cells and B cells [23]. Conversely, EV derived from tumor cells or from MSC were shown to exhibit inhibitory functions on T cells, NK cells and DCs [40,41]. Moreover, MSC derived EV promoted regulatory T cell activity and induced differentiation of monocytes into myeloid-derived suppressor cells (MDSCs).

In the setting of experimental autoimmune encephalomyelitis, MSC-derived EV inhibited autoreactive lymphocyte proliferation and induced tolerogenic signalling, via PD-L1, TGF-β, IL-10, and CD4+ CD25+ Foxp3+ Treg cells [41].

We recently found that EV derived from heterologous human bone marrow MSC mimic the immune-modulatory properties of the cells in type 1 diabetes by inducing a shift towards an anti-inflammatory and regulatory T cell profile [42].

After an integrin-mediated EV internalization in patient's PBMCs, a significant down-regulation of Th1 responses, detected as IFN-γ production, number of Th17 cells and levels of pro-inflammatory IL-17 was observed [42]. It is well established that Th17 effector cells participate in type 1 diabetes pathways paralleling Th1 cells, and their secreted signature cytokine IL-17 contributes to β cell death. T cells, upon interaction with EV, have shown to produce PGE2 and TGF-β, known to be involved in MSC effects, in an autocrine loop mediating and propagating the immunomodulation [43]. TGF-β was also conveyed as mRNA and as protein within and on the surface of EV [44]. TGF-β is known to mediate inhibition of lymphocyte proliferation and to promote Treg generation [18].

Functional miRNAs are also packaged into EV and can be transferred to target cells [31,32,33]. Among the EV-expressed miRNAs, miR-21, which is known to enhance TGF-β signalling was shown to be enriched in MSC-derived EV. We observed that RNA depletion of EV reduced the TGF-β transcript in PBMCs suggesting that the transfer of TGF-β mRNA or miRNA-21 enhances TGF-β production [42]. Hence EV may enhance the TGF-β activation pathway and TGF-β release, in a paracrine/autocrine manner in T lymphocytes. Furthermore, the study indicated that MSC-derived EVs may restore Th1/Th2 balance and preserve Treg cells in type 1 diabetes. In fact, EV increased the production of the regulatory cytokine IL-10, and induced higher frequencies of Foxp3+ Treg cells [42]. The production by PBMCs of IL-6 which is known to suppress maturation of inflammatory DC and mediate β cell repair [45] was also increased in the presence of EV. In preliminary experiments we found that MSC derived EV mimic also the effect of MSC on DC maturation impairing the antigen presentation. However the effect of EV depends on the cell of origin. At variance of EV derived from bone marrow MSC, those derived from islet of NOD mice stimulate immune response and contribute to islet immunopathology. In this experimental setting EV derived from MSC-like cells of diabetic mice may present β-cell antigens to DCs triggering islet injury [46]. In addition, the effect of EV may be detrimental depending on target cells and on metabolic states of the cell of origin. In fact, EV derived from MSC cultured in diabetic-like conditions induce features of diabetic retinopathy *in vitro* [47]. This effect is related to an impairment of retinal pericytes function. Taken together this observations may suggest that autologous EV obtained from diabetic patients may be ineffective or even detrimental.

Conclusions

In conclusion MSC-derived EV vicariate several immune-modulatory actions of MSC, and represent a potential non antigen-specific immune-regulatory approach to type 1 diabetes prevention therapy [4]. EV have some advantages in respect to the cells as they have minimal immunogenicity allowing an allogenic use and, being naturally occurring component of biological fluids, have a low inherent toxicity. Moreover, due to their small size they may easily diffuse across the biological barriers reaching target cells. EV, in respect to MSC may be more easily manipulated. Ongoing studies are focusing on engineering EV for specific targeting and for molecule delivery [48]. Several studies suggest that an immune-modulatory approach to restore tolerance to β cells may be achieved with combination of synergic immunotherapies, which include antigen-specific and non-specific approaches. EV released from MSC might represent an immune-modulator in MHC-mismatched recipients, overcoming the potential immunogenicity of MSC in an allogenic setting.

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