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Exosomes in the Pathogenesis, Diagnosis and Treatment of Pancreatic Diseases

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Abstract

Exosomes (EXOs) are small vesicles (30-200 nm) of endocytic origin, which are released by many different cell types into the extracellular space. They may play a key role in facilitating cell-cell communication, under both physiological and pathological conditions. EXOs contain a wide range of RNA molecules and proteins. Their specific molecular signatures make them promising candidates in early diagnosis and prognosis of pancreatic diseases. EXOs could also provide a new method to monitor treatment response in patients suffering from pancreatic cancer and other diseases of the pancreas. Additionally they may help to improve current treatments via personalized medicine approaches using them as therapeutic vehicles themselves.

Introduction

Exosomes (EXOs) are cell-specific, small double membrane extracellular microvesicles heterogeneous in size, ranging from 30 to 200 nm that are produced by different cell types[1]. Their classification has been based in their size, density, morphology (typical "cup-shaped" observed by electron microscopy) and the presence of common surface markers such as the CD63, CD81 and CD9 tetraspanins, fusion proteins (Flotilin, Annexins, GTPases), endosome-associated proteins (Alix,TSG101) and heat shock proteins (Hsc70, Hsp 90). So far they have been found in all biological fluids analyzed in different organisms and are released to media by most cells when cultured. The mechanism proposed for EXOs production is the fusion of multivesicular bodies with the plasma membrane in an exocytic manner. This route could serve as a directional packaging system of signaling molecules, in fact growing evidence indicate there is enrichment of certain RNA species such as some mRNAs, miRNAs and other ncRNAs with exclusion of rRNAs. Also, proteomic analysis suggests the presence of particular compositions related to tissue of origin and to physiological state. In this sense, the presence of certain surface markers has been linked to directed cell-cell communication processes and to the development of disease. Therefore, particular EXO cargos represent molecular signatures of pathological processes. In this review, we will focus on the current knowledge of EXOs production and cargo related to the pathogenesis of pancreatic diseases.

Exosomes in the pathogenesis of the pancreatic diseases

Pancreatic cancer

Pancreatic cancer is one of the most lethal human cancers, and the prognosis of patients is very poor. In addition pancreatic cancer has a non-specific clinical presentation, and therefore it is often diagnosed at an advanced stage. Early diagnostic tests and effective treatments that could improve patient survival are still required. In this sense, exosomes derived from pancreatic cells have the potential to serve as disease state sensors and might be also exploited as directional therapeutic carriers once specific surface markers to target pancreatic cells and curative treatment molecules are identified. In other types of cancer including ovarian and breast cancer, a role in cancer development, progression and drug resistance[2] has been linked to the presence

of EXOs. Paradoxically, pancreatic tumor-derived exosomes seem to have an anti-proliferative effect on tumor cells. For example human pancreatic cancer SOJ-6 cells secrete exosomes with mitochondria-dependent proapoptotic function by interacting with lipidic rafts leading to disorganization of the Notch-1 complex. This in turn activates PTEN and GSK3-β which inhibit the activity of the mitochondrial pyruvate dehydrogenase (PDH) increasing the pro-apoptotic factor Bax and decreasing the anti-apoptotic Bcl-2 function, thus activating the death machinery involving caspases and mitochondria after cell cycle arrest in G0G1 phase[3,4] . In addition human pancreatic derived exosomes initiate apoptosis in tumors through granzyme B release induced by activated NK cells. Furthermore, NK cytolytic activity was abrogated by Hsp70-specific antibody[5]. Similar studies with antigen-presenting cells (APCs) derived EXOs showed their capacity to prime naïve CD8+ T lymphocytes to eradicate tumors[6], indicating that the cell type or tissue of origin dictates the function of the produced EXOs upon the adaptive or the innate immune system.

Despite the antitumor effect observed by pancreatic tumor-derived EXOs it is still unclear whether the constant production of exosomes by tumor cells benefits or harms their own survival *in vivo* [7]. In fact cancer patients in an advanced state produce large amounts of EXOs arguing against an effective immunostimulatory or antitumor effects of these vesicles. In pancreatic tumor cells metastatic tumorigenesis has been reported to be facilitated by specific tetraspanins angiogenic signals [8,9]. Upregulation of rat D6.1A tetraspanin promotes tumor growth by its capacity to induce systemic angiogenesis that effectively and specifically induces endothelium sprouting. Similarly EXOs derived from rat pancreatic adenocarcinoma cells can efficiently induce angiogenesis in tumors and tumor free tissues through a mechanism dependent on the expression of tetraspanin8 (Tspan8). The uptake of Tspan8-CD49d complex-containing EXOs by endothelial cell (EC) was accompanied by enhanced ECs proliferation, migration, sprouting and maturation of EC progenitors. Unraveling exosome-initiated regulation of angiogenesis may provide ways to interfere with tumor growth. In addition to tumor growth, the capacity of tumor cell spreading to other tissues is a measure of tumor progression success. The metastasizing capacity of rat pancreatic adenocarcinoma cells has been linked to EXOs production through a mechanism dependent on both CD44 expression and the presence of a soluble matrix[10]. Tetraspanin interactions with platelets and leukocytes suggested EXOs provide tumor cells with a survival advantage in the hostile environment which they encounter during metastatic spread [11]. Thus, regulation of EXOs activity could serve as a modulatory strategy of tumor metastasis. The expression profile of miRNA and other ncRNAs has been associated not only the tissue of origin[12], but also to tumor type and the particular stage of progression. In particular, a subset of miRNAs has been shown to provide specific functions in the development of pancreatic cancer. The expression patterns of these miRNAs are useful in the diagnosis/prognosis of pancreatic cancer[13]. The study of EXO-contained miRNAs and other ncRNAs could be used as non-invasive biomarkers for the diagnosis, treatment and prognosis in pancreatic cancer patients. It has been observed that miR-17-5p (a poor prognosis marker) and miR-21 (a carcinogenic marker) may be useful serum exosome-based biomarkers for the screening of pancreatic cancer[14]. Also, both the epidermal growth factor receptor (EGFR) protein and DNA, essential for development of pancreatic cancer[15] has been found in exosomes from pancreatic cancer cells and serum of patients with pancreatic cancer[16,17]. The results suggest that exosomes are suitable markers for diagnosis and prognosis and also may be predictive biomarkers of pancreatic cancer.

Diabetes

Diabetes is the most common metabolic disease resulting from defects in glucose homeostasis associated to either insulin secretion, insulin action or both. Pancreatic beta cells play an important role in glucose homeostasis and their impairment is crucial in the development and progression of diabetes. Beta pancreatic cells release EXOs with a proteomic content that varies in relation to cytokine stimulation. Therefore, a role of EXOs in the development or the progression of diabetes can be envisaged as mediators of long range signaling within an individual[18]. An alternative mechanism of action attributed to EXOs in association to hyperglycemia and diabetes involves their production as a way to mediate direct translocation of the heat shock protein Hsp90 in endothelial cells required for endothelial nitric-oxide

synthase (eNOS) activity, resulting in changes of cell nitric oxide (NO) levels characteristic of the diabetic pathological state[19]. In addition to proteins, miRNAs and other ncRNA content in EXOs have been extensively associated to several human disorders including diabetes, as an additional mechanism for EXO mediated intercellular signaling[20].

Type I Diabetes

Type 1 diabetes mellitus (T1D) results from autoimmune destruction of the insulin-secreting cells in the pancreas. In this context, a role for cell-cell communication between cells of the immune system has been attributed to EXOs that could explain, in part, the development of T1D[21-23]. In particular Sheng et al[24] demonstrated that EXOs contain diabetes autoantigens such as glutamic acid decarboxylase 65 kDa (GAD65) that act as strong and innate stimuli, inducing inflammatory cytokine secretion through a MyD88-mediated TLR-signaling pathway and activate purified antigen presenting cells that results in T-cell proliferation. Therefore, they could serve as triggering factors for specific autoimmunity events leading to diabetes in susceptible individuals[25].

Type II Diabetes (T2D)

Type 2 diabetes (T2D), the most common type of diabetes in humans, is caused by the development of insulin resistance and/or relative insulin deficiency. EXOs have been implicated in the development of insulin resistance. As mediators of signaling cascades, EXOs released by adipose tissue of obese mice are enriched with the protein retinol-binding protein 4 (RBP4) that acts as a mediator of insulin resistance through mechanisms that involve macrophage activation and cytokine production[26]. Interestingly, transgenic mice overexpressing RBP4 or wild type C57BL/6 mice injected with purified RBP4 protein develop insulin resistance and RBP4 knockout mice exhibit enhanced insulin sensitivity. The concentration of RBP4 in serum is elevated in humans with insulin resistance, obesity, type 2 diabetes and impaired glucose tolerance[27,28]. It will be of interest to determine whether the high levels of RBP4 associate with EXOs in the serum of type 2 diabetes or impaired glucose tolerance subjects. Also associated with T2D are proteins particularly enriched in EXOs, like the pro-inflammatory secretory of lipopolysaccharide-activated macrophages cyclophilin A, which was found at higher levels in the plasma of patients with type 2 diabetes. The observation that monocytes from patients with T2D contain lower levels of this protein being their serum levels elevated could support the possibility that monocytes change their cyclophilin A content by a mechanism that involves the production and release of EXOs[29], similar to what has been observed for the redistribution of the Hsp90 protein in endothelial cells[30].

Gestational diabetes mellitus (GDM)

Gestational diabetes mellitus (GDM) is a type of diabetes of variable degree acquired during pregnancy and has been associated with alterations of placental anatomy and physiology. It affects 5% of all pregnancies and parallels the global increase in obesity and T2D worldwide[31]. Since GDM is a syndrome that leads to feto-placental vascular endothelial dysfunction involving higher nitric oxide (NO) concentrations and increases of oxidative state and vascular resistance, it will be of interest to determine whether endothelial cells in these patients release EXOs with an abnormal Hsp90 cargo similarly to the mechanism described for high glucose and diabetic endothelial cells under controlled culture conditions[32]. In addition, NO synthase activity is affected by miRNA-203 presence which has also been identified in EXOs[33]. On the other hand, EXOs appear to play an essential role in maternal-fetal communication preventing an excessive immune response and the developing of autoimmunity in human pregnancy[34]. Isolated placental EXOs have been shown to suppress T signaling components such as CD3-zeta andJAK3, while inducing SOCS-2[35-37]. Also, NKG2D ligand on placental exosomes induces reduction of the cognate receptor and thus impair the cytotoxicity of effector lymphocytes[38]. Furthermore, recent evidence provided by Frängsmyr et al[39] and Stenqvist et al[40] demonstrates that the mechanism linked to fetal tolerance involves secretion of EXOs containing bioactive Fas Ligand and Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) protein linking the immunomodulatory and protective role of human placenta to its EXOs-secreting ability. Interestingly, the number of EXOs present in maternal peripheral blood is significantly higher than that observed in non-pregnant women[41]. It will be required to better characterize yields, morphological features and particular molecular cargos of placental EXOs from women with normal and pathological pregnancies such as GDM to determine the specific mechanisms needing production of EXOs and/or signaling via these particular placental-derived vesicles.

Diabetic nephropathy

Diabetic nephropathy (DN) is a major complication of diabetes mellitus characterized by a progressive deterioration of renal function. The reduction of podocyte number is related to DN progression and it could be useful as early non-invasive marker for the disease. Wilm´s Tumor-1 (WT1) protein is a podocyte marker used to evaluate the podocyte lesion. Due to the low WT1 cellular levels, its presence in urine cannot be detected. However, WT1 has been detected in EXOs isolated and concentrated from urine. Similarly, soluble E-cadherin, whose up-regulation correlates with the disease state of DN, is detected in urine EXOs from DN patients, suggesting that kidney EXOs could participate in the pathogenic process by regulating signaling mechanisms[42-44]. In addition to proteins, renal-derived EXOs contain particular mRNAs and miRNAs that may control the expression profile of the target cells correlating with the disease state[45]. For example, urinary exosomal miRNA content has been found to be altered in type 1 diabetic patients with incipient DN. The abundance of different miRNAs is found to be increased (miRNA-130a, miRNA-145) or decreased (miRNA-155, miRNA-424), correlating with disease state. In particular, miRNA-145 has been proposed as a candidate regulator of DN by targeting the prosclerotic cytokine TGF-β1[46].

Diabetic retinopathy

Diabetic retinopathy is defined as the retinal damage caused by complications of diabetes. It is known that αB-crystallin proteins are released in EXOs from the apical surface retinal pigmented healthy epithelium (RPE)[47], accumulating in the interphotoreceptor matrix where they could protect neighboring cells[48]. In macular degeneration, the physical disturbance of the center of the retina called the macula has been linked to diabetes. In this pathological condition there is an abnormal growth of new blood vessels within or beneath the macula leading to irreversible blindness. This process is associated with up-regulation of the intrinsic stress response alpha-beta crystallin proteins in the retina and therefore EXOs production might be participating in the process. Interestingly, a correlation of angiogenic or anti-angiogenic effects has been associated with EXOs, depending on the layer of the retina where they were produced. For example, EXOs from retinal astroglyal cells (RACs) inhibit neovascularization in a laser-induced choroidal neovascularization animal model while EXOs from RPE do not[49].

Exosomes in diagnostics

EXOs and microvesicles have been identified as potential sources for clinical disease biomarkers. They are particularly useful for this application because EXOs can be isolated from different body fluids collected by non-invasive (urine, saliva, breast milk, etc.) or minimal-invasive (blood) methods[50]. In addition, EXOs provide advantages versus classical methods for the identification of biomarkers such as the quantification of soluble proteins or RNA molecules in plasma for several reasons: 1) they provide protease/nuclease controlled environment increasing molecule stability at the time, 2) allow for concentration of specific molecules of interest in easy to isolate particles, and 3) a subset can be isolated using a specific anti-cell surface marker antibody followed by analysis of specific cargo proteins/RNAs. In fact, the EXOcargo of proteins, mRNAs, miRNAs and other ncRNAs is determined by the state of the cell-type of origin and has been associated with some pathological condition with changes that correlate with specific stages of the disease. EXOs not only serve as carriers of biomarkers of the disease, but can serve as methods for monitoring prognosis and early diagnosis of a disease. Pancreatic ductal adenocarcinoma (PDAC) cells can be identified by the mis-localization of the cytoplasmic protein plectin to the plasmatic membrane of cancer cells. Very recently, this protein has been located to EXOs isolated from PDAC cells. Moreover, the production of EXOs required plectin expression that was linked to enhanced tumor growth in immunodeficient and in immunocompetent mice[51], suggesting that EXOs may be used as vehicles that evade endogenous defense systems of the individual leading to the spread of cancerous cells. Due to the important applications that robust biomarkers of the aggressive pancreatic cancer disease may have in the clinic, Lau et al[52] developed an EXOs-based disease-specific, validated salivary biomarker assay. The assay is based on a specific cancer transcriptome profile in the EXOs isolated from saliva detected only in animals that received orthopic injection of pancreatic cells and not in those receiving the same cells that had been engineered to block microvesicle release . Moreover, the fact that these tumor specific EXOs were detected in saliva allows for the possible detection of early onset of pancreatic cancer in a non-invasive manner.

In T2D, a disease associated with monocyte activation by achieving altered cyclophilin A content

through mechanisms that involve the production and release of EXOs, the release of the regulatory protein cyclophilin A in EXOs could be used as a screening biomarker for the disease[53]. Also, a signature pattern of microRNA expression has been shown to discriminate between Impaired Fasting Glucose (IFG) and T2D in blood samples of patients. Among the set of miRNAs found to be either up or down regulated in EXOs, the level of miR-144, which regulates expression of the insulin receptor substrate 1 (IRS1), correlated with the metabolic state of the cell[54]. This work exemplifies how serum-derived EXOs could be used for diagnostic/prognostic applications as well as treatment monitoring of patients with T2D. Placental miRNA specific profiles, such as miR-141, miR-149, miR-299-5p and miR-135b, have been detected in maternal plasma known to contain EXOs from various tissues and therefore could represent a source of biomarkers for disease detection and monitoring during pregnancy[55]. In regard to GDM, miR-29a, miR-222 and miR-132 have been proposed as biomarkers[56], however, as with the EXO-associated miRNA-203 targeting NO synthase, their possible role as biomarkers of GDM remain to be confirmed[57]. Currently, there is a need for specific and sensitive biomarkers of renal injury and disease stage of DN. Urinary EXOs protein composition from patients and normal donors have shown significant differences. Among the total amount of 254 different proteins analyzed, 25 were significantly altered in DN and validation studies confirmed that a panel of three of those proteins (AMBP, MLL3 and VDAC1) could constitute markers of the disease[58].

Exosomes in therapy

The relationship between the molecular cargo of EXOs, disease onset and/or progression of the disease makes them very valuable biomarkers in a clinical setting. It is clear that a deeper understanding of their specific content will help in the identification of the inherent molecular mechanisms leading to disease as well as for the development of more effective therapies. In addition, EXOs obtained from genetically modified cells designed to load particular molecular contents and/or directional targeting can serve as effective therapeutic vehicles. Alternatively, inhibition of formation and release of EXOs may be a novel strategy in the treatment of pancreatic cancer since it may inhibit tumor spread and/or growth. Islet transplantation is a viable option for the treatment for type I diabetes. EXOs derived from islet endothelial cells can activate angiogenesis that improves revascularization and beta pancreatic cell function[59]. EXOs derived from islet endothelial cells could be used for monitoring transplanted islets and for preventing ischemia-reperfusion injury in solid organs. Also, IL-4 gene delivery to beta cells has been shown to enhance β-cell proliferation and survival *in vivo* and reverse hyperglycemia in NOD mice with diabetes[60]. EXOs derived from immunosuppressive dendritic cells that express IL-4 are able to block progression of diabetes in NOD mice, and therefore EXOs from these cells could be used for the delivery of IL-4 to treat type I diabetes[61].

Similarly, EXOs derived from a specific type of cells, namely retinal astroglial cells (RACs) of normal mice, could be used for the treatment of macular degeneration due to their antiangiogenic potential. The results of Hajrasouliha et al[62] show a possible EXOs-based therapeutic approach in ocular delivery for diabetic retinopathy.

Conclusions

In summary, subsets of EXOs in body fluids represent an ideal source of cell or tissue type specific proteins and RNAs for the diagnosis, treatment and evaluation of the prognosis of pancreatic diseases. Further characterization of EXOs miRNA/protein profiles, their mechanisms of action and their relation with different stages of disease in the pancreas will undoubtedly provide the basis for a deeper understanding of the role of EXOs in pancreatic disease pathogenesis and their possible use for the benefit of patients suffering from pancreatic diseases. Finally, it is important to note that in order to establish EXO-based clinical approaches, a consistent set of practices for their isolation, characterization and manipulation must be adapted.

Conflict of Interest:

The Authors declare that they have no conflict of interests.

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