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Molecular and functional characterization of circulating extracellular vesicles from diabetic patients with and without retinopathy and healthy subjects

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ABSTRACT

Diabetic retinopathy is a sight-threatening complication of diabetes, characterized by loss of retinal pericytes and abnormal angiogenesis. We previously demonstrated that extracellular vesicles (EVs) derived from mesenchymal stem cells cultured in diabetic-like conditions are able to enter the pericytes, causing their detachment and migration, and stimulating angiogenesis *in vitro*. The purpose of this work was the molecular and functional characterization of EVs derived from diabetic subjects with or without diabetic retinopathy, compared with healthy controls. Characterization of EVs extracted from serum/plasma of diabetic patients with or without retinopathy, and healthy controls, was performed by FACS and microarray analysis of microRNA (miRNA) content. Relevant miRNA expression was validated through qRT-PCR. EV influence on pericyte detachment, angiogenesis and permeability of the blood-retinal barrier was also investigated. Diabetic subjects had a 2.5 fold higher EV concentration than controls, while expression of surface molecules was unchanged. Microarray analysis revealed 11 differentially expressed miRNAs. Three of them (miR-150-5p, miR-21-3p and miR-30b-5p) were confirmed by qRT-PCR. Plasma EVs from subjects with diabetic retinopathy induced pericyte detachment and pericyte/endothelial cell migration, increased the permeability of pericyte/endothelial cell bilayers and the formation of vessel-like structures, when compared with EVs from controls. In conclusion, circulating EVs show differences between diabetic patients and healthy subjects. EVs extracted from plasma of diabetic retinopathy patients are able to induce features of retinopathy in *in vitro* models of retinal microvasculature. Our data suggest a role for miR-150-5p, miR-21-3p and miR-30b-5p as potential biomarkers of the onset of diabetic retinopathy.

1. Introduction

The pathogenesis of diabetic microvascular complications is closely linked to vessel abnormalities, due to altered interactions between pericytes and endothelial cells (ECs) (Raza et al., 2010). ECs are primarily exposed to the complex signalling from the blood flow and can influence pericytes, while pericytes transmit to the endothelium signals from neighbouring tissues (Armulik et al., 2005). This is further complicated by the *angiogenesis paradox* in diabetes: while diabetic retinopathy eventually leads to increasing hypoxia which stimulates abnormal neovascularization in the retina, potentially useful angiogenesis is inhibited in other ischaemic organs, such as the heart and limbs (Costa and Soares, 2013).

Therapeutic use of autologous/donor material is considered a potential option for the treatment of patients with multifactorial diseases. Extracellular vesicles (EVs) are released by different cell types in the vascular environment. EV surface antigens are specific of the donor cells and can help identify their origin. EVs have a regenerative potential, since they contain lipids, proteins, RNA and microRNAs (miRNA), and shuttle information that regulate the functions of target cells (Camussi et al., 2010). On the other hand, important pathophysiologic mechanisms associated with endothelial dysfunction in vascular disease (diabetes, atherosclerosis and hypertension), could be orchestrated by circulating EVs, or EVs from surrounding tissues acting in a paracrine way. As EVs can modulate vascular permeability, tone and angiogenesis, they might contribute to vascular complications, in

Abbreviations: CTR, group of healthy controls; DR, group of diabetic patients with retinopathy; EC, endothelial cell; ECM, extracellular matrix; EV, extracellular vesicles; HMEC, human microvascular endothelial cells; HRP, human retinal pericytes; HIF-1α, hypoxia-inducible factor 1 α; KEGG, Kyoto Encyclopedia of Genes and Genomes; miRNA, miR, microRNA; MSC, mesenchymal stem cells; noDR, group of diabetic patients without retinopathy; PS, phosphatidyl-serine; VEGF, vascular endothelial growth factor; VEGFR1/2, vascular endothelial growth factor receptor 1/2

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particular diabetic retinopathy (Müller, 2012). EV secretion depends on the status of donor cells; therefore they could represent promising biomarkers in patients with metabolic diseases, such as type 2 diabetes (Müller, 2012). An increased concentration of circulating EVs has been reported in diabetic animals (Müller, 2012) and type 2 diabetic individuals (Koga et al., 2005; Feng et al., 2010; Helal et al., 2010).

MicroRNAs (miRNAs) are small non-coding sequences of 18–24 nucleotides, which interfere with stability and translation of target mRNAs by coupling with complementary sequences and thus exerting a negative regulatory effect (Fabbri et al., 2008). They can circulate freely in the blood flow or be embedded inside EVs, which can transfer them from cell to cell (Valadi et al., 2007). Circulating miRNAs are correlated with the disease states and are currently studied as putative biomarkers of cancer and chronic diseases, such as type 1 and 2 diabetes (Guay and Regazzi, 2013; Snowhite et al., 2017), and diabetic retinopathy (Mastropasqua et al., 2014).

We have recently demonstrated that EVs derived from the mesenchymal stem cells (MSCs) enter human retinal pericytes (HRPs), causing their detachment from substrate and migration. They also induce angiogenesis *in vitro* and increase blood-retinal barrier permeability. These effects are worsened by culturing MSCs in high glucose and hypoxia, conditions similar to those described in the diabetic microvasculature (Beltramo et al., 2014). Subsequently, we showed that the expression of miR-126, which plays a prominent role in angiogenesis (Mastropasqua et al., 2014) and is involved in diabetic retinopathy (Bai et al., 2011; Ye et al., 2013), is down-regulated in HRPs exposed to MSC-derived EVs obtained in hyperglycaemic/hypoxic conditions, leading to increased expression of angiogenic molecules, such as vascular endothelial growth factor (VEGF) and hypoxia-inducible factor 1 α (HIF-1α) (Mazzeo et al., 2015). Therefore, we concluded that diabetic-like conditions may influence vessel stability through EV paracrine signalling.

Our present hypothesis is that circulating EVs, affected by the diabetic condition, may influence small vessel homeostasis, and that identification of molecular differences in EVs from healthy controls and diabetic subjects, with and without microvascular complications, could represent a predictive option for diagnostic purposes. The objectives of this work are therefore the molecular and functional characterization of circulating EVs from serum and plasma of diabetic patients with or without retinopathy and healthy controls, and to investigate their role in the regulation of small vessel homeostasis and angiogenesis.

2. Materials and methods

2.1. Subjects

Seven type 1 diabetic subjects with proliferative diabetic retinopathy, but without other diabetic complications, systemic diseases limiting life expectancy (eg cancer, cirrhosis), or other autoimmune diseases, were included in the DR group. They were age- and gender-matched with 7 healthy controls (CTR group) and 7 diabetic subjects without retinopathy (noDR group) (Table 1). All diabetic patients were on multiple daily insulin injections and performed self-blood glucose monitoring, but took no other medication. Participants were asked to sign an informed consent. Ethical clearance for involvement of human subjects in research was obtained from the *Comitato Etico Interaziendale A.O.U. Città della Salute e della Scienza di Torino - A.O. Ordine Mauriziano - A.S.L. TO1*.

Overnight fasting venous blood samples were collected in tubes containing EDTA for plasma separation and clot activator for serum.

2.2. Cell cultures

Human retinal pericytes (HRPs) were stabilized in our laboratory, as previously described (Berrone et al., 2009). Human bone marrow MSCs, and human microvascular ECs (HMECs) were purchased from Lonza (Basel, Switzerland). HRPs and MSCs were maintained in DMEM +10% FCS, while HMECs in EBM-2 growth medium (Lonza) supplemented with angiogenic factors, according to the instructions. When subcultured for the experiments they were grown in DMEM +10% FCS. Reagents for cell cultures were purchased from Sigma-Aldrich (St Louis, MO, USA).

2.3. EV isolation and characterization

Collection of EVs from serum, plasma and MSCs was performed by centrifugation at 3000*g* for 30 min to remove debris, apoptotic bodies and platelets, followed by ultracentrifugation at 100,000 g for 3 h at 4 °C of the cell-free supernatants (ultracentrifuge: Optima L-100K, Beckman Coulter, Brea, CA, USA; rotor: 90 Ti, 90000 rpm, fixed angle, Beckman Coulter). EVs were either used immediately or stored at −80 °C in DMEM +5% dimethyl-sulfoxide. No differences in biological activity were observed between fresh and stored EVs. EV size, distribution and number were assessed using a NanoSight LM10 (NanoSight Ltd, Minton Park, UK), running the Nanoparticle Tracking Analysis 2.3 software. For all *in vitro* experiments we used an EV concentration similar to the one measured in peripheral blood of CTR group, according to our preliminary data (8-10 × 10^8 EV/ml), in order to rule out possible dose-dependent effects on microvascular cells.

2.4. Expression of surface molecules

EV expression of surface molecules was measured by FACS analysis using Guava easyCyte™ Flow Cytometer (Millipore, Burlington, MA, USA) with a panel of antibodies (Abcam, Cambridge, UK) against adhesion molecules (CD29, CD44, CD81), VEGF-receptor 1 and 2 (VEGFR-1 and VEGFR-2), marker proteins for: MSCs (CD73, CD29, CD90, CD105, CD44), exosomes (CD63, CD81), ECs (CD105, CD31), platelets (CD42), monocytes (CD14), lymphocytes (CD3, CD4, CD20, CD45, CD81). Staining with FITC-conjugated annexin-V was used as a marker for phosphatidyl-serine (PS).

2.5. Cell survival parameters

We evaluated the ability of EVs from the 3 groups to influence viability and proliferation of microvascular cells. HMECs and HRPs cultured in serum-deprived DMEM were exposed for 24 h to serum/plasma EVs from the different subjects and from MSCs, when appropriate. To evaluate HRP detachment, cells remained attached to wells after washing were trypsinized and counted by 2 individual operators in Bürker chambers after Trypan blue staining. Proliferation was measured as DNA synthesis (*Cell Proliferation ELISA BrdU* kit, Roche Diagnostics, Basel, Switzerland) and apoptosis as DNA fragmentation (*Cell Death Detection ELISA^{PLUS}* kit, Roche), according to the manufacturer's instructions.

2.6. Permeability

40,000 ECs/well were seeded on the inner surface of 0.45-μm pore-transwell inserts (Corning, New York, USA) and let adhere for 24 h. 40,000 HRPs were subsequently added into the same insert. After further 24 h, inserts were washed and moved to clean wells. 600 μl DMEM without red phenol and FCS were added in the lower chamber, while 200 μl of the same medium supplemented with serum/plasma EVs were added into the inserts. After 2 h, FITC-dextran (100 μg/ml final concentration) was added into the upper chamber, and fluorescence measured in the lower chamber after further 30', 1, 2, 3 and 4 hrs, through a Victor-3 Multilabel Plate Reader (Perkin Elmer, Waltham, MA, USA).

2.7. Cell migration

HRP and HMEC migration rate following 24hr exposure to plasma