Detection of Circulating Microparticles in Patients with Proliferative Diabetic Retinopathy

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Abstract: Background: The development of vasculopathies in diabetes involves multifactorial processes including pathological activation of vascular cells. Release of microparticles by activated cells has been reported in diseases associated with thrombotic risk, but few data are available in diabetes. Diabetic retinopathy is associated with increased local activation or apoptosis of retinal, neural, and vascular `endothelial cells in the eye which indicate that microparticles (MPs) of different cellular origin might be locally generated in the eye of diabetic patients. Aim: The aim of this study is to investigate the presence of endothelial, platelet, and retinal-derived microparticles both in the vitreous and in the plasma of diabetic patients compared with that of non diabetic ones. Subjects and methods: In a case-control study, this study included 45 patients: 25 diabetic patients with non proliferative diabetic retinopathy (NPDR), and 20 diabetic patients with proliferative diabetic retinopathy (PDR) compared with control group consists of non diabetic 10 subjects. Blood samples were analyzed by flow cytometry. Microparticles present in plasma and vitreous were analyzed according to their parameters of size and fluorescence. Results: As regard plasma samples, there was significant increase in CD144 and CD41 in groups II, III as compared with control group (p = 0.001). Peanut agglutinin PNA was not detected in plasma sample among all studied groups. Whereas, as regard to the vitreous sample, there was significant increase in CD144, CD41 and PNA in groups II,III as compared with control group (p= 0.048, 0.009, 0.048), (P = 0.001) respectively. Conclusion: microparticles appear as a new prognostic potential of type 2 diabetes in the early detection of vascular complications. Moreover significant increase of different types of microparticles in vitreous fluid of membrane in patients with PDR, may contribute to disease progression.

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1. Introduction

Long term micro- and macrovascular complications represent the main cause of morbidity and mortality in both type 1 and type 2 diabetes. Microangiopathy is a common feature of both types of diabetes, whereas macroangiopathy occurs more frequently in type 2 diabetes on account of the clustering of other traditional risk factors of atherosclerosis, i.e., hypertension, dyslipidemia, or obesity. The pathogenesis of diabetic vascular complications is complex and multifactorial. Early alterations of endothelial function may be involved in the development of both micro- or macroangiopathy in diabetic patients [(1)] .

Diabetic patients also show hypercoagulability and platelet hyperaggregability with increased levels of platelet activation-markers such as P-selectin, soluble CD40 ligand and microparticles (MPs) [(2)] .

It is well accepted that in response to activation or apoptosis, all eukariotic cells shed microparticles (MPs). These elements are produced from the plasma membrane after the flip-flop of the membrane phospholipids leading to a loss of membrane asymmetry. The blebs formed at the cell surface are then shed in the circulation under the form of vesicles ranging in size from 0.1 to 1 m. Increased levels of microparticles, mainly derived from platelets and to a lesser extent from leukocytes and endothelial cells [(3)] .

Microparticles have been identified not only in human plasma but also in other tissues with high cellular activation, inflammation, or apoptosis, such as human atherosclerotic plaques or synovial fluid in rheumatoid arthritis [(4,5)].

Endothelial microparticles (EMPs) are an emerging marker of endothelial cell (EC) dysfunction, and their circulating numbers are elevated in a number of pathologic states including cardiovascular disease [(6)]. Since vascular-endothelial cadherin is exclusively expressed by endothelial cells, CD144-positive microparticles may be regarded as endothelium-derived microparticles (EMPs), directly reflecting endothelial damage. However, it is unknown whether circulating EMPs are cause or consequence of CVD, and whether their occurrence associates with CVD per se or, rather,

with diabetes-related metabolic abnormalities [2] .

Platelet-derived MPs contain surface receptors for both factor VIII [7], and FVa, which combines with FXa to form the prothrombinase complex [8]. High- and low-affinity binding sites for activated FIX are also present on platelet-derived MPs [9]. These findings suggest that platelet-derived MPs can exert procoagulant distant effects from the site of platelet activation, and for a period longer than that of activated platelets. In addition, **Sinauridze *et al.*,** reported that platelet-derived MPs have 50- to 100-fold higher specific procoagulant activity than activated platelets [10].

Despite current treatments and existing knowledge, diabetic retinopathy remains to be a major cause of blindness in patients. New evidence indicates that diabetic retinopathy may be an inflammatory disease. The retinal vasculature of diabetic humans contains increased numbers of leukocytes, a finding that coincides with the increased expression of ICAM-1 in retinal vasculature. The increased density of leukocytes in the retinal vasculature results in injury to the endothelium via a FasL-mediated mechanism; a process that leads to breakdown of the blood-retinal barrier . Blood-retinal barrier breakdown develops early in the course of diabetic retinopathy in humans and, as a long term lesion is the major pathology leading to macular edema and the risk of subsequent visual loss [11] .

Diabetic retinopathy is associated with increased local activation or apoptosis of retinal, neural, and vascular endothelial cells in the eye . These finding indicate that microparticles of different cellular origin might be locally generated in the eye of diabetic patients. [12]

The aim of this study is to investigate the presence of endothelial, platelet, and retinal-derived microparticles both in the vitreous and in the plasma of diabetic patients compared with that of non diabetic ones.

2. Patients and Methods

This study was performed on 45 patients (age range 36–69 years, mean 57) who underwent vitrectomy at Tanta University Hospital. All patients signed a written informed consent. Prior to surgery, diabetic retinopathy was evaluated according to the simplified international diabetic retinopathy classification [13], made on the basis of clinical data, intraoperative assessment by the surgeon, and review of fundus and fluorescein angiography.

The patients of the study were divided into three groups:

Group I: control group consists of non diabetic 10 subjects.

Group II: 25 diabetic patients with non proliferative diabetic retinopathy (NPDR).

Group III: 20 diabetic patients with proliferative diabetic retinopathy (PDR).

Venous blood samples (10 ml) were collected on EDTA tubes before vitrectomy and platelet-free plasma (PFP) from 55 subjects was immediately prepared by successive centrifugations according to the methodology of **Amabile *et al.*,** [14]

Undiluted vitreous fluid samples (300–400 l) were collected from patients' eyes at the start of a standard three-port pars plana vitrectomy for the treatment of retinal diseases. A core vitrectomy was performed using the vitreous cutter (Accurus 800, Fortworth, Texas). Vitreous samples were collected at the beginning of vitrectomy before opening the balanced salt solution infusion line to maintain intraocular pressure. The tube vacuum connection was then disconnected and the vitreous was aspirated using sterile syringe. The vitreous sample is then homogenized by gently pipetting the suspension up and down several times. Samples were collected from the eyes of the patients with diabetic retinopathy.The control group consisted of vitreous samples from 10 eyes of 10 non diabetic patients with an idiopathic macular hole, an idiopathic epiretinal membrane, a rhegmatogenous retinal detachment, or age-related macular degeneration. Vitreous microparticles were isolated from fresh vitreous drawn at beginning of the surgery. Vitreous was separated from cells and platelets after two centrifugations (500*g* for 15 min and 13,500*g* for 5 min).

Reagents

Fluorescein isothyocyanate (FITC)-conjugated monoclonal antibody (mAb) against vascular endothelial (VE)-Cadherin (CD144) and phycoerythrine (PE)-conjugated mAb against platelet glycoprotein GPIIbIIIa (PE-CD41, clone P2) were used to identify endothelial microparticle (EMP) and platelet microparticle (PMP), respectively. Lectins from arachis hypogaea peanut agglutinin (PNA) conjugated with FITC were from Sigma Aldrich, France PE- and FITC-conjugated isotype controls (PEIgG2, FITC- IgG1) were used to define the background noise of the labeling. Microparticle absolute values were determined using Flowcount beads (Beckman Coulter, Margency, France).

Microparticle quantitation

Numeration of platelet- and endothelial-derived microparticles was performed, as previously described [15], using anti-CD41 and anti-CD144 labeling, respectively. After thawing, 30 l of plasma was incubated with either FITC-CD41 or

with PE-CD144 PE and their corresponding isotype control. FITC-conjugated lectins were diluted in PBS to reach the final concentration of 100 g/ml. Human vitreous (60 l) was incubated with lectin from PNA FITC (20 g. Its respective control was preincubated with D-galactose (80 mmol/l for 30 min) (Sigma-Aldrich). Then, 30 l of Flowcount beads was added to each sample for calculation of microparticle absolute value. Microparticles were gated as events with a 0.1-to 1.0-m diameter identified in forward-scatter and sidescatter intensity dot-plot representation using standards synthetic beads of 1 m in diameter (Polyscience).

Flow cytometric analysis.

After labeling, samples were analyzed by Facs caliber flow cytometry. Microparticles present in plasma and vitreous were analyzed according to their parameters of size and fluorescence.

Statistical analysis:

Statistical presentation and analysis of the present study was conducted, using the mean, standard deviation, t. test and Linear Correlation Coefficient by SPSS V.16.[16].

3. Results

This study was performed on 45 patients with type 2 diabetes mellitus, 25 patients with NPDR as group II and 20 patients with PDR as group III and compared with 10 non diabetic subjects (group I).

As regard plasma samples, there was significant increase in CD144 and CD41 in group II as compared with control group and in group III as compared with group II (p=0.001). PNA was not detected in plasma sample among all studied groups as shown in table 1

Whereas, as regard to the vitreous sample, there was significant increase in CD144, CD41 and PNA in group II as compared with control group (p= 0.048, 0.009, 0.048) respectively. There was also significant increase in CD144, CD41 and PNA in group III as compared with control group (p= 0.001) and in group III as compared with group II (0.001). Table 2

Table 3 demonstrate that in group II, there was significant positive correlations between CD144 and CD 41in plasma sample (p= 0.004) and in vitreous sample, there was significant positive correlation between CD144 and CD 41, CD41 and PNA and between CD 144 and PNA (p=0.014, 0.035 and 0.059) respectively.

Table 4 demonstrate that in group III, there was significant positive correlations between CD144 and CD 41in plasma sample (p=0.032) and in vitreous sample, there was significant positive correlation between CD144 and CD 41, CD41 and PNA and between CD 144 and PNA (p=0.029, 0.028 and 0.037) respectively.

Table (1): Plasma levels of endothelial, platelet and photoreceptor derived micoparticles in all studied groups:

	Group I (n=10)	Group II (n=25)	Group III (n=20)	P1	P2	P3
CD 144	125.9 ± 12	355.6 ± 64.8	912 ± 89.4	0.001*	0.001*	0.001*
CD 41	530.8 ± 35.7	1440 ±79.17	2170.4 ± 109	0.001*	0.001*	0.001*
CD PNA	0	0	0	-	-	-

* Highly Significant (p< 0.001)

P1 comparison between GI&GII, P2: GI&GIII, P3: GII&GIII

Table (2): Vitreous levels of endothelial, platelet and photoreceptor derived micoparticles in all studied groups:

	Group I (n=10)	Group II (n=25)	Group III (n=20)	P1	P2	P3
CD 144	35.44 ± 9.3	42.7 ± 12.10	213.2 ± 77.1	0.048**	0.001*	0.001*
CD 41	23.25 ± 10	46 ±21.32	158.44 ± 56.1	0.009**	0.001*	0.001*
CD PNA	13.32 ± 4.4	15.77 ± 3	178.30 ± 34.4	0.048**	0.001*	0.001*

* Highly significant (p< 0.001) **Significant (p< 0.05)

P1 comparison between GI&GII, P2: GI&GIII, P3: GII&GIII,

Table (3): Correlation between all studied parameters in group II:

	Plasma sample	Vitreous sample	
	CD41	CD144	CD41
CD144	r.0.569 p0.004*	-	r.0.358 P0.014*
CD PNA	-	r.0.214 p0.059	r.0.299 P0.035*

** Highly significant (p< 0.001) *Significant (p< 0.05)

Table (4): Correlation between all studied parameters in group III:

	Plasma sample	Vitreous sample	
	CD41	CD144	CD41
CD144	r.0.310 p0.032*	-	r.0.417 P0.029*
PNA		r.352 P0.037*	r0.410 P0.028*

** Highly Significant (p< 0.001) *Significant (p< 0.05

4. Discussion

DM is associated with increased levels of circulating EMPs. This agrees with previous studies where levels of endothelial microparticles (EMPs) were associated with microalbuminuria and microvascular complications in patients with diabetes, suggesting that EMPs could be a marker of diabetes associated endothelial dysfunction [17,18].

This study revealed significant increase in EMPs (CD144) and platelet derived MPs (CD 41) in the studied diabetic patients as compared with control group and significant increase in diabetic patients with PDR as compared with diabetic patients without PDR in venous sample. Also, PNA was not detected in plasma sample among all studied groups.

These results were in agreement with **Tramontano *et al.,*** who found that the absolute median number of EMP was significantly increased in DM population [19]. However, diabetic patients differ by the procoagulant activity and the cellular origin of microparticles. Indeed, EMP and PMP, levels and procoagulant activity were elevated in type 1 diabetes. Interestingly, this procoagulant activity was correlated with levels of HbA1c [18].

The immunophenotype of MPs depends on whether they are released by cell activation or by apoptotic stimulus [20]. Cellular apoptosis is associated with an increase in cytosolic calcium, with changes in the transmembrane steady state leading to the cleavage of cytoskeleton filaments. These phenomena result in the blebbing and shedding of membrane-derived MPs into the extracellular fluid [21].

MPs released from apoptotic cells may be different in lipid and protein composition from membrane vesicles shed following cell activation and could possibly have different patho-physiological effects. Blebbing of cellular membrane occurs rapidly after cells enter the apoptotic process [20].

The reason that cells shed MPs from their main body may be an attempt to reverse the apoptotic process by getting rid of unwanted signaling molecules like the proapoptotic caspase 3 [22]. The release of MPs would also allow cells to escape phagocytosis by removing quickly from the cell surface "eat-me-signals," such as phosphatidylserine [23]. Alternatively, membrane shedding could constitute a signaling entity to phagocytes and neighbor cells, because their interaction modulates inflammation, immune responses, and repair mechanisms [24].

Actually, the release of membrane vesicles to signal to neighbor or remote target cells is not a specific property of eukaryotic cells [25,26]. These results might be interpreted as an indication of enhanced endothelial cell apoptosis, rather than activation in those having DM [19].

Enodothelial cell-derived MPs express many receptors and components of the parent endothelial cell including tissue factor (TF) and can support thrombin generation by the TF/FVIIa pathway [27,28]. That is to say, endothelial cell-derived MPs provide a source of TF, as well as a catalytic surface, for assembly of prothrombinase complex. Furthermore, endothelial cell-derived MPs are possible to be a marker of endothelial activation in patients with the metabolic syndrome [29,30].

Microparticles can also directly affect vascular endothelial cells by increasing leukocyte adhesion, triggering cytokine production, and exposing tissue factor or P-selectin[31,32]. In addition, microparticles promote endothelial dysfunction by impairing the endothelial NO pathway and inducing proinflammatory responses [33-35].

In this study , it was found that in vitreous sample , there was significant increase in CD144, CD41 and PNA in diabetic group without PDR (group II) as compared with control group and in diabetic group with PDR (group III) as compared with group II

These results were in agreement with a study by **Chaded *et al.*,** who reported that there were vitreous levels of platelet CD41 and endothelial CD144 microparticles were all markedly increased in diabetic compared with control. Microparticles of endothelial origin, identified as expressing VE-cadherin (CD144), were the most abundant microparticle subpopulation in vitreous samples from diabetic patients. Vitreous levels of endothelial CD144, and platelet CD41 microparticles were increased in association with PDR compared with non-PDR (NPDR) [36].

Significant numbers of endothelial microparticles found in the vitreous fluid could be generated from local microvascular endothelial cells in PDR or that clearance of endothelial microparticles was abnormal. On the contrary, the ratio for platelet CD41 microparticles was lower than unity, favoring the interpretation that platelet microparticles present in PDR vitreous fluid likely originate from the plasma [36].

Sabatier *et al.*, reported that levels of platelet-derived MPs and monocyte derived MPs have been shown to correlate with diabetic complications or the extent of diabetic retinopathy, which is associated with microvascular damage [28].

PDR is associated with ocular increases in oxidative stress, protein glycation, growth factors, inflammatory cytokines, and cell apoptosis, all of which stimulate the shedding of membrane microparticles from retinal or vascular cells [5,37]. In addition, this demonstrates the presence of microparticles positively labeled with either PNA in vitreous samples but not in plasma, indicating their photoreceptor or microglial origin, respectively. These microparticles originate from cells localized in deeper retinal layers and may be released in vitreous fluid following the tear of the retinal internal limiting membrane[38].

In conclusion, MPs appears as a new prognostic potential of type 2 diabetes in the early detection of vascular complications. Elevation of plasma MPs levels, particularly those of endothelial origin, reflects cellular injury and may be a useful as a surrogate marker of vascular dysfunction. Moreover the presence of different types of microparticles in vitreous fluid of membrane shed from retinal, endothelial, and circulating cells and their significant increase in patients with PDR, may contribute to disease progression.

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