Platelet Hyperaggregation and Increased Plasma Level of Von Willebrand Factor in Diabetics with Retinopathy* **

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Summary. In 18 insulin-dependent diabetics (6 without retinopathy, 6 with proliferative retinopathy and 6 with proliferative retinopathy treated by hypophysectomy) matched for age and duration of diabetes, in vitro haemostasis was studied using ADP induced platelet aggregation, ristocetin induced platelet aggregation which allows von Willebrand factor (VIII VWF) assay, and determination of antihemophilic factor procoagulant activity (VIII AHF). Using gel filtration-isolated platelets, the ADP induced hyperaggregation previously reported in diabetics with severe retinopathy untreated by hypophysectomy appeared to be related to a platelet and not a

plasma factor; the normal results of thrombin induced aggregation suggests that the presumed abnormal platelet factor is related to the platelet plasma membrane. High level of plasma VIII VWF was observed in diabetics with proliferative retinopathy while the VIII AHF level was within normal limits.

Key words: Diabetes, retinopathy, hypophysectomy, ADP induced platelet aggregation, ristocetin induced platelet aggregation, Von Willebrand factor, antihemophilic procoagulant factor.

Platelet hyperaggregation induced in vitro by low doses of adenosine-diphosphate (ADP) and decrease in the rate of disaggregation have been reported in diabetics with severe retinopathy [1–3]; this abnormality has not been observed in diabetics without retinopathy [1, 3], and has not been noticed in diabetics with severe retinopathy treated by hypophysectomy [3]. The present study using platelets isolated by gel filtration was undertaken to determine if the factor enhancing the ADP-induced hyperaggregation in diabetics lies in the plasma or in the platelets.

On the other hand a plasma factor necessary for ristocetin-induced platelet aggregation [4] and deficient in Von Willebrand's disease has been recently reported; a quantitative assay of this "Von Willebrand factor" (VIII VWF) has been described using platelet aggregation induced by ristocetin [5, 6]. In this study we have determined the VIII VWF plasma level in diabetics. As the VIII VWF and the VIII clotting factor (VIII AHF) activities are carried by 2 subunits of the same molecule [5, 6], the VIII AHF assay was also performed and the VWF : AHF ratio was calculated.

Materials and Methods

A. Patients

Three groups of 6 insulin-dependent diabetics matched for age and duration of diabetes (Table 1) were studied:

- group I: 6 patients of average age 41 years (range 22 to 60 years), of mean duration of diabetes 15 years (range 4 to 22 years), without retinopathy (that is with a normal ocular fundus after careful ophthalmoscopic examination and fluorescein angiography).
- group II: 6 patients of average 40 years (range 18 to 60 years), of mean duration of diabetes 17 years (range 6 to 25 years), with proliferative retinopathy (that is with new vessels in one or both eyes).
- group III: 6 patients of average age 43 years (range 27 to 57 years), of mean duration of diabetes 21 years (range 17 to 25 years), having undergone surgical hypophysectomy for proliferative retinopathy.

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Abbreviations used in this paper:

PRP: platelet rich plasma

PPP: platelet poor plasma

GFP: gel filtered platelets

VWF or VIII VWF: Von Willebrand factor associated with factor VIII molecule

AHF or VIII AHF: antihemophilic factor procoagulant activity

Table 1. Sex, age and duration of diabetes in the three groups of diabetics

Group I				Group II				Group III			
Cases	Sex	Age (years)	Duration of diabetes (years)	Cases	Sex	Age (years)	Duration of diabetes (years)	Cases	Sex	Age (years)	Duration of diabetes (years)
1	F	22	4	7	F	35	17	13	M	34	21
2	M	40	15	8	M	60	25	14	M	50	25
3	M	25	10	9	M	55	21	15	F	27	25
4	M	50	20	10	M	45	18	16	F	57	21
5	F	60	22	11	M	25	15	17	M	43	17
6	M	50	18	12	M	18	6	18	M	45	18
Mean		41	15	Mean		40	17	Mean		43	21

The tests were carried out with control subjects selected from a group of blood donors of the same sex and age.

B. Methods

a) Preparation of platelet-rich plasma (PRP), platelet-poor plasma (PPP) and gel-filtered platelets (GFP). For the preparation of PRP, 9 volumes of blood were withdrawn from the antecubital vein into plastic tubes containing 1 volume of 3.8% w/v sodium citrate; the mixture was centrifuged at 90 g for 10 min at 15° C. After decanting the PRP, the PPP was prepared by centrifugation at 27500 g for 20 min at 10° C, using a Sorvall RC 2 B centrifuge. The PRP from each patient and from each control subject was reconstituted to the same platelet count by dilution with autologous PPP.

For the preparation of isolated platelets, 9 volumes of blood were withdrawn into plastic tubes containing 1 volume of ACD (0.8% citric acid; 2.2% sodium citrate; 2.45% dextrose); gel-filtration on Sepharose 2 B was carried out according to a modification [7] of the method of Tangen *et al.* [8].

Isolation of platelets was necessary for determining if the ADP-induced hyperaggregation was dependent on a plasma or on a platelet factor. Therefore in a first experiment GFP from control subjects were added to PPP from diabetic patients; in a second experiment GFP aggregation rate in control subjects and in diabetics was measured in the absence of PPP, and the following cofactors were then used [7]: 0.05 ml of gamma-globulins (from a 16 mg/ml solution), 0.05 ml of human fibrinogen (from a 10 mg/ml solution) and calcium chloride at a final concentration of 2.10^{-3} M.

b) Platelet aggregation. The determination was carried out by the photometric method of Born [9] using

an aggregometer at 37° C with constant stirring. Two types of aggregometer were used: 1) the Born aggregometer MK 3 for the study of the PRP where the final volume in the tube was 1 ml, to which 10 µl of the aggregation inducing agent were added; 2) the Born mini-aggregometer for the study of the GFP where the final volume in the tubes was 0.2 ml, consisting of 0.1 ml of GFP and 0.1 ml of PPP, to which 10 µl of the aggregation inducing agent were added.

Two methods were used for the platelet aggregation determination. In one the rate was calculated by measuring the tangent of the curve at the maximum of its slope during the first 30 sec; in the other the intensity (fall of optical density) was obtained by measuring the height of the curve 2 min after addition of the inducer. Rate and intensity values were expressed as a percentage: the differences in optical density (O.D.) between the PRP and the corresponding PPP or the GFP + PPP and blank + PPP were arbitrarily designated as 100%.

Platelet aggregation was determined in the presence of three inducers: 1) ADP (Sigma) at a final concentration of 2.5×10^{-6} M for the GFP aggregation, 2) Thrombin (Roche) at a final concentration of 0.2 units/ml for the PRP aggregation, and of 0.03 units/ml for the GFP aggregation, 3) Ristocetin (Lundbaeck) at a final concentration of 1000 µg/ml for both PRP and GFP aggregation.

c) VWF assay. This determination was carried out with the method described by Weiss *et al.* [6], but in order to avoid the centrifugations and resuspensions from the 4 washings during the platelet preparation, the following modification was used: 5 ml of GFP were collected after Sepharose 2 B gel filtration of 5 ml of PRP; GFP were centrifuged at 750 g for 15 min at 15° C and resuspended in 4 ml of the eluant; the suspension contained about 200 000 platelets per

mm³; a single washing was sufficient for discarding the high molecular weight VIII VWF which is eluted with the GFP. The aggregation system contained 0.1 ml of washed GFP, 0.1 ml of various PPP dilutions ($1/2$, $1/10$, $1/20$ and $1/40$) and 10 μ l of ristocetin solution, with a final concentration of 1000 μ g/ml. On log-log paper the aggregation rates plotted against the various dilutions of added plasma were linear. By definition the aggregation rate determined with a pool of normal half-diluted plasma represents 100 U per 100 ml of VWF activity.

d) VIII AHF assay. A method previously reported [10] was used.

Results

a. ADP Induced Aggregation

The effect of diabetic PPP on the ADP induced aggregation of control subjects GFP is shown in the left part of Fig. 1. The group II diabetic PPP did not induce hyperaggregation in control subjects GFP with the exception of one case, while these patients exhibited the previously reported ADP induced hyperaggregation using PRP [3]; the same result was observed with group I and group III diabetic patients who did not exhibit hyperaggregation. This finding suggests, at least with the method used in our laboratory, that diabetic PPP has no clear effect on the ADP induced aggregation of control subject's GFP.

The rate of ADP induced aggregation on diabetic GFP in the absence of PPP as compared with control subjects GFP in the same conditions is shown in the right part of Fig. 1. The rate was normal or diminished in group I diabetics, elevated in group II and lowered in group III. These findings suggest that

Fig. 1. Left part: effect of diabetic PPP on ADP induced aggregation of control subject's GFP. Results are expressed as a percentage, the value obtained with control subjects PPP representing $100 \pm 15\%$. Right part: rate of ADP induced aggregation on diabetic GFP in the absence of PPP (gamma-globulins, fibrinogen and calcium chloride being used as cofactors), as compared with control GFP in the same conditions

Table 2. ADP and thrombin induced aggregation rate with diabetic PRP. Results are expressed as percentage compared with the aggregation rate with PRP from control subjects

Group II			Group III		
Case	ADP 0.5 μ M	Thrombin	Case	ADP 0.5 μ M	Thrombin
7	414	148	13	87	101
8	400	232	14	92	72
9	280	192	15	88	137
10	177	112	16	12	35
11	583	115	17	43	40
12	828	146	18	38	90
Mean	447	157	Mean	60	79

Fig. 2. VWF activity. Results are expressed in arbitrary units (see text)

the ADP induced hyperaggregation previously reported in diabetics with severe retinopathy is related to a platelet and not to a plasma factor (with the exception of one out of the 6 cases in this series).

b. Thrombin Induced Aggregation

Using PRP, thrombin induced aggregation was determined in group II and group III diabetics. As shown in Table 2, two out of the 6 patients in group II exhibited hyperaggregation (cases 8 and 9); in two other cases (cases 7 and 12) the aggregation rate was slightly elevated, but this elevation may be related to ADP released and not to thrombin. In group III the aggregation rate was within normal limits, with the exception of two cases (cases 16 and 17) with a diminished rate.

Using GFP, results were similar.

c. Assay of VWF and VIII AHF

As shown in Fig. 2, VWF activity was normal or slightly elevated in group I diabetics and significantly elevated in group II and group III diabetics with retinopathy.

Fig. 3. AHF activity. Results are expressed as percentage of the control (mean of 20 normal subjects)

Table 3. VWF: AHF ratio. The average value in normal subjects is 1

Group I		Group II		Group III	
Case	Ratio	Case	Ratio	Case	Ratio
1	1.18	7	1.57	13	2.66
2	0.73	8	2.82	14	3.75
3	1.33	9	2.15	15	4.35
4	1.29	10	2.59	16	0.90
5	2.12	11	2.80	17	1.75
6	1.56	12	3.47	18	2.66
Mean	1.37	Mean	2.56	Mean	2.68

VIII AHF activity was normal in the three groups, with the exception of one case in group III (Fig. 3).

As shown in Table 3, the VWF : AHF ratio was within normal limits in group I diabetics and elevated in group II and group III diabetics.

Discussion

Using platelets isolated by gel filtration these results suggest that ADP hyperaggregation in diabetics with severe retinopathy is related to a platelet factor. This finding is not in agreement with the data from Kwaan *et al.* [2] and from Leone *et al.* [11] who reported that hyperaggregation is due to a plasma factor. This discrepancy is difficult to explain; it might stem from the difference in the method employed for platelet separation, which involved repeated washing [2, 11]. Isolation from plasma by washing appears to be too vigorous a method, since microscopic examination of platelets prepared with this technique has shown morphological alterations not observed with gel filtration [7]; it is thus possible that after the washing-separation technique, the presence of a platelet factor may have been missed. Recently Metcalf and Lyman [12] have shown that using polylysine as a platelet aggregating agent, the need for a plasma cofactor for platelet aggregation varied as a function of the conformational variation of the polymer; since in our studies ADP was used as an aggregating agent, variations in the conformation may not explain the discrepancy between our results and those of Kwaan and Leone. The existence of an abnormal plasma factor was not supported in the present study.

The platelet factor which interacts in ADP hyperaggregation may originate inside the cell and be released from its cytoplasmic pool by ADP, or may be related to a platelet membrane abnormality. In this study thrombin induced aggregation was in most cases within normal limits; as thrombin acts in releasing intra-

platelet ADP after acting on a surface receptor, this finding suggests that the origin of the platelet factor is not inside the platelet, and hence is located at the plasma membrane, probably at the site of action of ADP. Its nature is still to be determined; it has been reported that in Glanzmann's disease ADP non-reactive platelets are associated with the absence of a platelet membrane glycoprotein [13]; in diabetic microangiopathy chemical alterations in the glomerular basement membrane are already known [14]; thus, the present findings suggest the hypothesis that the nature of the platelet factor may be related to some abnormality, possibly of the glycoproteins, in the platelet membrane.

The absence of ADP induced hyperaggregation in diabetics with proliferative retinopathy treated by hypophysectomy has been reported [3] and has so far no explanation. It is of interest to note that in the present study no hyperaggregability was found in the platelets isolated from blood obtained from these patients.

Beside the abnormalities of ADP induced aggregation, a high plasma level of VWF was observed in diabetics with severe retinopathy, while a normal level was observed in diabetics without retinopathy; this finding has not previously been reported. In patients with Von Willebrand's disease the low platelet retention to glass beads appeared to be better corrected with plasma from diabetics than from normals [15].

The VIII AHF level was within normal limits in our 3 groups of diabetics while a high level has previously been reported [16, 17].

An elevation of the VWF: AHF ratio was therefore noticed in diabetics with retinopathy. Such a dissociation between these two factors is an unexpected finding and its significance remains to be determined. Factor VIII is a high molecular weight complex of two proteins which may be dissociated into several oligomers by modifications of ionic strength [18] and which consists of VIII VWF and VIII AHF; these two factors may be complexed in a single molecule or may be two different and strongly associated molecules [18]; VIII VWF appears to be synthesised in endothelial cells, which do not elaborate VIII AHF [19, 20]. It therefore seems that a dissociation between these 2 factors is possible, as was observed here.

The generally accepted scheme for primary haemostasis consists in platelet adhesion to collagen followed by release of several components including ADP. It has also been suggested that two non-dependent platelet-plasma-vessel systems may provide the white thrombus formation; the one including platelets, fibrinogen, ADP and collagen, the other including platelets, VWF and basement membrane [21]. Haemostatic abnormalities observed in diabetics with

retinopathy may therefore be concerned with both systems.

Haemostatic abnormalities may be one of the mechanisms in the genesis of diabetic microangiopathy. If abnormalities in platelet aggregation take place *in vivo*, intracapillary microthrombi could occur and might be the cause of non-vascular areas recognised by fluorescein angiography in the early stage of retinopathy [22, 23]; this would support the role of thrombosis as a factor of diabetic microangiopathy [24].

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