Platelet Enzyme Activities in Diabetes Mellitus in Relation to Endothelial Damage

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SUMMARY

Increased platelet reactivity has been suggested in the pathogenesis of both arteriosclerosis and diabetic microangiopathy. Therefore, platelet function and platelet enzyme activities were assessed in a large group of 357 diabetics (256 patients with IDDM, aged 16–49 and 101 patients with NIDDM, aged 50–78) and 163 matched controls, and related to photographically documented retinopathy (Rd) and to peripheral vascular disease (PVD) as well as to plasma levels of von Willebrand factor (VIII R:Ag) as an indicator of endothelial damage.

Patients with IDDM had increased platelet aggregation (PA, expressed as μM ADP threshold concentration) before Rd was detectable in comparison to control subjects ($P < 0.01$). PA was further increased in patients with advanced Rd ($P < 0.01$), whereas 20 newly diagnosed diabetics with IDDM exhibited normal PA. Patients with minimal Rd did not differ from patients without Rd. Plasma β-thromboglobulin (reflecting platelet consumption in vivo) was enhanced significantly in patients with Rd only ($P < 0.05$), as was malondialdehyde (MDA) production of platelets (as a measure of platelet endoperoxide formation).

Factor VIII-related antigen in plasma was already increased in patients without Rd ($P < 0.05$), yet more so in patients with Rd ($P < 0.01$). Prostacyclin-stimulated adenylate cyclase activity (ACA) of platelets (as an antiaggregatory enzyme system) was twice as high in diabetics with advanced Rd compared with patients without Rd and with controls ($P < 0.01$). Significant correlations were found between PA and plasma F VIII R: Hg, MDA production, and ACA of platelets.

Increased PA was also found in subjects with NIDDM ($P < 0.05$). Patients with PVD, however, did not differ from patients without PVD when subjects with Rd were excluded.

These results indicate that increased platelet activity may precede clinically detectable vascular disease, but is associated with increased levels of plasma von Willebrand factor protein, suggesting that endothelial damage is already present. Increases of PA platelet enzyme activities and plasma von Willebrand factor are particularly prominent in diabetics with Rd, reflecting highly active and perhaps younger platelets in this condition. DIABETES *32* (Suppl. 2):47–51, 1983.

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Platelet–vessel wall interactions have been implicated in the pathogenesis of arteriosclerosis as well as in the development of microangiopathic complications in diabetes mellitus.[1–12] The degree of platelet functional ability to adhere to endothelium, to aggregate, and to disaggregate, is important in this context. In the past there have been several reports of increased sensitivity of platelets to aggregating agents in diabetics, especially in patients with retinopathy, nephropathy, or neuropathy.[1–3,7–12] It is not established, however, whether the abnormal function of platelets might be the cause or consequence of underlying angiopathy. Furthermore, the reported findings of platelet hyperaggregability in diabetics could not be confirmed by others.[13–16] These discrepancies may be explained by the well-known high coefficient of variation of platelet function tests and by the small number of patients studied in most of these reports (Table 1). This study was designed as a careful reevaluation of platelet aggregation data and plasma β-thromboglobulin levels in a great number of diabetic patients and control subjects with all degrees of vessel damage. Endoperoxide production rate and the activity of prostacyclin-sensitive adenylate cyclase were measured to determine the biochemical basis of altered platelet reactivity. The results were correlated with von Willebrand factor (VIII R:Ag) as an indicator of endothelial damage.[17]

PATIENTS AND METHODS

Subjects. A total of 357 diabetic patients from the Schwabing City Hospital were included in this study. One hundred sixty-three control subjects were matched for age, sex, and weight.

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TABLE 1
Platelet aggregation in diabetics

Author	Year	Number of patients	Result*	Comments
Hannen[13]	1968	47	–	
Heath[11]	1971	72	+	Only microangiopathy
Bensoussan[12]	1975	18	+	Microangiopathy
O'Malley[8]	1975	20	+	Neuropathy
Colwell[2]	1976	51	+	All
Davis[15]	1978	56	–	
Petersen[14]	1978	38	–	
Porta[16]	1980	35	–	

*Aggregation: + = increased; – = not increased.

In age group 1 (16–49 yr) type I diabetics of different disease duration were studied. All degrees of retinopathy were present. Diabetic retinopathy (Rd) was assessed ophthalmoscopically and by fundus photography and classified into no Rd, minimal and more severe background Rd, and proliferative Rd.

In age group 2 (50–78 yr) insulin-treated as well as orally treated diabetics were included, since results of platelet tests were similar in both treatment groups. Forty-six diabetics and 23 nondiabetics exhibited signs of peripheral vascular disease (PVD) as diagnosed by palpation, auscultation, electronic oscillometry, and Doppler-ultrasonic measurements. All patients were in good metabolic control.[18] Excluded were patients on drugs that may have had an influence on platelet function. Many of the older patients, however, were receiving digitalis or antihypertensive therapy that could not be omitted during the platelet tests.

Platelet aggregation. Platelet aggregation was performed according to Born as previously described.[19] Platelet-rich plasma (PRP) was diluted with the patient's own platelet-poor plasma to give a final platelet concentration of 200,000/μl. The sequence of aggregation agents was kept constant: ADP 0.5, 1.0, 2.0, 5.0, 10.0 μM. Percentage changes in optical density were measured comparing PRP 4 min after induction of platelet aggregation with the curve obtained after adding 10 μM ADP (maximal aggregation). The concentration that induced 50% of maximal aggregation was referred to as the threshold concentration.

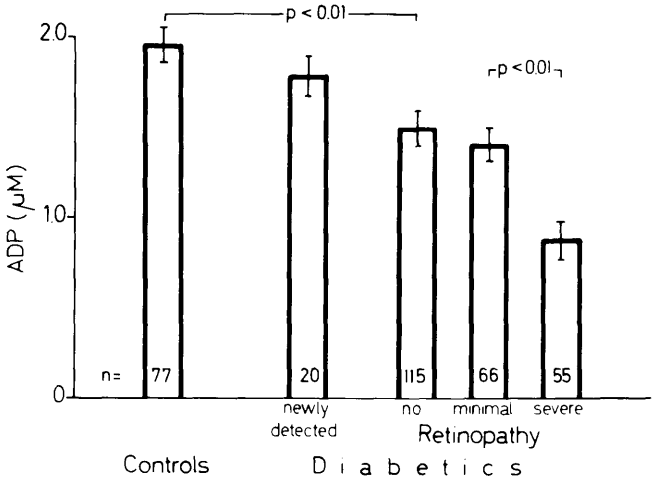

FIGURE 1. ADP-induced platelet aggregation (threshold concentrations) in insulin-dependent diabetics and controls (age 16–49 yr; x̄ ± SEM).

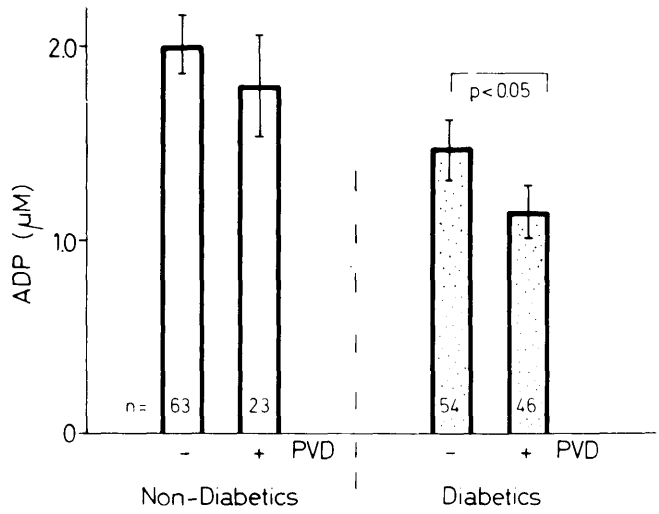

FIGURE 2. ADP-induced platelet aggregation (threshold concentrations) in diabetics and nondiabetics, aged 50–78 yr, with and without peripheral vascular disease (PVD).

Platelet adenylate cyclase activity (ACA). The anti- and disaggregatory ACA was measured by the increase of cAMP content in PRP after incubation with 0.28 μM prostacyclin (PGI_2).[20] Platelet phosphodiesterase was blocked by 1 mM iso-butyl-methyl-xanthine (IBMX) and cAMP was determined by a radioimmunoassay (Amersham Corp., Arlington Heights, Illinois).[21] ACA was expressed as multiples of basal cAMP concentration. PGI_2 was a gift of the Upjohn Company (Kalamazoo, Michigan).

β-Thromboglobulin. Plasma β-thromboglobulin was measured in 75 subjects according to Ludlam[22] using the Amersham radioimmunoassay; 0.5 ml of plasma (meniscus layer) was taken for assay.

Malondialdehyde production. Malondialdehyde (MDA) is an end product of platelet endoperoxides.[23] Stimulation of platelets with 1 mM n-ethyl-maleimide (NEM) induces a marked increase of MDA production. In this study MDA was determined according to Okuma.[23] Absorbance from the pink thiobarbituric acid reaction was read in a Zeiss photometer

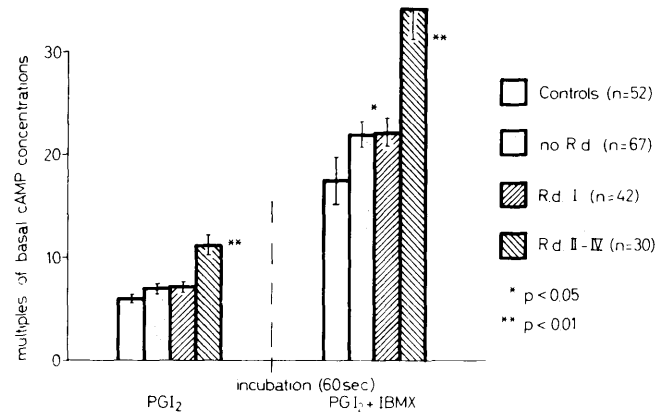

FIGURE 3. Effects of 0.28 μM PGI_2 and 1 mM iso-butyl-methyl-xanthine (IBMX) on cAMP content in platelet-rich plasma of diabetics with and without retinopathy (Rd).

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(wave length: 532 nm). Results were expressed as MDA/10^9 platelets.

Von Willebrand factor (VIII R:Ag). Von Willebrand factor was determined as factor VIII-related antigen (VIII R:Ag) by quantitative immune electrophoresis[24] and expressed as percent of a standard reference (100%).

RESULTS

ADP threshold concentration. In both age groups diabetics showed increased platelet aggregation in comparison to control subjects. In age group 1 ADP threshold concentration (μM ADP final concentration; $\bar{x} \pm$ SEM) was 1.94 ± 0.12 in 77 control subjects and 1.45 ± 0.06 in 115 diabetics without Rd. Mean values for all diabetics, with the exception of 20 newly detected diabetics, differed highly significantly from controls (Figure 1). Diabetics with advanced Rd showed an even further increase of platelet aggregation (0.84 ± 0.10 μM ADP), while patients with minimal or ophthalmoscopically absent Rd did not differ.

In 101 diabetics beyond the age of 50 (age group 2) ADP threshold concentrations were significantly lower than in 86 control subjects (1.38 ± 0.10 versus 1.92 ± 0.14; $P < 0.001$). Separated into patients with and without PVD, ADP concentrations in diabetics with macroangiopathy were significantly lower than in diabetics without PVD (1.16 ± 0.10 versus 1.60 ± 0.16; $P < 0.05$) (Figure 2).

Platelet ACA. Determinations of cAMP in PRP before and after incubation with PGI_2 and IBMX could be made in a great number of subjects of age group I. cAMP concentrations in PRP (adjusted to 200,000 platelets/μl) were comparable in diabetics and controls (Rd 0: 43.4 ± 2.4; minimal Rd: 43.2 ± 2.5; advanced Rd: 43.0 ± 3.0 pmol/ml). Incubation of PRP with 0.28 μM PGI_2 induces an enormous increase of cAMP that is further exaggerated when platelet phosphodiesterase was blocked by IBMX (Figure 3). Patients with advanced Rd showed the highest ACA. In 57 diabetics with Rd both ADP threshold concentration and ACA were obtained. In this group a significant negative correlation of ADP threshold concentration and ACA (ln) was noted (Figure 4).

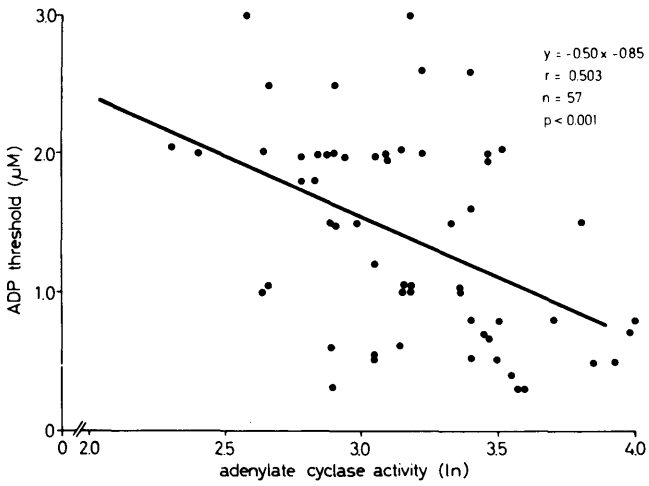

FIGURE 4. ADP-induced platelet aggregation (threshold concentrations) and platelet adenylate cyclase activity in diabetic patients with retinopathy.

TABLE 2
NEM-(1 mM)-stimulated platelet malondialdehyde production and platelet aggregation (ADP threshold concentration) in diabetics with and without retinopathy (Rd; $\bar{x} \pm$ SEM)

	Rd ⊕ (N = 22)	Rd ⊖ (N = 18)
MDA (nM/10^9 platelets)	7.27 ± 0.52	5.15 ± 0.26
ADP concentration (μM)	0.80 ± 0.12	1.45 ± 0.23

Correlation: N = 40; r = −0.32; y = −0.12 × +1.9; $P < 0.05$.

β-Thromboglobulin. In 75 subjects of age group 1 plasma β-thromboglobulin levels were measured. A relatively large variation of values was present in all groups: controls, 58.3 ± 10.0 ng/ml ($\bar{x} \pm$ SEM); diabetics without Rd, 74.3 ± 11.8 ng/ml; and diabetics with Rd, 95.3 ± 10.1 ng/ml. The difference between the group of diabetics with Rd and controls was significant ($P < 0.05$).

MDA production. Platelet MDA production was measured in 48 subjects of age group 1. The mean values after NEM incubation were significantly higher in 22 diabetics with Rd than in patients without Rd (7.27 ± 0.52 versus 5.15 ± 0.26 nmol/10^9 platelets; $\bar{x} \pm$ SEM; $P < 0.05$). In eight controls this value was 5.32 ± 0.47. In Table 2 the mean value of the corresponding ADP threshold concentrations is also incorporated. The correlation between these values was significant (r = −0.32; y = −0.12x + 1.90, $P < 0.05$).

Von Willebrand factor. In 91 subjects of age group 1 von Willebrand factor (VIII R:Ag) in plasma was determined. Diabetics with Rd exhibited the highest values (Table 3), although diabetics without Rd also showed a slight, yet significant increase. A highly significant association of in vitro platelet aggregation (ADP threshold concentration) was found with the concentration of von Willebrand factor (Figure 5).

DISCUSSION

The pathogenesis of diabetic microangiopathy remains to be determined in detail. The observations of microthrombi[25] and capillary obliterations[7] in diabetic retinopathy and nephropathy taken together with the findings of enhanced platelet aggregability[1–3,9–12] point to the platelets as an important contributory factor. The results of this study indicate that increased platelet activity is an early feature in the course of diabetes and may precede clinically detectable vascular disease. It is particularly prominent in patients with advanced Rd.

The normal reactivity of platelets in the group of newly detected diabetics, however, suggests that platelets change their function only after a certain period of overt diabetes mellitus. In this context it cannot be excluded that, in an unknown proportion of patients classified as being free of Rd, clinically undetected microangiopathy was present. On the other hand, the definitive morphologic-pathologic vas-

TABLE 3
Factor VIII-related antigen in diabetics and controls ($\bar{x} \pm$ SEM) of age group 16–29 yr

Diabetics with Rd	(N = 29)	236 ± 16%	$P < 0.01$
Diabetics without Rd	(N = 42)	143 ± 10%	$P < 0.05$
Controls	(N = 20)	103 ± 10%	

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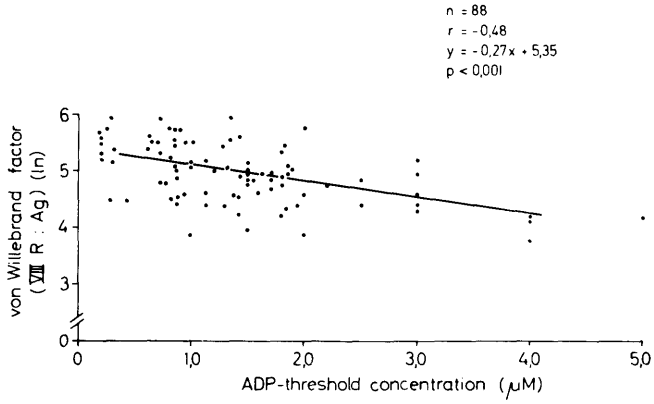

FIGURE 5. Correlation of ADP-induced platelet aggregation in vitro (ADP threshold concentration) with von Willebrand factor in plasma (in percent of 100% reference activity, ln) in normals and diabetics with and without retinopathy.

cular changes of microangiopathy are preceded by a period of variable and reversible processes that have been termed functional angiopathy[26,27] but that could very well have some influence on platelet–vessel wall interactions.

Mechanical effects related to changes of the vessel lining together with a possible increased shear stress of blood might then "activate" platelets even further in patients with more advanced microangiopathy.

In diabetics with macroangiopathy, platelet aggregation was increased only to a minor degree. The mean aggregation values never reached the levels of diabetics with Rd. Furthermore, when patients who suffered concomitantly from advanced Rd were eliminated, diabetics with and without PVD did not significantly differ with respect to platelet aggregation, suggesting that in the older age group (group 2), the primary association of platelet aggregation might be with Rd. This is not too surprising in view of the far greater vascular bed of small blood vessels compared with that of large vessels.

Our understanding of platelet function has been enlarged by the detection of the vessel- and platelet-active prostaglandins and thromboxanes. Prostaglandin endoperoxides and their product thromboxane A_2 have an important role in platelet function and thrombus formation. One of the end products of platelet endoperoxides is malondialdehyde. In this study diabetics with Rd exhibited an increased rate of MDA production. These results agree with a recently published paper by Betteridge et al.[28] and with recent thromboxane data obtained by Halushka et al.[29] The finding of increased MDA formation provides further evidence that platelet activation may be a feature of diabetic microangiopathy. The results on β-thromboglobulin concentrations in plasma are probably also to be interpreted along the same line. Since β-thromboglobulin is liberated into the plasma from the α-granules of platelets during the release reaction of clot formation, increased circulating levels of β-thromboglobulin in plasma of young diabetics with Rd appear to indicate increased activation and consumption of platelets in vivo in this condition, confirming previous work.[9,10,28,30–32] In addition, we and others have found a shortened life span of platelets in patients with Rd, again suggesting an increased turnover of platelets.[10,14,33–35]

Surprisingly, not only the "aggregatory pathways" of platelets seemed to be more active in relation to Rd, but also the anti- or disaggregatory system of prostacyclin-stimulated adenylate cyclase. There was a close correlation of ADP-induced platelet aggregation in vitro with prostacyclin-stimulated adenylate cyclase activity of platelets. In other words, all measured enzyme activities of platelets in vitro were increasingly enhanced in connection with the increasing extent of Rd and concomitantly with signs of an enhanced turnover of platelets in vivo. It is noteworthy in this context that Karpatkin has shown increased enzyme activities in younger platelets and in megathrombocytes[36,37] and that increased numbers of megathrombocytes have been reported in diabetics with Rd.[1] Relatively younger platelets, however, seem likely to be present in such patients, in view of their signs of an increased platelet turnover. Hence, it might be concluded that the overall increased platelet function in relation to the severity of Rd is a secondary event, yet probably an important one in contributing to the final capillary occlusion.

It is of interest that platelet aggregation was also significantly associated with circulating levels of von Willebrand factor (VIII R:Ag) in the present study (Figure 5). VIII R:Ag is essential for normal platelet adhesion to the vessel wall and is mainly produced by endothelial cells; increased levels of VIII R:Ag in blood have been thought to reflect endothelial damage. A number of groups have reported raised levels of von Willebrand factor in diabetes, especially in patients with severe Rd.[12,38–41] Our results indicate a modest increase of VIII R:Ag even in diabetics without detectible Rd, suggesting that endothelial damage might already occur in the period of functional microangiopathy. Porta et al. have recently found evidence for functional endothelial cell damage in terms of an increased fluorescence in leakage from the retinal capillaries in connection with enhanced blood concentrations of von Wiilebrand factor.[42] Thus, multiple repeated injuries to the capillary wall might be related to both direct capillary dysfunction and accelerated platelet turnover with its subsequent implications.

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