Activated platelets in subjects at increased risk of IDDM

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Summary Activated platelets respond to activated leukocytes and endothelial cells via adhesion molecules linking inflammation and thrombosis. Platelets of recent-onset insulin-dependent diabetic (IDDM) patients have been shown to be activated independent of metabolic control. This study evaluates the levels of circulating activated platelets exposing adhesion molecules in healthy subjects at increased risk of IDDM (surface markers were: P-selectin (CD62), thrombospondin, lysosomal GP53 (CD63)). From the DENIS and the ENDIT screening programmes 19 identified islet cell antibody positive (titre ≥ 20 Juvenile Diabetes Foundation units) first degree relatives of IDDM patients (male/female 9/10; age 22 ± 15 years; body mass index (BMI): 20.0 ± 4.3 kg/m²) with clearly normal metabolism (HbA_{1c}: 6.1 ± 0.8 %; fasting blood glucose: 4.95 ± 0.67 mmol/l) were available for this investigation. Platelet CD62 as well as thrombospondin and CD63

expression were determined by flow cytometry. We matched 50 normal volunteers for age (29 ± 6 years), anthropometric measures (male/female 26/24; BMI: 22.3 ± 2.8 kg/m²) and metabolic parameters (HbA_{1c}: $5.8\% \pm 0.3$; fasting blood glucose: 4.41 ± 0.53 mmol/l) served as control subjects. The mean number of CD62⁺ platelets was increased 3.2-times in prediabetic patients: $1.94 \times 2.91^{\pm 1}$ vs $0.60 \times 1.83^{\pm 1}$ %, $p < 0.0001$. Thrombospondin⁺ and CD63⁺ platelet levels were concomitantly increased ($1.45 \times 2.38^{\pm 1}$ / $5.97 \times 2.89^{\pm 1}$ % vs $0.52 \times 2.01^{\pm 1}$ / $1.64 \times 2.26^{\pm 1}$ %, $p < 0.0001$ for both comparisons). Thus, intravascular platelet activation is already present in potentially prediabetic subjects representing an antecedent, potentially pathogenic feature of IDDM. [Diabetologia (1997) 40: 573–577]

Keywords Activated platelets, prediabetic, islet cell antibodies.

A change in the bone marrow thrombocytopoiesis of diabetic patients accounts for the release of platelets

Received: 6 September 1996
and in revised form: 16 January 1997

* See acknowledgements

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Abbreviations: ICA, Islet cell antibodies; JDF-units, Juvenile Diabetes Foundation Units; DENIS, Deutsche Nikotinamid Interventionsstudie; ENDIT, European Nicotinamide Intervention Trial; TSP, thrombospondin; IDDM, insulin-dependent diabetes mellitus; GM, geometric mean; Sdf, standard deviation factor.

with increased numbers of constitutive adhesion receptors such as the GPIIb/IIIa ($\alpha_2\beta_3$) integrin [1]. More reactive platelets could become more easily activated in vivo and which may contribute to the evolution of diabetic vascular complications from the beginning of the disease rather than being a consequence of it. In line with this we recently reported an increased exposure of adhesion molecules such as P-selectin to circulating platelets of recent-onset insulin-dependent diabetic (IDDM) patients who appeared independent from metabolic control [2]. By exposure to P-selectin activated platelets become involved in the cross-talk between activated endothelial cells and leukocytes. Furthermore, the finding of increased levels of soluble intercellular adhesion molecules (ICAM)-1 and L-selectin in islet cell antibody

(ICA) positive healthy relatives of IDDM patients strongly suggests a condition of up-regulated adhesion molecules in persons with developing autoimmune-mediated beta-cell destruction [3]. Platelet activation may be a part of or a response to such inflammatory cell reactions in the pathogenesis of IDDM.

This study was designed to clarify whether activated platelets exposing adhesion molecules circulate in immunologically affected (ICA positive), but metabolically still healthy individuals at increased risk of IDDM.

Individuals and methods

Individuals and study design

Two multicentre screening programmes for the Deutsche Nikotinamid Interventionsstudie – DENIS and the European Nicotinamide Intervention Trial – ENDIT – Germany studies were used to identify non-diabetic subjects at increased risk of IDDM as defined by ICA of 20 Juvenile Diabetes Foundation (JDF) units or more. Both studies were approved by local ethical committees of participating centres and informed consent was obtained from all individuals or their parents. To date 2844 first degree relatives of IDDM patients have been screened for ICA as recently reviewed [4]. On two or more occasions 111 relatives were found to be ICA positive (≥ 20 JDF units). Out of these, 19 individuals presenting without any signs of inflammation (Table 1) were chosen for the present study according to availability of blood samples at the haemostasis laboratory within 12 h. Five received the intervention medication of the nicotinamide trials, i. e. $1.2 \text{ g} \cdot \text{m}^{-2} \cdot \text{day}^{-1}$ nicotinamide when investigated. All subjects had a clearly normal glucose metabolism (Table 1). Reference values were derived from 50 healthy volunteers [2]. The use of any platelet-affecting medication 10 days prior to blood sampling was prohibited for all individuals studied.

Blood sampling, staining and flow cytometry. Venous blood was taken for platelet analysis without tourniquet from a peripheral vein of resting subjects and directly put into platelet stabilizing reagent with immediate fixation (paraformaldehyde 0.5%, pH = 7.4) [5]. Fixed platelets were washed and finally diluted to 50000/ μl and labelled by P10 (anti-thrombospondin, TSP), 2.17 (anti-CD62) and 2.28 (anti-CD63) monoclonal antibody. These monoclonal antibodies react specifically with activated platelets. Matched mouse-IgG was used as non-specific control. Sheep anti-mouse F(ab)₂-fluorescein isothiocyanate conjugated fragments were used as second antibody. Immediately after staining the fluorescence of 10000 platelets was measured with a Fluorescence activated cell sorter (FACS)-Star instrument (Becton-Dickinson, San José, Calif., USA). The percentage of specific fluorescence positive platelets was obtained after channel-by-channel subtraction of unspecific mouse IgG binding (list mode).

Islet cell antibody assay. An indirect immunofluorescence assay on human pancreas cryo-sections was used as previously published [6]. The detection limit of the assay is 2.5 JDF units. A titre of 20 JDF units or more was considered as an ICA positive status according to the DENIS and ENDIT study protocols.

Table 1. Clinical documentation parameters of control subjects and ICA-positive patients at risk for IDDM

		Control subjects	ICA ^a -subjects
Sex ratio	(female/male)	24/26	10/9
Age	(years)	29.0 $\pm$ 6.0	22.4 $\pm$ 15.3
Body mass index	(kg/m ²)	22.3 $\pm$ 2.8	20.1 $\pm$ 4.3
Smoking	(yes/no)	0/50	3/19
Hypertension	(yes/no)	0/19	0/19
HbA _{1c}	(%)	4.8 $\pm$ 0.4	5.3 $\pm$ 0.6
Triglycerides	(mmol/l)	1.2 $\pm$ 0.7	1.0 $\pm$ 0.6
Cholesterol	(mmol/l)	5.0 $\pm$ 1.1	4.6 $\pm$ 0.8
Creatinine	($\mu\text{mol/l}$)	70.7 $\pm$ 8.8	61.9 $\pm$ 17.7
Fibrinogen	(g/l)	2.7 $\pm$ 0.4	2.4 $\pm$ 3.9
QUICK-value	(%)	94.0 $\pm$ 8.0	83.4 $\pm$ 13.5
Partial prothrombin time	(s)	30.7 $\pm$ 2.9	33.9 $\pm$ 6.1
Thrombin time	(s)	9.6 $\pm$ 0.7	9.1 $\pm$ 0.3
Leukocyte count	($\times 10^9/\text{l}$)	5.58 $\pm$ 1.47	5.82 $\pm$ 2.31
Platelet count	($\times 10^9/\text{l}$)	240.0 $\pm$ 54.4	244.2 $\pm$ 52.9
Mean platelet volume	(fl)	8.3 $\pm$ 1.6	8.9 $\pm$ 1.7

Table 2. Levels of P-selectin, thrombospondin and lysosomal GP53 positive platelets in individuals at increased risk of IDDM (first degree relatives of IDDM patients; ICA ≥ 20 JDF units) (geometric mean $\times$ standard deviation factor^{±1})

	P-selectin CD62	Thrombospon- din	Lysosomal GP53	CD63
Control subjects	0.60 $\times$ 1.83 ^{±1} %	0.52 $\times$ 2.01 ^{±1} %	1.64 $\times$ 2.26 ^{±1} %	
Individuals at increased risk of IDDM	1.94 $\times$ 2.91 ^{±1} %	1.45 $\times$ 2.38 ^{±1} %	5.97 $\times$ 2.89 ^{±1} %	

Other laboratory investigations. Platelet counts were determined by a Coulter Counter (Hialeah, USA). All other parameters documented were measured with routine methods using reagent grade chemicals (Boehringer, Mannheim, Mannheim, Germany).

Statistical analysis. The parameters measured CD62, thrombospondin and CD63 were log normal distributed in a normal control population. Thus, geometric mean (GM) $\times$ standard deviation factor (Sdf)^{±1} was used for descriptive statistics of the data (i. e. GM/Sdf = upper 95 % confidence value; GM $\times$ Sdf = lower 5 % confidence value). Clinical parameters of Table 1 were expressed as arithmetic mean $\pm$ standard deviation as usual for easy comparison.

Statistical comparison between groups was calculated by Student's *t*-test of log transformed measurements. Level of significance was set at *p* less than 0.05.

Results

In this study 19 out of 111 ICA positive individuals from more than 2800 screened first degree relatives of patients with IDDM were available for the flow cytometric study of platelet activation. The exposure of the activation dependent adhesion molecules P-selectin (CD62), thrombospondin and lysosomal GP53 (CD63) was measured. All probands and control subjects were clearly healthy with regard to their

Fig. 1. Activated platelets in peripheral blood of healthy but ICA positive first degree relatives of IDDM patients (i.e. individuals at increased risk of IDDM) and normal control subjects. Each symbol represents individual counts for the percentage of platelets positive for CD62, thrombospondin and CD63, respectively

metabolic status and clinical parameters that may interfere with platelet activation.

Levels of activated platelets were significantly increased (Table 1). Mean levels in ICA positive subjects corresponded to a 3.2-fold (CD62), 2.8-fold (thrombospondin) and a 3.6-fold (CD63) increase from mean normal levels. About one half of the ICA positive subjects exceeded the 99 % confidence interval (CD62⁺: 8/19 above geometric mean + 2SD of control subjects; TSP⁺ 6/19 and CD63⁺ 9/19) (Fig. 1). Elevation of all investigated markers of platelet activation was highly correlated between each other (Fig. 2). As expected, nicotinamide intake in five study individuals did not have any influence on the

Fig. 2. Numbers of ICA positive healthy first degree relatives of IDDM patients exceeding 99 % confidence limit of control subjects for one, two or all three investigated markers of platelet activation

activation marker status ($2.39 \times 3.14^{\pm 1} \%$ CD62⁺ platelets, $1.23 \times 2.57^{\pm 1} \%$ TSP⁺ platelets, $9.86 \times 2.03^{\pm 1} \%$ CD63⁺ platelets in nicotinamide-treated individuals compared to $1.81 \times 2.94^{\pm 1} \%$ CD62⁺ platelets, $1.54 \times 2.38^{\pm 1} \%$ TSP⁺ platelets, $5.00 \times 3.10^{\pm 1} \%$ CD63⁺ platelets in untreated individuals; all differences non significant).

Discussion

The present study showed for the first time in a cohort of metabolically healthy, ICA positive first degree relatives of IDDM patients that there is a significant increase in activated platelets exposing adhesion molecules such as P-selectin (CD62), thrombospondin and lysosomal GP53 (CD63). These molecules become exocytosed (GP53, P-selectin) or rebound (thrombospondin) to the outer membrane of platelets (thrombogenic membrane transformation) which continue to circulate during the intermediate phase before consumption occurs by functional aggregation. The detection of platelets positive for these molecules by means of immune-phenotyping thus indicates a state of ongoing platelet activation/secretion which hallmarks a tendency toward thrombosis [7]. The flow cytometric technique allowed identification of about one half of the individual study probands with elevated numbers of activated platelets in peripheral blood. Thus, activation of the cellular haemostasis system occurs in a substantial part of healthy individuals at increased risk of IDDM prior to any metabolic abnormalities being demonstrable. This finding is in line with the recently observed independence of platelet activation from metabolic control in recent-onset diabetes [2]. The individuals investigated here have a 30–50 % risk of developing diabetes within 5 years. Whether individuals with activated platelets represent a distinct subpopulation among ICA positive persons at increased risk

of developing the disease could not yet be judged with the limited follow-up time. So far, individuals with elevated activation markers do not have any other different clinical features.

Recruitment of leukocytes adhering to activated endothelial cells is known to be mediated by activated cytoadhesive receptors and their complementary ligands. In that regard, P-selectin (CD62) mediates platelet adhesion to neutrophils binding to the up-regulated P-selectin on activated endothelial cells which allows the initial rolling of these cells at the vessel wall. Conversely, P-selectin mediates neutrophil binding to activated platelets adhering in response to vessel wall injury. Thus, P-selectin (CD62) could act as the initial molecular interface between inflammation and thrombosis [8, 9]. Similar to IDDM elevated numbers of activated platelets [10] and thrombotic occlusions of functional end-arteries with consecutive hypoxic mucosal lesions were described in inflammatory bowel disease [11] confirming an immune modulatory role of activated platelets as shown in vitro [13, 14].

Alternatively, increased activation of platelets may be part of the predisposition to IDDM. Disease transfer by bone marrow was demonstrated in the BB rat and NOD mouse model [15, 16]. We recently reported a controlled case of IDDM transfer with bone marrow transplantation between HLA-identical siblings [17]. These observations have so far only been interpreted with respect to macrophage or lymphatic bone marrow derived cells. But platelets with more functional constitutive adhesion receptors such as GPIIb/IIIa were released from a primarily altered bone marrow thrombopoiesis in BB rats [1].

Whether the increase in platelet activation in ICA positive individuals is a reflection of inflammation and endothelial cell injury in affected islets of Langerhans or an inherent bone marrow derived disturbance should be a matter of further investigation.

However, activated platelets are able to contribute to the local destruction of the beta cells by causing microcirculatory flow disturbances. Considering that each islet is supplied with blood by a single arteriole this mechanism may cause randomly distributed events leading to destruction of islets. Indeed, simultaneous appearance of end-stage islets, heavily infiltrated islets, islets with peri-insulitis or normal islets are a characteristic feature of the pancreas in human IDDM and animal models of the disease [14]. In line with this, antagonism of the platelet activating factor which acts as co-factor of P-selectin mediated integrin activation on leukocytes adherent to activated endothelial cells (e.g. by thrombin), has been proven to decrease insulitis and to protect from diabetes manifestation in the BB rat model of IDDM [18].

In conclusion, the results encourage evaluation of the suitability of the well-documented flow cytometric platelet test for the exposure of adhesion molecules

as an additional easy-to-use marker in subjects at increased risk of the development of IDDM. Finally, antagonism of platelet activation may be envisaged for the prevention of IDDM in such individuals.

Acknowledgements. The technical assistance of Ms. M. Tubes and Ms. C. Schmidt-Soltau is gratefully acknowledged. We thank Dr. H. K. Nieuwenhuis, University Hospital of Utrecht, The Netherlands, for generous supply of specified antibody material. This work was supported by grants from the "Wilhelm Sander-Stiftung", Neustadt a.d. Donau, Germany "Die Stiftung das zuckerkranken Kind", Ulm, Germany, and the Bundesminister für Gesundheit, Bonn, and the Minister für Wissenschaft und Forschung des Landes Nordrhein-Westfalen, Düsseldorf, Germany.

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