Diabetologia (2004) 47:537–540
DOI 10.1007/s00125-004-1352-4

Diabetologia

Short Communication

Enhanced P-selectin expression and increased soluble CD40 Ligand in patients with Type 1 diabetes mellitus and microangiopathy: evidence for platelet hyperactivity and chronic inflammation

M. Yngen[1] · **C.-G. Östenson**[2] · **H. Hu**[1] · **N. Li**[1] · **P. Hjemdahl**[1] · **N. H. Wallén**[1, 3]

[1] Department of Medicine, Clinical Pharmacology Unit, Karolinska Hospital, Stockholm, Sweden
[2] Department of Molecular Medicine, Endocrine and Diabetes Unit, Karolinska Hospital, Stockholm, Sweden
[3] Department of Internal Medicine, Danderyd University Hospital, Stockholm, Sweden

Abstract

Aims/hypothesis. Platelet activation, endothelial dysfunction and inflammation may be involved in early stages of diabetic microangiopathy. We therefore investigated patients with Type 1 diabetes mellitus, without (n=19) and with (n=20) microangiopathy, matched for glycaemic control and duration of disease, and matched with healthy control subjects (n=27).
Methods. Platelet activation was measured as platelet P-selectin expression using whole blood flow cytometry and as soluble P-selectin by immunoassay. Von Willebrand factor antigen in plasma, serum soluble E-selectin, CD40 ligand (sCD40L) and C-reactive protein (CRP) served as markers for endothelial function and inflammation.
Results. Thrombin-induced platelet P-selectin expression was enhanced, and soluble P-selectin and sCD40L concentrations were increased in patients with microangiopathy compared with the control subjects (p<0.01 for both) and with patients without microangiopathy (p<0.05 for P-selectin expression and sP-selectin), whereas all three parameters were similar in patients without microangiopathy and in the control subjects. CRP and soluble E-selectin were increased in patients with microangiopathy, compared with the control subjects (p<0.01 and p<0.05), whereas von Willebrand factor did not differ between the groups.
Conclusions/interpretation. Microangiopathy in Type 1 diabetes is associated with platelet hyperactivity, endothelial dysfunction and low-grade inflammation, indicating an increased risk for cardiovascular disease. [Diabetologia (2004) 47:537–540]

Keywords Type 1 diabetes mellitus · Microangiopathy · Platelets · Endothelium · Inflammation · P-selectin · Soluble CD40 Ligand · C-reactive protein

Diabetes is a strong risk factor for atherothrombotic complications [1] and diabetic vascular complications often begin as microvascular injury. Recently, epidemiological studies have shown that Type 1 diabetes mellitus is at least as great a risk factor for cardiovascular mortality as Type 2 diabetes, and that this also can occur at a young age [2]. Thus, early detection and treatment of risk factors for cardiovascular disease in Type 1 diabetes are warranted.

P-selectin, an adhesion molecule and a constituent of the platelet α-granule membrane and the Weibel-palade bodies in endothelial cells, is expressed on activated platelets and endothelium and is shed into plasma in a soluble form. P-selectin mediates the rolling of monocytes on activated endothelium, induces procoagulant microparticle formation [3] and promotes atherosclerotic lesion development [4]. Increased platelet P-selectin expression and increased plasma concentrations of soluble P-selectin have been reported in diabetes mellitus and in atherosclerotic

Received: 19 September 2003 / Revised: 22 December 2003
Published online: 13 February 2004

M. Yngen (✉)
Department of Medicine, Clinical Pharmacology Unit,
Karolinska Hospital, 171 76 Stockholm, Sweden
E-mail: marianne.yngen@ks.se

Abbreviations: sP-selectin, Soluble P-selectin · sCD40L, soluble CD40 Ligand · CRP, C-reactive protein

disease, but few studies have focused on the role of P-selectin in Type 1 diabetic microangiopathy.

The CD40-CD40 Ligand (CD40L) system is involved in immune reactions, but could also be an important proinflammatory mediator in atherosclerosis [5]. Interestingly, CD40 is expressed on platelets, and 95% of CD40L in blood is platelet associated. CD40L on activated platelets triggers an inflammatory reaction of endothelial cells [5] and is rapidly shed from the platelets into a biologically active soluble form, sCD40L. Soluble CD40L can be a predictive marker in patients with acute coronary syndromes [6]. One study has reported increased plasma concentrations of sCD40L in Type 1 diabetes, but its relation to vascular complications was not studied [7].

The aim of this study was to investigate alterations of platelet and endothelial function, as well as inflammatory markers, in diabetic microangiopathy, by comparing well controlled Type 1 diabetic patients with early stages of retinopathy, to patients free from microangiopathy, and healthy control subjects. The patient groups were matched for duration of disease and glycaemic control, the two strongest predictors for developing retinopathy.

Methods

Subjects. A total of 39 patients with Type 1 diabetes mellitus and with a duration of the disease over 10 years, selected from a register at the Department of Endocrinology and Diabetology at Karolinska Hospital participated in the study. Twenty patients were diagnosed as having microvascular complications. Among these, 13 patients had ophtalmoscopically verified background retinopathy, 6 had non-proliferative retinopathy and 1 had proliferative retinopathy.

Nineteen patients had no history or signs of microvascular complications or cardiovascular disease. Patients with severely impaired metabolic control (HbA_1c >9.0%) and patients with other concomitant diseases were excluded. The two patient groups were matched for sex, age, duration of disease, metabolic control and BMI. The use of medical treatment (statins, ACE-inhibitors, HRT) was equally distributed among the groups.

The patients were compared with 27 healthy individuals matched for sex and age, recruited among the staff of the hospital. The study was approved by the Ethics Committee of the Karolinska Hospital, and informed consent was obtained from all subjects.

Blood sampling. The subjects were instructed not to take any platelet inhibiting drugs during 14 days preceding the sampling, and to abstain from tobacco and caffeine-containing beverages on the day of the sampling. Blood was collected in the morning after an overnight fast, (before breakfast and morning injection of insulin) after a 30-min rest in a semi-recumbent position. Sampling was done without stasis, using the vacutainer technique.

Blood chemistry analysis. HbA_1c was measured by an immunoturbidimetric method (TINIA; Roche diagnostics, Mannheim, Germany). Microalbuminuria was analysed using Beckmann IMMAGE Immunochemistry Systems (Beckman Instruments, Richmond, Calif., USA). Enzyme immunoassays (EIA) were used to determine concentrations of sP-selectin, sE-selectin, sCD40L (R&D Systems, Abingdon, UK), and von Willebrand factor antigen (Asserachrom, Diagnostica Stago, France). High sensitivity CRP was determined by means of particle enhanced immunonephelometry (BN Systems, Dade Behring, Marburg, Germany). Urinary 11-dehydro-TxB_2 was analysed using EIA (Cayman Chemical, Ann Arbor, Mich., USA), and a sample work-up procedure developed by us. Thrombin generation was measured as prothrombin fragment 1+2 (Enzygnost F1+2; Behring Diagnostics, Marbourg, Germany) and plasma fibrinogen by the clotting method of Clauss (Diagnostica Stago, Asnieres-sur-Seine, France).

Platelet P-selectin expression. Platelets were identified with a fluorescein isothiocyanate (FITC) conjugated anti-CD42a monoclonal antibody. Platelet P-selectin expression was determined by a R-phycoerythrin (RPE)-conjugated anti-P-selectin monoclonal antibody (Becton Dickinson, San Jose, Calif., USA). FITC and RPE conjugated isotypic monoclonal antibody DAK-GO1 (Dakopatts) were used as negative controls. Agonists used were ADP (1 µmol/l) and thrombin (0.02, 0.03, 0.04, 0.08 IU/ml). When thrombin was used, clotting was prevented by the peptide gly-pro-arg-pro. Within 3 min of collection, 5 µl citrated whole blood was added to 45 µl of Hepes-buffered saline containing antibodies and agonists, and incubated at room temperature for 20 min. Samples were then diluted and fixed with 0.5% formaldehyde saline before analysis using a Coulter EPICS XL-MCL flow cytometer (Coulter, Hialeah, Fla., USA). Platelets were discriminated from other blood cells by their light scattering characteristics and live gated in an electronic bit map. The gated cells were subsequently subjected to single colour analysis of RPE-CD62P fluorescence to obtain the percentage of P-selectin positive cells in the platelet population.

Statistical analysis. Data are expressed as mean ± standard error of the mean, or median and interquartiles. Between-group comparisons were analysed by Student's unpaired two-tailed *t* test or Mann-Whitney *U* test. The effects of agonist stimulation were analysed by two factor repeated measures analysis of variance, ANOVA. Correlations were assessed by Spearman test. A *p* value of less than 0.05 was considered statistically significant. The software used was Statistica (1999 edition, version 5.5; Statsoft, Tulsa, Okla., USA).

Results

Characteristics and biochemical analyses (Table 1). Age, sex, triglycerides, and diastolic blood pressure were similar in the three groups. Duration of disease, metabolic control and BMI did not differ between the two patient groups. Systolic blood pressure and urinary albumin excretion were higher among patients with microangiopathy compared with the control subjects ($p<0.05$ for both), but were not significantly different between patients without complications and the control subjects.

Plasma concentrations of sP-selectin were higher in patients with microangiopathy compared with the control subjects ($p<0.01$) and to patients without microangiopathy ($p<0.05$), but were not significantly different between patients without microangiopathy and the control subjects.

Table 1. Characteristics and biochemical analyses in control subjects and patients

	Control subjects (n=27)	Type 1 diabetes w/o microangiopathy (n=19)	Type 1 diabetes with microangiopathy (n=20)
Age (years)	43.5±2.2	42.3±2.8	42.4±2.8
Sex (female/male)	14/13	9/10	10/10
Smoking (yes/occasionally/no)	6/1/20	5/3/11	3/0/17
Duration of disease (years)		14.7±0.7	15.9±0.7
BMI (kg/m^2)	23.1±0.4	25.4±0.7[b]	25.0±0.7[a]
Blood pressure (mmHg)			
Systolic	116±2	121±3	128±3[b]
Diastolic	74±1	73±1	76±2
HbA_1c (%)[d]	4.6±0.05	6.5±0.2[c]	6.9±0.2[c]
U-albumin (µg/mg of creatinine)	3.8 (2.9; 5.4)	4.2 (3.8; 7.0)	5.9 (4.4; 14.4)[a]
Triglycerides (mmol/l)	1.1±0.1	0.9±0.05	0.9±0.1
HDL-cholesterol (mmol/l)	1.4±0.1	1.6±0.1	1.7±0.1[a]
LDL-cholesterol (mmol/l)	3.0±0.1	2.8±0.2	2.5±0.2
sP-selectin (ng/ml)	40.8±1.7	43.0±2.5	50.5±2.3[b]
sCD40L (ng/ml)	3.95±0.32	4.09±0.40	5.36±0.64[a]
CRP (mg/l)	1.1±0.4	1.6±0.3	3.1±0.8[b]
sE-selectin (ng/ml)	37.1±2.2	38.7±3.4	46.3±3.1[a]
von Willebrand factor (KIU/l)	1.00±0.07	1.12±0.08	1.19±0.10

Values are presented as means ± SEM, except for U-albumin where median with 25th and 75th percentiles are shown.
[a] p<0.05, [b] p<0.01, [c] p<0.001; patients vs control subjects
[d] Reference <5.2% for non-diabetic subjects

Serum concentrations of sCD40L were increased in patients with microangiopathy compared with the control subjects (p<0.05), but did not differ between patients without microangiopathy and the control subjects. CRP was increased in patients with microangiopathy compared with the control subjects (p<0.01), but did not differ between patients without microangiopathy to the control subjects.

sE-selectin was increased among patients with microangiopathy (p=0.02; compared with the control subjects), but did not differ between patient groups, or between patients without microangiopathy and the control subjects. von Willebrand factor antigen did not differ between the three groups.

Monocyte counts were higher in patients with microangiopathy compared with the control subjects (p<0.05). Plasma concentrations of prothrombin fragment F1+2 (a marker of thrombin generation), fibrinogen and urinary 11-dehydro-thromboxane B_2 excretion did not differ between the groups.

Platelet P-selectin expression (Fig. 1). Platelet P-selectin expression in unstimulated samples did not differ between the groups. Thrombin induced platelet P-selectin expression was significantly enhanced in patients with microangiopathy compared with the control subjects (p<0.01), as well as with patients without angiopathy (p<0.05). Platelet P-selectin expression responses to thrombin did not differ between patients without microangiopathy and the control subjects. Responses to ADP (1 µmol/l) did not differ between the three groups.

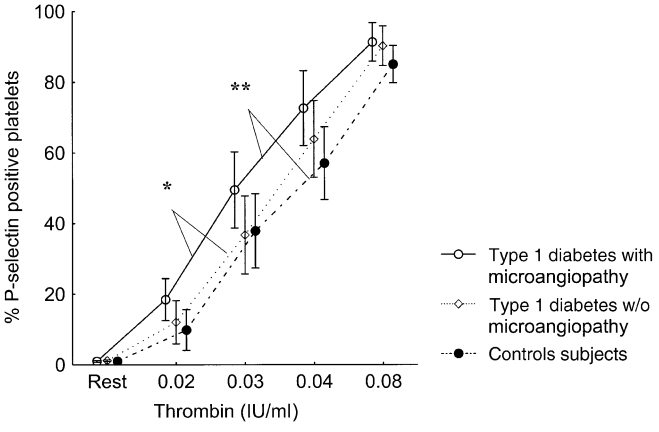

Fig. 1. Platelet responsiveness to thrombin stimulation. Blood samples were incubated in the absence or presence of thrombin. Platelet activation in control subjects and in Type 1 diabetic patients without or with microangiopathy, was monitored by platelet P-selectin expression using whole blood flow cytometry. Data are means ± SEM. Repeated measures ANOVA. *p<0.05; **p<0.01

Correlations. Serum concentrations of sCD40L were correlated with thrombin-induced P-selectin expression (R=0.30; p<0.05; all subjects and R=0.46; p<0.05; patients with microangiopathy), but not with sP-selectin.

Discussion

Our study shows that mild microangiopathy (i.e. early stage retinopathy) in Type 1 diabetes is associated

with platelet hyperactivity, even though the patients were metabolically well controlled. Thrombin induced platelet P-selectin expression was enhanced and soluble P-selectin in plasma was increased in patients with microangiopathy compared with healthy control subjects. Furthermore, we found signs of enhanced inflammatory activity and endothelial dysfunction with higher concentrations of sCD40L, CRP and sE-selectin in serum among the patients with microangiopathy. The findings that soluble P-selectin was increased and platelet P-selectin expression was enhanced among Type 1 diabetic patients with microvascular complications, but not in patients without complications, indicate that platelet activation is related to the presence of microangiopathy, rather than to diabetes per se, and that diabetic microangiopathy is associated with accelerated hemostasis.

Soluble CD40L was increased among our patients with microangiopathy compared with the control subjects. It has been shown that platelets release CD40L upon thrombin stimulation in vitro and during thrombus formation in vivo [5]. Accordingly, in the present study, serum sCD40L concentrations correlated with the thrombin induced P-selectin expression. Recently, increased concentrations of sCD40L were reported in patients with acute coronary syndromes, and concentrations above 5.0 µg/l indicated an increased risk for cardiac events [6]. Notably, our Type 1 diabetic patients with microangiopathy had a mean value of 5.3 µg/l for sCD40L, i.e. in the same range as high risk patients with coronary artery disease.

Both platelet derived P-selectin and CD40L may activate endothelial cells and leukocytes [4, 5]. Thus, platelet activation is not only involved in hemostasis, but could initiate a persistent inflammatory response of the vessel wall, inducing a vicious circle of endothelial perturbation and secondary platelet activation. Our finding of increased circulating CRP among patients with microangiopathy supports the presence of ongoing low-grade chronic inflammation in the Type 1 diabetic vasculature [8] and indicates a high risk for developing macrovascular complications [9].

We found no differences in von Willebrand factor antigen in plasma between groups, which could indicate that endothelial function was only mildly disturbed in the diabetic patients investigated. However, the increase in sE-selectin in our study show that there is an early endothelial perturbation in Type 1 diabetic patients with microangiopathy. Our data are in agreement with the suggestion that von Willebrand factor is a less sensitive marker for endothelial dysfunction than sE-selectin.

In conclusion, our study shows that Type 1 diabetic patients with microvascular complications and good metabolic control have hyper-reactive platelets, as well as signs of endothelial-cell activation and low-grade chronic inflammation. Taken together, the present and a previous study suggest that generalised platelet activation does not precede the development of microvascular complications [10], but this needs to be proven in a longitudinal study. However, our study indicates that Type 1 diabetic patients with early stages of microangiopathy are at increased risk for developing cardiovascular disease.

Acknowledgements. We are grateful to M. Daleskog, M.-C. Johansson, P. Hillelson and L. Sandlund for their expert technical assistance. We also wish to acknowledge Dr. C. Perneby for supervision of the thromboxane metabolite assay and Dr. T. Bystedt for help with defining the patient data. This work was supported by grants from the Swedish Research Council (5930 and 00034), the Swedish Heart Lung Foundation, the Coagulation Research Foundation, Karolinska Institute, the Swedish Society for Medical Research, the Swedish Society of Medicine and the Stockholm County Council.

References

1. Biondi-Zoccai GG, Abbate A, Liuzzo G, Biasucci LM (2003) Atherothrombosis, inflammation, and diabetes. J Am Coll Cardiol 41:1071–1077
2. Laing SP, Swerdlow AJ, Slater SD et al. (2003) Mortality from heart disease in a cohort of 23,000 patients with insulin-treated diabetes. Diabetologia 46:760–765
3. André P, Hartwell D, Hrachovinová I, Saffaripour S, Wagner DD (2000) Pro-coagulant state resulting from high levels of soluble P-selectin in blood. Proc Natl Acad Sci USA 97:13835–13840
4. Burger PC, Wagner DD (2003) Platelet P-selectin facilitates atherosclerotic lesion development. Blood 101:2661–2666
5. Henn V, Slupsky JR, Gräfe M et al. (1998) CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. Nature 391:591–594
6. Heeschen C, Dimmeler S, Hamm CW et al. (2003) Soluble CD40 ligand in acute coronary syndromes. N Engl J Med 348:1104–1111
7. Varo N, Vicent D, Libby P et al. (2003) Elevated plasma levels of the atherogenic mediator soluble CD40 ligand in diabetic patients: A novel target of thiazolidinediones. Circulation 107:2664–2669
8. Schalkwijk CG, Poland DC, Dijk W van et al. (1999) Plasma concentration of C-reactive protein is increased in type 1 diabetic patients without clinical macroangiopathy and correlates with markers of endothelial dysfunction: Evidence for chronic inflammation. Diabetologia 42:351–357
9. Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH (1997) Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. N Engl J Med 336:973–979
10. Alessandrini P, McRae J, Feman S, Fitzgerald GA (1988) Thromboxane biosynthesis and platelet function in type 1 diabetes mellitus. N Engl J Med 319:208–212