Markers of Platelet Activation Are Increased in Adolescents With Type 2 Diabetes

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OBJECTIVE
In adults with diabetes, in vivo platelet activation is a marker for atherosclerosis and cardiovascular disease (CVD). This pilot study investigated whether adolescents with diabetes had evidence of increased in vivo platelet activation.

RESEARCH DESIGN AND METHODS
In vivo platelet activation was compared in four groups of age-matched adolescents: type 1 diabetes (T1D, n = 15), type 2 diabetes (T2D; n = 15), control subjects with normal BMI (n = 14), and overweight/obese control subjects (n = 13). Platelet surface activation markers and plasma levels of soluble activation markers were measured and compared among groups.

RESULTS
Increased expression of all activation markers was observed in T2D compared with either control group (P < 0.05); levels of soluble markers were also higher in T2D than in T1D (P < 0.05). There were no differences in marker expression between the nondiabetic control groups.

CONCLUSIONS
Platelet activation in adolescents with T2D may be a marker for the risk of CVD development in early adulthood.

Cardiovascular disease (CVD) is the primary cause of morbidity and mortality among adults with type 1 (T1D) and type 2 diabetes (T2D). Adults with diabetes have hyperactive platelets that are involved in the initiation and progression of atherosclerosis and in acute arterial thrombosis (1,2). The relationship among diabetes, platelet hyperactivity, and CVD is well established in adults (1,2). The same connection has not been made in adolescents with diabetes, despite observations that some of these adolescents, particularly those with T2D, have risk factors for accelerated progression of atherosclerosis and onset of CVD in early adulthood (3,4).

The purpose of this pilot study was to determine whether we could identify in vivo platelet activation in adolescents with T1D or T2D compared with age- and BMI-matched nondiabetic control subjects by examining the expression of surface-expressed and soluble platelet activation markers.

RESEARCH DESIGN AND METHODS
Study Participants
Adolescents, ages 12–18 years, with T1D (n = 15), T2D (n = 15), and nondiabetic control subjects (n = 27: 14 with a normal BMI; 13 overweight or obese [OB]) were
recruited from a single pediatric center. The diagnosis of diabetes was made according to Canadian Diabetes Association criteria (5). Classification of T2D was based on the absence of diabetes-associated antibodies and clinical criteria (5). Subjects with T1D had been instructed to match their rapid insulin doses to carbohydrate intake using an insulin-to-carbohydrate ratio and encouraged to follow national healthy nutrition guidelines. Subjects with T2D had been counseled to work toward achieving a healthy body weight with decreased portion sizing and elimination of sugared drinks from their diets. Control subjects were recruited from a hospital-based pediatric clinic. They were excluded if they had a diagnosis of T1D or T2D but were not prescreened for other components of the metabolic syndrome. The normal-weight (NW) control subjects had BMIs within the 5th and 85th percentile for age and sex, and the OB control subjects had BMIs >85th percentile for age and sex. Subjects in the control groups were on no specific diet.

Subjects who met inclusion criteria were recruited on a consecutive basis from the respective clinics. Exclusion criteria for all subjects included Prader-Willi syndrome, hypothyroidism, alcohol or drug abuse, congenital CVD, pregnancy, and use of glucocorticoids, lipid-lowering, or platelet-inhibitory medications. The study was approved by the University of Manitoba Human Research Ethics Board and conducted in accordance with the principles of the Declaration of Helsinki 2001. Informed assent and consent were obtained from participants and their parents or guardians.

Age, height, weight, blood pressure, duration of diabetes (where applicable), and medication use data were collected. BMI z scores were calculated from the Centers for Disease Control reference population. Overweight and obesity were classified on BMI z score specific cut points published by the International Task Force on Obesity (6).

Venous blood was collected into citrate tubes after an overnight fast. Blood for ELISA analysis was centrifuged at 1,100g for 20 min at 4°C, and the platelet-poor plasma removed and frozen in aliquots at −70°C until testing. Blood for flow cytometry was processed at room temperature within 60 min of collection. Fasting lipid profile and HbA1c were analyzed in the Clinical Biochemistry Laboratory at the Winnipeg Health Sciences Centre.

**Measurement of Platelet Surface and Soluble Activation Markers**

Flow cytometric analysis was performed on whole blood using the FACS Calibur flow cytometer (BD Biosciences, Mississauga, Ontario, Canada) (7). Fluorophore-conjugated monoclonal antibodies (MoAb) to the following platelet surface antigens were used: CD41 (platelet integrin αⅡbβ3), CD42b (platelet glycoprotein Ib), CD63 (platelet dense granule and lysosomal membrane protein), CD62P (platelet α-granule membrane protein), and MoAb PAC-1 (directed to an activation-induced neoepitope on integrin αⅡbβ3). CD41-positive or CD42b-positive single platelets were identified. For each sample, 10,000 events were acquired and analyzed using Cell Quest Pro software (BD Biosciences). Results are shown as the percentage of platelets expressing CD62P, CD63, or binding PAC-1 MoAb.

Plasma concentrations of platelet-derived activation markers, soluble CD62P (sCD62P), soluble CD40 ligand (sCD40L, a proinflammatory mediator), and platelet factor 4 (PF4, a chemokine stored in platelet α-granules) were measured in triplicate by ELISA (R&D Systems Europe Ltd., Lille, France; or AbCys, Paris, France) (8). Coefficients of variation for all assays were ≤10%.

**Statistical Analysis**

Minimum group sample sizes of 14 subjects were chosen to detect an effect size of 1.2 of diabetes, compared with no diabetes, on each of the assays performed, with a power of 80% and a significance level of 0.05.

Medians and interquartile ranges are reported. Clinical characteristics and markers of platelet activation were compared using the nonparametric Mann-Whitney U test or Kruskal-Wallis ANOVA, followed by the Dunn post hoc test. Spearman rank correlation (r_s) was used to examine relationships among clinical characteristics and the expression of platelet activation markers for all 57 subjects. A P value of <0.05 was considered significant.

**RESULTS**

**Clinical Characteristics**

The clinical characteristics of the participants are reported in Table 1. Duration of diabetes was longer in those with T1D (P = 0.02). All subjects with T1D and five with T2D were prescribed insulin. One subject with T2D was prescribed metformin. The median BMI z scores of the participants with T2D and the OB control subjects were not statistically different but were significantly higher than the BMI z scores of the T1D and the NW control groups. Both groups with diabetes had significantly higher levels of HbA1c and higher mean systolic blood pressures than the control groups. Participants with T2D also had significantly higher levels of triglyceride and lower levels of HDL-cholesterol than the other three groups.

**Expression of Platelet Activation Markers**

There was no difference in the expression of surface activation markers between the NW and the OB control groups (Table 1). Expression levels were significantly higher for all three activation markers in subjects with T2D compared with either control group. Expression levels in subjects with T1D were intermediate between the control and T2D groups, but these differences did not reach statistical significance.

Significantly higher concentrations of the three soluble mediators (sCD62P, sCD40L, PF4) were measured in plasma from subjects with T2D compared with either control group or the T1D group. There were no significant differences in plasma concentrations of soluble mediators among the other three groups.

**Correlation of Clinical Characteristics with Platelet Activation Markers**

HbA1c was positively correlated with CD63 (r_s = 0.49, P < 0.001), CD62P (r_s = 0.37, P < 0.01), and sCD40L (r_s = 0.38, P < 0.01) expression. The BMI z score was positively correlated with PF4 (r_s = 0.48, P < 0.001), sCD62P (r_s = 0.32, P < 0.05), and sCD40L (r_s = 0.27, P < 0.05). A significant negative correlation was observed between HDL-cholesterol and sCD62P (r_s = −0.27, P < 0.05). No relationship was found between duration of diabetes and expression of platelet activation markers.

**CONCLUSIONS**

This study provides evidence of increased in vivo platelet activation in adolescents with T2D. Increased numbers of circulating activated platelets...
have previously been reported in adults with T2D, activation marker expression being highest in patients with detectable vascular disease (2). In vivo platelet activation was not increased in participants with T1D compared with control subjects, although there was a trend toward increased surface marker expression. However, the small sample size may have affected our ability to detect a significant difference, because children with T1D have been shown to have increased carotid intima-media thickness, a marker of early onset atherosclerosis (4).

Increased platelet expression of CD63, CD62P, and sCD40L was positively associated with HbA1c. This has previously been observed in obese adults and is most robust in subjects with the metabolic syndrome (11,12). Obese children and adolescents may have increased circulating levels of inflammatory cytokines, markers of oxidative stress, endothelial dysfunction, and a procoagulant state, even in the absence of diabetes (13,14). Desideri et al. (15) showed that plasma levels of sCD62P and sCD40L were elevated in a group of obese youth compared with age-matched nonobese controls. The current study shows a correlation of levels of sCD62P and sCD40L with BMI z score, although there was no significant difference between the NW and OB control groups for any of the platelet activation markers. In contrast, the group with T2D, with a similar median BMI z score as the OB controls, had increased levels of all platelet activation markers. Additional characteristics of the metabolic syndrome in many of the participants in the T2D group, but not in the OB control subjects, may explain the differences in platelet activation between those with T2D and the OB control subjects (2). These results suggest that not all children with an elevated BMI are equally at risk for platelet hyperactivity but that those with additional risk factors that characterize T2D and/or the metabolic syndrome are more likely to demonstrate markers of oxidative stress, inflammation, and platelet activation.

In summary, we have demonstrated in vivo platelet activation in adolescents with T2D, potentially a marker for the risk of CVD in early adulthood. Longitudinal studies are required to determine if in vivo platelet activation is a clinically useful biomarker for CVD risk in this population and whether platelet activation can be modified by therapeutic interventions that address glycemic control and/or obesity and hypertension.

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