Elevation of monocyte–platelet aggregates is an early marker of type 2 diabetes

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(Received: July 23, 2012; Accepted after revision: November 5, 2012)

Abstract: Background: Diabetes has been shown to be an accelerating factor in the progression of atherosclerosis. The metabolic changes in diabetes contribute to modified platelet function and enhanced leukocyte–platelet aggregate formation. The attachment of activated platelets leads to the activation of leukocytes causing enhanced cytokine production and upregulation of surface adhesion molecules. Therefore, platelet–leukocyte aggregates may be of great importance in the development of cardiovascular complications. Materials and Methods: Monocyte–platelet aggregates and monocyte Mac-1 expression were measured by flow cytometry to obtain differences between type 2 diabetic and healthy subjects. Inflammatory mediators were evaluated to assess the presence of inflammation. Results: We found no signs of inflammation in type 2 diabetes; however, we observed enhanced aggregation level of monocytes and platelets. The expression of Mac-1 did not differ between diabetic and control subjects, but it was significantly higher on monocytes bearing platelets in both groups. Conclusions: Elevation of monocyte–platelet aggregates is an early marker of diabetes, which precedes the signs of inflammation. Enhanced Mac-1 expression can be observed on monocytes bearing platelets, independent from the presence of diabetes.

Keywords: diabetes mellitus, inflammation, monocytes, platelets, cell aggregation, adhesive receptor Mac-1

Introduction

In advanced atherosclerosis featured by intensive plaque formation, plasma levels of inflammatory mediators get increased and circulating leukocytes and platelets become activated [1–7]. Since activated platelets are prone to form aggregates with leukocytes, mostly monocytes, the measurement of heteroaggregates has been shown to be a very sensitive marker of platelet activation [8]. Elevated leukocyte–platelet aggregate levels were found in progressive cardiovascular diseases such as myocardial infarction, angina pectoris and stroke [4, 5, 8–11], as well as in hypercholesterolemia and diabetes [12–14]. Interestingly, monocyte–platelet aggregates have shown a more prominent and permanent increase than neutrophil–platelet aggregates [15] and were associated with acute changes in plasma glucose levels and with carotid atherosclerotic progression in both diabetic and non-diabetic populations [3].

The first step in the formation of heteroaggregates is the binding of P-selectin on platelets to leukocyte surface PSGL-1 (P-selectin glycoprotein ligand) [15]. The second important event is the ligation of αMβ2 integrin (Mac-1, CD11b/CD18) on leukocytes [15], which stabilizes the complex and, hence, is crucial in the survival of monocyte–platelet aggregates in the circulation. Mac-1 is also crucial in the firm adhesion of leukocytes to endothel cells [16, 17].

In advanced atherosclerosis, the level of inflammatory mediators (e.g. CRP, IL-6) proved to be elevated [18]. Therefore, in our study, we evaluated the changes of inflammatory mediators and leukocyte–platelet interactions in type 2 diabetic patients. We investigated whether there are any changes in the expression of Mac-1, the adhesive receptor known to be responsible for the survival of heteroaggregates, and studied if there is any relationship between the expression of Mac-1 and elevated monocyte–platelet levels. Platelet-derived soluble CD40L (CD40 ligand, CD154) is often associated with enhanced atherosclerosis, elevated aggregate formation and platelet activation [19], but there are few and controversial results about its association with diabetes. We also measured soluble CD40L concentrations to analyze its association with type 2 diabetes.

Materials and Methods

Study population

The study population included 14 type 2 diabetic patients (age: 52.31±3.14; 6 males and 8 females), diagnosed at least 6 months before the study with oral glucose tolerance test, with HbA1c levels above 6.5%. Patients were nonsmokers, treated with diet alone or with oral antidiabetic agents and fasted overnight before
sampling. Patients who were receiving insulin or had clinically expressed evidence of coronary artery disease (a history of previous myocardial infarction or angina), renal or hepatic disease were excluded. None of the patients received anti-inflammatory or anti-thrombotic medications within 14 days before sampling. The control group consisted of 14 healthy adult volunteers (age: 43 ± 4.14; 5 males and 9 females) who had not taken any anti-platelet or anti-inflammatory medications for at least 14 days before sampling. The local ethical committee approved the study and written informed consent form was obtained from all subjects.

**Laboratory measurements**

After overnight fasting, blood samples were taken for laboratory measurements. Plasma glucose, HbA1c, cholesterol (total cholesterol, HDL and LDL), triglycerides, monocyte count, platelet count and inflammatory mediators: erythrocyte sedimentation rate (ESR), white blood cell count (WBC) and CRP were measured by standard laboratory techniques in the laboratory of the National Health Institute Budapest (Table I).

| Table I | Laboratory parameters |
|---------|-----------------------|
|         | Diabetes               | Control               | p value |
| Age     | 52.31 ± 3.14           | 43 ± 4.14             | 0.09    |
| Plasma glucose (mmol/L) | 8.66 ± 1.21            | 4.99 ± 0.21           | 0.01    |
| HbA1c (%) | 8.9 ± 0.65            |
| Total cholesterol (mmol/L) | 5.23 ± 0.33            | 5.08 ± 0.31           | 0.74    |
| HDL cholesterol (mmol/L) | 1.4 ± 0.16             | 1.37 ± 0.2            | 0.9     |
| LDL cholesterol (mmol/L) | 3.15 ± 0.44            | 3.04 ± 0.39           | 0.85    |
| Triglycerides (mmol/L) | 2.23 ± 0.46            | 1.37 ± 0.18           | 0.17    |
| WBC (G/L) | 6.71 ± 0.41            | 6.43 ± 0.5            | 0.71    |
| Monocytes (G/L) | 0.33 ± 0.01           | 0.38 ± 0.03           | 0.1     |
| Platelets (G/L) | 281.35 ± 12.38         | 258.63 ± 20.2         | 0.75    |
| CRP (mg/L) | 3.78 ± 1.02            | 2.57 ± 0.94           | 0.59    |
| ESR (mm/h) | 15.83 ± 1.9            | 9.3 ± 3.35            | 0.08    |
| IL-6 (pg/mL) | 15.9 ± 1.92            | 16.95 ± 0.52          | 0.71    |
| sCD40L (ng/mL) | 1.21 ± 0.14            | 1.19 ± 0.2            | 0.97    |
| Mac-1 | 226.73 ± 47            | 206.89 ± 32.8         | 0.73    |
| MPA | 43.8% ± 4.3%           | 31.2% ± 3.11%         | 0.027   |

**Flow cytometry**

Monocyte–platelet aggregates and monocyte Mac-1 (CD11b/CD18) expression were assessed by flow cytometry with FACScalibur (BD Biosciences Heidelberg, Germany) CellQuest Software. For flow cytometric assay, blood samples were prepared as follows: blood was drawn from peripheral vein into commercially available sodium-citrate containing tubes.

Fifty microliters of citrated blood was incubated with monoclonal antibodies for 30 min and then fixed with 0.5% paraformaldehyde. To classify monocyte populations, we used PC-5 conjugated anti-CD14 monoclonal antibody. For the identification of platelets, we used fluorescein isothiocyanate (FITC) conjugated non-blocking anti-CD41 monoclonal antibody. Double positive events were identified as monocyte–platelet aggregates. A PE conjugated non-blocking anti-CD11b antibody identified Mac-1 receptor. All monoclonal antibodies were from BD Biosciences Heidelberg, Germany. For measurements of Mac-1 and aggregates, 3000 CD14 positive events were counted.

**Plasma IL-6 and sCD40L concentrations**

Samples for cytokine analysis were drawn into native tubes; plasma was obtained and stored at −70 °C until further analysis. Plasma concentrations of sCD40L and IL-6 from diabetic and control subjects were determined by enzyme-linked immunosorbent assay using commercially available ELISA kits (IL-6: Diagnosticum Zrt. Budapest, Hungary, sCD40L: R&D Systems, Abingdon, United Kingdom).

**Statistical analysis**

Student’s t-tests or Mann–Whitney test and Pearson’s correlation analyses were used to evaluate the associations between the measured parameters. Results were presented as mean ± SEM.

**Results**

**Laboratory parameters**

There were no significant differences in the laboratory parameters of diabetic and healthy subjects, except for plasma glucose levels, which was significantly higher in the diabetic group (8.66 ± 1.21 vs. 4.99 ± 0.21 mmol/L in the diabetic and control group, p = 0.01), (Table I). We found no significant differences regarding inflammatory parameters (erythrocyte sedimentation rate
(ESR), white blood cell count (WBC), CRP and IL-6) between the type 2 diabetic and the control group.

Formation of monocyte–platelet aggregates in diabetic and control subjects

Our results showed that monocyte–platelet aggregates were significantly elevated in the diabetic group when compared with healthy subjects (43.8%±4.3% vs. 31.2%±3.11% in the type 2 diabetic and control group, \( p = 0.027 \), Fig. 1). Monocyte–platelet aggregate levels showed a correlation with triglyceride levels \( (r = 0.6) \) and with plasma glucose \( (r = 0.52) \) but not with total cholesterol \( (r = 0.18) \), HDL \( (r = -0.49) \), LDL \( (r = 0.39) \) or sCD40L \( (r = 0.19) \).

Monocyte Mac-1 expression in diabetic and control subjects

We also examined the expression of Mac-1 on the monocytes in diabetic and control subjects. We found no significant difference \( (226.73±47 \text{ vs. } 206.89±32.8, p = 0.73 \), Fig. 2). The level of Mac-1 did not correlate with any of the laboratory parameters or sCD40L.

Expression of Mac-1 (CD11b/CD18) on single monocytes and monocytes binding platelets

When we examined the expression of Mac-1, we found that it was present in a significantly higher amount on monocytes binding platelets than on monocytes without platelets (about 40% greater for monocytes binding platelets, \( p = 0.005 \), Fig. 3). The same difference was found in both study groups and in the same manner, regardless of the presence of diabetes.
Discussion

Diabetes is a predisposing factor for atherosclerosis. In advanced disease, significant alterations can be observed in the vessel wall. At the same time, the level of inflammatory mediators get increased, and circulating leukocytes and platelets become activated. Because of these findings, atherosclerosis has been recently described as an inflammatory process [1, 2]. Indeed, in advanced disease featured by intensive plaque formation, acute cardiovascular and cerebrovascular events, a remarkable increase of IL-6, CRP and other inflammatory markers can be detected. This process, which normally proceeds also in healthy individuals with aging, is drastically enhanced by metabolic changes such as diabetes or lipid disorders. Yet, it is not entirely clarified, whether inflammation has a causative role in the process or it is a consequence of the disease, but some results suggest a diagnostic value to inflammatory markers, e.g. CRP [18]. Leukocyte–platelet heteroaggregates have also been shown to be elevated in acute atherosclerotic events such as angina pectoris [5], acute cardiovascular and cerebrovascular events [11, 20] and diabetic angiopathy [13]. In our study, we evaluated inflammatory mediators in type 2 diabetic patients without vascular complications and found no signs of inflammation. In contrast, we found elevated monocyte–platelet aggregate level in diabetic patients compared to healthy subjects. This suggests that the elevation of monocyte–platelet aggregates is a very early marker of the disease reacting rapidly on the metabolic alterations in diabetes. However, the triggering effect of heteroaggregate formation is still not clarified. Leukocyte–platelet heteroaggregate formation has been associated with glucose levels after acute glucose load in healthy and diabetic subjects [13]. In our study, the level of monocyte–platelet aggregates showed no association with laboratory parameters, except for triglyceride and plasma glucose levels.

Platelets attach to monocytes via P-selectin–PSGL-1 interaction, which is strengthened by the ligand binding of Mac-1 [16]. While P-selectin engagement results in a short, reversible interaction, the ligand binding of Mac-1 leads to a stabilization and a longer (15–20 min) survival of the complex, as well as an enhanced adhesiveness to the vessel wall. In this process, Mac-1 plays again a crucial role.

Elevated level of adhesive integrin Mac-1 has been associated with acute cardiovascular and cerebrovascular events and advanced diabetes. In diabetic patients without any signs of vascular complications, we and others [21] found no elevation of Mac-1 in spite of the different basal glucose levels. In contrast, we found a significantly higher expression of Mac-1 on monocytes binding platelets, compared to single monocytes. This difference seems to be independent from the presence of the disease as it could be observed in both diabetic and control subjects and in the same manner.

Platelets have been shown to contribute to inflammatory and atherogenic processes. Activated platelets bind to leukocytes above all monocytes [15] and direct them to attach on vascular endothelium [22]. Moreover, formation of monocyte–platelet aggregates has been shown to be a sensitive marker of platelet activation [8]. On the other hand, sCD40L, which is released from platelets upon activation and has been found to be associated with atherosclerosis was not elevated in our study in type 2 diabetic patients. Evaluation of other markers of platelet activation in early diabetes as well as a long term follow-up of leukocyte–platelet aggregate levels would be of interest.

Conclusions

Elevated monocyte–platelet aggregates can be observed in type 2 diabetes when no signs of inflammation are present. This may indicate a more sensitive predictive value for heteroaggregates than inflammatory markers. In our study, the elevation of Mac-1 on monocytes was independent from the presence of the disease.

Abbreviations

CRP: C-reactive protein; FITC: fluorescein isothiocyanate; HDL: high density lipoprotein; LDL: low density lipoprotein; PE: phycoerythrin

Acknowledgements

We would like to thank the laboratory of the Hungarian Military Hospital, especially Prof. Susan Lakatos and Dr. János Fent for the support of this work and the methodological instructions.

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