Platelet Degranulation Is Associated With Progression of Intima-Media Thickness of the Common Carotid Artery in Patients With Diabetes Mellitus Type 2

Suzanne Fateh-Moghadam, Zonyang Li, Simon Ersel, Thomas Reuter, Patrik Htun, Ursula Plöckinger, Wolfgang Bocksch, Rainer Dietz, Meinrad Gawaz

Background—Platelets play a key role in atherogenesis and thromboembolic complications in patients with type 2 diabetes.

Methods and Results—We prospectively examined the relationship between systemic platelet activation and progression of carotid wall thickness within 1 year in 105 patients with type 2 diabetes. The intima-media thickness (IMT) of the common carotid artery was measured bilaterally at study entry and after 1 year. Platelet activation was assessed with the use of immunologic markers of platelet activation (CD62P, CD63, and CD40L) and flow cytometry. The prevalence for progression of atherosclerotic carotid disease in this population was 55.2%. We found that platelet degranulation (CD63 and CD40L) correlated with progression of IMT within 1 year (CD63: \( r = 0.231, P = 0.022 \); CD40L: \( r = 0.230, P = 0.029 \)). Diabetic patients with progression of IMT had a significantly increased expression of CD63 compared with patients with stable carotid disease (mean intensity of immunofluorescence; median, interquartile range: 17.1 [12.4, 25.8] versus 11.9 [7.7, 19.8]; \( P = 0.004 \)). Multivariate logistic regression analysis revealed that degranulation of platelet CD63 is a predictor for progression of IMT independently of classical cardiovascular risk factors and hemoglobin A1c in diabetic patients (\( P = 0.017 \)).


Key Words: atherosclerosis • cell adhesion molecules • diabetes mellitus • platelets

Accelerated atherosclerosis and diabetic microvascular disease make diabetes a leading cause of coronary artery disease, ischemic stroke, retinopathy, and chronic renal failure.

Diabetes has a number of effects on platelet function that may predispose to atherosclerosis. These include increased primary and secondary platelet aggregation, increased platelet activation with release of the contents of \( \alpha \)-granules, including \( \beta \)-thromboglobulin and platelet factor 4, and enhanced surface expression and activation of platelet glycoprotein IIb-IIIa (GPIIb-IIIa) complex. Moreover, platelet NO synthase activity is reduced in diabetes. Furthermore, a hypersensitivity of platelets to collagen and increased platelet Fc receptor expression in diabetes have been described.

An increase in systemic platelet activation has been described for a variety of atherosclerotic diseases, including coronary artery disease, transplant vasculopathy, and cerebrovascular disease. In a mouse model of atherosclerosis, enhanced platelet adhesion to the carotid vessel wall promotes atherogenesis. It was found recently that activation of circulating platelets is associated with enhanced wall thickness of the carotid artery in humans. However, a prospective analysis of the role of systemic platelet degranulation and progression of carotid artery disease in patients with diabetes mellitus type 2 is missing.

In the present study, we prospectively analyzed whether systemic platelet activation is associated with progression of carotid artery disease in type 2 diabetes using intima-media thickness (IMT).

Methods

Patients
A total of 105 patients who underwent routine clinical follow-up at the outpatient clinic were consecutively and prospectively studied...
Peripheral arterial disease was present or significant lesions (53; 50.5%). Macroangiopathy was defined when symptomatic coronary artery disease or angiography was performed. Values above normal range were controlled twice. In patients with albuminuria, which was measured in every patient at study entry. Onset of macroangiopathy was confirmed by an ophthalmologist. Nephropathy was defined depending on the albumin excretion (presence of macroalbuminuria or microalbuminuria), which was measured in every patient at study entry. Hypertension, defined as weight in kilograms divided by the square of height in meters, family history (first-degree relatives with symptomatic coronary heart disease). Microangiopathy was defined when either retinopathy or nephropathy were present. Retinopathy was confirmed by an ophthalmologist. Nephropathy was defined depending on the albumin excretion (presence of macroalbuminuria or microalbuminuria), which was measured in every patient at study entry. Values above normal range were controlled twice. In patients with suspected coronary artery disease, angiography was performed (n=53; 50.5%). Macroangiopathy was defined when symptomatic peripheral arterial disease was present or significant lesions (>50% lumen narrowing) in at least 1 major epicardial coronary artery were identified by angiography.

**Ultrasound Evaluation**

All patients (n=105) were evaluated twice by ultrasonographic scanning of the carotid artery at time of study entry and after 1 year. Ultrasonographic scanning was performed using an ultrasonic phase-locked echotracking system, which was equipped with a high-resolution real-time 8-MHz linear 2D scanner (System Five; GE-Vingmed). The IMT was measured bilaterally at a defined location: 30 mm proximally to the bifurcation at the far wall of the common carotid artery (CCA) provided that this point was free of calcification and the internal and external carotid arteries. We marked the definite sites where we did the IMT measurements.

**Figure 1.** Schematic representation of the CCA, the bifurcation, and the internal and external carotid arteries. We marked the definite sites where we did the IMT measurements.
variability of 0.03±0.02 mm. After 1 year, the patients were re-evaluated and determination of the IMT was done in the same way as at time of study entry. Hence, the IMT measurements were performed prospectively. Mean IMT was calculated for the right and left carotid artery in the following way: mean IMT=IMTleft+IMTright. The difference of mean ΔIMT (mean ΔIMT=mean IMT after 1 year−mean IMT at study entry) within 1 year was calculated and used as index of disease progression. A mean ΔIMT of >0 indicates disease progression over 1 year.

**Platelet Analysis**

For flow cytometric analysis, 0.5 mL of blood was collected into a polystyrene syringe with 1.0 mL of a fixative that contained methacrolein (CyfixII). Evaluation of surface expression of platelet membrane glycoproteins (CD62P, CD40L, and CD63) was performed with specific monoclonal antibodies and 2-color whole blood flow cytometry, as described in detail previously. Specific monoclonal antibody binding was expressed as relative intensity of immunofluorescence and was used as a quantitative measurement of glycoprotein surface expression. The platelet assay used in the present study obtains reproducible results without significant artificial platelet activation and has proved suitable for platelet analysis in a variety of clinical settings. To establish reference values, control samples obtained from healthy individuals were always run and processed simultaneously with patient samples. Correlation of markers was: CD62P versus CD40L, $r=0.274$, $P=0.009$; CD62P versus CD63, $r=0.379$, $P=0.001$; and CD40L versus CD63, $r=0.622$, $P=0.0001$. Coefficients of variance (CV) for the flow cytometric parameters were: CV (CD62P)$=9.4$%; CV (CD63)$=9.6$%; and CV (CD40L)$=12.2$%.

**Statistical Analyses**

Results of flow cytometric parameters are reported as median (interquartile range) unless otherwise indicated. Differences between groups were evaluated by using appropriate unpaired nonparametric tests (Mann–Whitney U test). A multiple logistic regression analysis implementing an automatic stepwise selection algorithm for risk factor inclusion was performed to assess independent risk factors for the progression of disease. A value of $P<0.05$ was regarded as significant (SPSS; Windows version 10.0).

**Results**

Between November 2001 and February 2003, we consecutively investigated 105 patients with type 2 diabetes who presented for optimization of antidiabetic therapy. At 1-year follow-up, paired ultrasound data were available for all 105 enrolled patients. The basic demographic and clinical characteristics are given in Table 1. There was no statistically significant difference in clinical and disease-related laboratory parameters nor medication between patients that presented with progression of carotid artery disease and patients with stable carotid artery disease (Table 1). During the observation period, medical treatment was kept almost constant. All patients were seen in defined intervals for follow-up evaluation. There were no significant differences in medical treatment at study entry and during follow-up (data not shown).

At time of enrollment, surface expression of membrane glycoproteins CD62P, CD63, or CD40L on circulating platelets was not associated with the degree of mean IMT of the carotid artery (CD63: $r=0.185$; $P=0.069$; CD40L: $r=0.053$; $P=0.621$; CD62P: $r=0.132$; $P=0.196$). However, we found that platelet degranulation of CD40L and CD63 correlated with mean ΔIMT as a marker of disease progression (CD63: $r=0.231$, $P=0.022$; CD40L: $r=0.230$, $P=0.029$; Figure 2). Patients were divided in 2 groups: patients with progression of carotid disease (n=58; mean ΔIMT=0.255 [0.110 and 0.423]; median [25th and 75th quartile]) and those with stable carotid artery disease (n=47; mean ΔIMT=−0.005 [−0.25 and −0.01]). Patients with progression had a significantly higher platelet expression of CD63 than patients with stable carotid artery disease (mean intensity of immunofluorescence; median, interquartile range: 17.1 [12.4, 25.8] versus 11.9 [7.7, 19.8]; $P=0.004$).

To test whether systemic platelet activation is associated with disease progression independent of cardiovascular risk factors and metabolic control of the disease, we performed a multivariate logistic regression analysis including the parameters age, gender, hemoglobin A1c (HbA1c), obesity, smoking, hypertension, hypercholesterolemia, family history, CD40L, CD62P, and CD63, statin medication, and mean IMT at study entry (Table 2). Among the variables tested, only CD63 immunoreactivity was associated independently with the progression of carotid artery disease ($P=0.017$). Thus, type 2 diabetes patients with enhanced surface expression of CD63 are at risk for progression of carotid artery disease. To predict progression of carotid disease for an individual patient with type 2 diabetes by using the CD63 immunoreactivity, we calculated the receiver operating characteristic (ROC) curve. The area under the ROC curve was 0.668. The optimum cutoff value was 12.4, with a sensitivity of 78.2% and a specificity of 58.1% (95% CI, 55.9 to 77.8). The prevalence for progression of carotid disease in this population was 55.2%. Thus, patients with enhanced CD63 levels (> 12.4)
had a 1.6-fold relative risk (95% CI, 1.1% and 2.2) for carotid artery progression.

**Discussion**

The findings of this study indicate that patients with type 2 diabetes and an increased systemic platelet activation have accelerated disease progression of atherosclerosis in the carotid arteries. Moreover, the platelet degranulation marker CD63 is an independent risk factor for progression of carotid artery disease.

Atherosclerosis is a chronic inflammatory disease influenced by circulating cells, including platelets. Platelet adhesion to the carotid artery occurs early during atherogenesis, which, in turn, leads to several steps in the development of atherosclerosis as release of proinflammatory cytokines, chemoattractants, and leukocyte infiltration. Chronic inhibition of systemic platelet activation reduces leukocyte accumulation and attenuates the progression of atherosclerotic lesions in cholesterol-fed apolipoprotein E–deficient mice. Platelets may mediate such effects by means of products released after adhesion and activation. Indeed, platelet-derived chemokines such as platelet factor 4 and other platelet-derived growth factors are found in atherosclerotic plaques, where they express biologic activities that may contribute to several aspects of the disease. Platelets have been shown recently to stimulate the atherogenic properties of endothelial cells via CD40 ligand and interleukin-1β.

We reported previously that systemic platelet activation is associated with an accelerated progression of transplant vasculopathy, suggesting that platelet activation is also critical for atherogenesis in humans. In the present study, we show that in patients with type 2 diabetes, systemic enhanced platelet activation as indicated by release of the contents of granules (CD63 and CD40L) is associated with progression of carotid artery disease, which strongly suggests an important role of systemic platelet activation in the course of the disease. It has been demonstrated previously that platelet degranulation is associated with atherosclerotic wall thickness in humans with cardiovascular risk factors. Our data suggest that platelet degranulation is not only associated with carotid wall thickness but is a risk factor for progression of carotid artery disease in type 2 diabetes. Thus, our study further supports and strengthens the importance of platelets for atherogenesis in diabetes.

In contrast to Koyama, we could not show a strong correlation between initial IMT at study entry and platelet degranulation of P-selectin. This discrepancy may be attributable to important differences in the study groups and to methodological differences in ultrasonographic scanning of the IMT of the CCA. Our study group consisted exclusively of type 2 diabetic patients, whereas in the group of Koyama, only 36.1% were diabetic patients. Further differences of the patients in the Koyama group were the lower mean body mass index of 23.1±3.2 kg/m² (versus 30.9±7.9 kg/m²), a lower HbA1c of 5.7±1.4% (versus 7.5±1.7%), and a slightly younger age (56.8±12.0 versus 62.0±8.1 years), as well as a smaller proportion of hypertensive patients (35.6 versus 82.9%).

Further, the ultrasonographic scanning of the IMT was performed differently from our study. Koyama measured the site of the most advanced atherosclerotic lesion of the CCA and hence correlated the maximum IMT of the CCA with P-selectin expression. Because we were interested mainly in progression, we did not record the maximum IMT but measured IMT bilaterally at a defined location, irrespective of being the most advanced lesion or not at study entry and after 1 year. Hence, the measured IMT of Koyama et al is hardly comparable to our IMT. In our study, neither initial IMT nor baseline HbA1c was an independent predictor for progression of IMT as described by Yamasaki et al. As mentioned above, because of differences in study design and patient characteristics, both studies are hardly comparable. Similar to the study of Koyama et al, the mean body mass index of the patient cohort in Yamasaki’s study was also significantly lower (22.6±0.17 kg/m² versus 30.9±7.9 kg/m²). Furthermore, Yamasaki et al excluded all patients with microangiopathy and macroangiopathy.

Enhanced platelet activation in type 2 diabetes may enhance the risk of athero-progression and manifestation of atherothrombotic complications. It is tempting to speculate that diabetic patients might benefit from an intensified antiplatelet treatment in primary and secondary prevention. For example, it has been suggested that in patients with coronary artery disease, soluble CD40L is a risk factor in acute coronary syndromes, and that elevation of soluble CD40 ligand identifies a subgroup of patients at high risk who are likely to benefit from antiplatelet treatment with GPIIb-IIIa antagonists.

Indeed, some clinical treatment studies already exist that show a causal role for platelet activity and progression of IMT of the carotid artery. Ranke et al showed that aspirin treatment slowed carotid plaque growth in a dose-dependent manner, with a dose of 900 mg daily more efficient than 50 mg daily. Kodama et al evaluated the effectiveness of long-term antiplatelet therapy in attenuating progression of IMT of the carotid artery of subjects with type 2 diabetes.

**Table 2. Multivariate Logistic Regression Modeling for Predictors of Progression of Carotid Artery Disease in Type 2 Diabetes**

<table>
<thead>
<tr>
<th>Parameters Tested</th>
<th>Regression Coefficient B</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predictive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD63</td>
<td>−0.109</td>
<td>0.017*</td>
</tr>
<tr>
<td>CD62P</td>
<td>0.037</td>
<td>0.624</td>
</tr>
<tr>
<td>CD40L</td>
<td>0.006</td>
<td>0.416</td>
</tr>
<tr>
<td>Mean IMT (study entry)</td>
<td>1.568</td>
<td>0.068</td>
</tr>
<tr>
<td>HbA1c</td>
<td>−0.194</td>
<td>0.292</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>1.210</td>
<td>0.194</td>
</tr>
<tr>
<td>Systemic hypertension</td>
<td>−0.994</td>
<td>0.233</td>
</tr>
<tr>
<td>Active smokers</td>
<td>0.322</td>
<td>0.695</td>
</tr>
<tr>
<td>Family history</td>
<td>0.055</td>
<td>0.924</td>
</tr>
<tr>
<td>Obesity</td>
<td>0.357</td>
<td>0.695</td>
</tr>
<tr>
<td>Age</td>
<td>−0.032</td>
<td>0.472</td>
</tr>
<tr>
<td>Gender (male)</td>
<td>−0.228</td>
<td>0.684</td>
</tr>
<tr>
<td>Statin medication</td>
<td>−0.275</td>
<td>0.695</td>
</tr>
</tbody>
</table>
Subjects who had an IMT over the threshold of normal subjects but showed no vascular events were randomly divided into groups given antiplatelet drugs, ticlopidine (n = 34), a small dose of aspirin (n = 40), or no drugs (control group; n = 74). After the follow-up period of 3.0 ± 0.06 years, the control group showed a significantly higher progression of IMT (0.067 ± 0.009 mm/year) than those given ticlopidine (0.034 ± 0.013 mm/year) or aspirin (0.033 ± 0.010 mm/year). These data indicated that antiplatelet drugs, irrespective of their pharmacological mechanisms, could attenuate the progression of the IMT of the carotid artery wall of asymptomatic type 2 diabetes.

Acknowledgments

This study was supported in part by grants from the Charité Research (n/H11005 40), or no drugs (control 34), a small dose of aspirin (n/H11006 40). The authors thank Professor Ries for the opportunity to use his laboratory facilities.

References

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Arterioscler Thromb Vasc Biol. 2005;25:1299-1303; originally published online April 7, 2005; doi: 10.1161/01.ATV.0000165699.41301.c5
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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