Plasma Fibrinopeptide A, β-Thromboglobulin, and Platelet Factor 4 in Diabetic Retinopathy

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We examined plasma levels of fibrinopeptide A, β-thromboglobulin, and platelet factor 4 in diabetic patients. Among diabetic patients (n = 33) plasma levels of fibrinopeptide A, β-thromboglobulin and platelet factor 4 were significantly higher than in controls (n = 41). In the subgroups of diabetic patients with (1) minimal (n = 13), and (2) moderate-severe (n = 14) retinopathy only plasma fibrinopeptide A levels were significantly higher than in controls. Among the total group of diabetic patients plasma levels of fibrinopeptide A increased significantly with increasing severity of retinopathy. These results suggest that diabetic retinopathy is associated with in vivo activation of blood coagulation factors and that this activation increases with advancing retinopathy. Invest Ophthalmol Vis Sci 29:856–860, 1988

There have been many studies investigating the possible role of altered platelet function and blood coagulation in the pathogenesis of the vascular complications found among diabetic patients. The results of these studies have been conflicting. For instance, platelet and von Willebrand factors have been reported to be increased, decreased or normal among diabetic patients. Such diverse results may be partly due to differing assay methodologies. Also, the interpretation of blood changes has been difficult because such changes could be the result, rather than the cause, of the microvascular lesions found among diabetic patients.

Fibrinopeptide A (FPA) is a small peptide that is specifically cleaved from the A α chain of fibrinogen by the action of thrombin. Thus, its plasma levels reflect in vivo activation of the coagulation cascade. In a preliminary study we observed an elevation of plasma FPA in three insulin-dependent diabetic patients with only one or two small retinal cotton wool spots. These observations led to the suggestion that isolated small retinal cotton wool spots—histopathologically retinal microinfarcts—may be an early finding in diabetic retinopathy and that there might have been activation of the coagulation cascade early on in the course of their development. Thus, the purpose of the present study was to investigate in vivo plasma coagulation and platelet factors in diabetic patients and to examine for possible relationship to severity of the retinopathy.

Materials and Methods

Twenty-one Type I and 12 Type II, insulin-using diabetic outpatients of the National Eye Institute (Bethesda, MD) were studied. Excluded were patients receiving salicylate or anticoagulant medication or with renal disease (serum creatinine > 1.2 mg/dl and proteinuria). All patients had a complete eye examination, including fundus examination by direct and indirect ophthalmoscopy, and Goldmann 3 mirror contact lens examination. Grading of diabetic retinopathy was based on clinical examination and seven stereo fundus color photographs, as defined by the Diabetic Retinopathy Study. Additionally, in 23 of the 33 patients retinal fluorescein angiography was performed.

Diabetic patients were divided into three groups on the basis of the severity of retinopathy in the worse eye. Group I (n = 6) had no evidence of diabetic retinopathy. Group II (N = 13) had minimal retinopathy: nine patients had one to five microaneurysms, three had one to three 200 μm retinal cotton wool spots in the absence of microaneurysm, and one had one 250 μm retinal cotton wool spot and three microaneurysms. Group III (n = 14) had moderate-severe retinopathy (either severe background diabetic retinopathy (n = 9), or preproliferative or proliferative retinopathy (n = 5)).

The control group consisted of 41 normal volunteers who were not diabetic, who were medication-free for at least 2 weeks, and who had no history of bleeding disorder or systemic disease. In all subjects blood was collected for coagulation studies by a dou-
ble syringe technique, without stasis, from an antecubital vein. This was done by one of the investigators (MER) who was unaware of the retinopathy status of the patient. Subjects were included only if a clean venipuncture and free flow of venous blood were obtained. In all subjects informed consent was obtained after the nature of the procedures had been fully explained.

Plasma levels of FPA were measured using a radioimmunoassay (Mallinkrodt, St. Louis, MO) and plasma levels of β-thromboglobulin (β-TG) and platelet factor 4 (PF4) by radioimmunoassay (Amer sham, Arlington Heights, IL and Abbott, North Chicago, IL, respectively). Platelet size was determined with a Particle Data Cellozone computerized system (Particle Data Inc., Elmhurst, IL). Plasma levels of factor VIII and von Willebrand factor were assessed using a one-stage VIII:C assay, a Laurell assay for von Willebrand factor antigen (vWF Ag) and a ristocetin cofactor activity assay. Analysis of von Willebrand factor multimers was carried out by the method of Hoyer and Shainoff. In the diabetic patients levels of glycosylated hemoglobin using ion exchange chromatography and plasma fibrinogen were determined.

In the statistical analysis, analysis of variance (ANOVA), nonparametric Kruskal-Wallis test, Pearson’s and Kendall’s methods of correlation were used. Plasma FPA values were log transformed to normalize the distributions and stabilize the variances. P values from post hoc analyses of the ANOVA or Kruskal-Wallis tests were adjusted for multiple comparisons by multiplying the obtained P values by 6, the number of pairwise comparisons from the four groups (control group and three diabetic groups). Associations of continuous variables and severity of retinopathy were tested with the Jonckheere statistic and, to adjust for other covariates, with polychotomous logistic regression. All P values were two-tailed.

Three diabetic patients with moderate-severe retinopathy (Group III) had an elevation of plasma levels of PF4 comparable to that of plasma levels of β-TG, indicating that in vitro platelet activation had occurred. Thus, these patients were excluded from the analyses of the β-TG and PF4 results.

### Results

There were no significant differences between diabetic patients and controls, or between diabetic subgroups, for age, sex, age of onset of diabetes or glycosylated hemoglobin levels. However, the severity of retinopathy was significantly associated with increasing duration of diabetes (Jonckheere statistic P < 0.001) (Table 1).

#### Plasma Fibrinopeptide A

There were significant differences among the four groups (controls, patients with no, minimal and moderate-severe retinopathy) for plasma FPA values (F = 9.34, df = 3,28, P < 0.001); specifically, diabetic patients with minimal (n = 13) or moderate-severe (n = 14) retinopathy had higher plasma FPA levels than controls (n = 35) (P = 0.04 and P = 0.002, respectively) (Table 2, Fig. 1). Similar results were obtained using the nonparametric Kruskal-Wallis test.

In the total group of patients, increasing plasma FPA levels were significantly associated with increasing severity of retinopathy (P = 0.03). This remained significant (P = 0.04) even after adjusting for age, sex and duration of diabetes with logistic regression. There was no significant association of plasma FPA levels and duration of diabetes (Kendall rank correlation = 0.14, P > 0.10) (Fig. 2).

Plasma FPA levels were not significantly associated with age or sex in either diabetic or control groups. Among the diabetic patients plasma FPA levels were not significantly associated with plasma fibrinogen (r = 0.13, n = 26, NS) or glycosylated hemoglobin (r = 0.08, n = 32, NS) levels.
Table 2. Plasma coagulation and platelet factors in diabetic patients, by retinopathy status, and controls

<table>
<thead>
<tr>
<th>Diabetic patients by grade of retinopathy</th>
<th>None</th>
<th>Minimal</th>
<th>Moderate-severe</th>
<th>Total</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>Fibrinopeptide A (ng/ml)*</td>
<td>5</td>
<td>1.4 (0.1)</td>
<td>13</td>
<td>3.1 (1.1)†</td>
<td>14</td>
</tr>
<tr>
<td>β-thromboglobulin (ng/ml)*</td>
<td>6</td>
<td>34.6 (6.3)</td>
<td>12</td>
<td>37.6 (4.3)</td>
<td>11</td>
</tr>
<tr>
<td>Platelet factor 4 (ng/ml)</td>
<td>6</td>
<td>5.8 (2.9)</td>
<td>12</td>
<td>7.2 (3.1)</td>
<td>11</td>
</tr>
<tr>
<td>Platelet size (µ3)</td>
<td>6</td>
<td>7.7 (1.0)</td>
<td>11</td>
<td>7.5 (1.1)</td>
<td>12</td>
</tr>
<tr>
<td>Factor VIII C (%)*</td>
<td>6</td>
<td>110 (34)</td>
<td>13</td>
<td>103 (14)</td>
<td>13</td>
</tr>
<tr>
<td>Ristocetin cofactor (%)*</td>
<td>6</td>
<td>129 (48)</td>
<td>13</td>
<td>128 (28)</td>
<td>13</td>
</tr>
</tbody>
</table>

Values are expressed as either mean (SD) or, for data not normally distributed, as median (interquartile range/2).

Normal ranges in our laboratory: for platelet size (3SD) 4.1-9.02 µ3; for Factor VIII C 50-200%; for ristocetin cofactor 59-143%; for von Willebrand factor antigen 55-168%.

† Patients with (1) minimal or (2) moderate-severe retinopathy had significantly higher plasma FPA levels than controls (P = 0.04, P = 0.002 respectively).

‡ The total group of diabetic patients compared with controls had significantly higher plasma levels of FPA (P < 0.001), β-TG (P < 0.001) and PF4 (P < 0.001).

Platelet Release Proteins, β-Thromboglobulin and Platelet Factor 4, and Platelet Size

The total group of diabetic patients had significantly higher plasma levels of both β-TG (Kruskal-Wallis X2 = 12.16, df = 1, P < 0.001) and PF4 (Kruskal-Wallis X2 = 10.94, df = 1, P < 0.001) than controls. There were no significant differences between any diabetic subgroup and controls for plasma levels of β-TG and PF4 even after adjusting for age and sex (Table 2). Also, these factors were not associated with the severity of diabetic retinopathy.

In the total group of diabetic patients, plasma levels of β-TG and FPA were significantly associated (r = 0.28, n = 28, P < 0.05). There was no significant association between platelet size and severity of diabetic retinopathy even after adjusting for age, sex and duration of diabetes (Table 2).

Factor VIII and von Willebrand Factor

Plasma levels of factor VIII coagulant activity, ristocetin cofactor or vWF Ag were not significantly associated with severity of diabetic retinopathy after adjustment for age, sex and duration of diabetes (Table 2) or with plasma glycosylated hemoglobin levels. Analysis of von Willebrand factor multimers showed normal patterns with no increase or decrease in the largest multimers (n = 22, data not shown).

Discussion

In the present study we found that plasma FPA levels were significantly higher in diabetic patients with minimal and moderate-severe retinopathy than in controls. Furthermore, among the diabetic patients plasma FPA levels increased significantly with increasing severity of the retinopathy.

Other investigators have also reported elevated plasma FPA levels in diabetic patients with macro- or microangiopathy. However, except for the study of Jones, the retinopathy status of the diabetic patients was either not documented at all or not carefully determined, in contrast to the present study.

Two other studies did not find raised plasma FPA levels in diabetic patients, with or without microangiopathy. However, in one of these studies the range of normal values of plasma FPA (1.6–19.2 ng/ml) was greater than that found among controls either in the present study (0.8–4.6 ng/ml) or in other reports. False elevation of plasma FPA levels may occur from either difficult blood collection or sample processing.
All of the current systems to grade the severity of diabetic retinopathy have shortcomings.\textsuperscript{26} We chose not to use the Wisconsin system,\textsuperscript{27} as it tends to be relatively insensitive to early changes such as small isolated cotton wool spots. In our study Groups I to III represent definite increases in severity of the diabetic retinopathy. Furthermore, the FPA results remain unchanged when the four patients with isolated cotton wool spots are excluded from the analysis.

The lack of significant association between FPA and fibrinogen levels found in the present study suggests that in the diabetic patients the elevated FPA levels reflect an activation of the coagulation factors earlier in the cascade with resultant increased thrombin production rather than an increase in circulating fibrinogen.

Jones\textsuperscript{22} has hypothesized that the hemodynamic changes associated with hypo- or hyperglycemia may lead to vessel damage and secondary activation of the coagulation cascade. As we have not had the opportunity to assess short-term metabolic control in our diabetic patients we have to date been unable to test this hypothesis. However, we did not demonstrate a previously reported association between plasma FPA levels and long-term diabetic control, as assessed by plasma glycosylated hemoglobin levels.\textsuperscript{21,23}

In diabetic patients actual thrombosis in the retinal vessels has not been observed on pathological examination. Usually, the initial clinicopathological change seen is that of a patchy and localized retinal capillary pericyte loss.\textsuperscript{28} While this could result from metabolic cell death,\textsuperscript{29} the clustering of the cell loss within a retinal capillary unit might also indicate a localized change in the blood supply to that unit.\textsuperscript{30} Indeed, clinical observations of the natural history of diabetic retinopathy suggest that the primary pathologic event seen is that of a patchy and localized retinal capillary unit might also indicate a localized change in the blood supply to that unit.\textsuperscript{31} The role of platelet abnormalities in relation to diabetic retinopathy remains controversial.\textsuperscript{1-3} We chose not to perform platelet aggregation studies because this in vitro phenomenon is not a quantitative method to evaluate platelet activation. However, in vivo platelet activation can be assessed by measuring plasma levels of the \( \alpha \)-granule secretion proteins \( \beta \)-TG and PF\(_4\).\textsuperscript{3} In the present study we found, as have other investigators,\textsuperscript{21,22,24,25,34-36} increased plasma levels of \( \beta \)-TG and PF\(_4\) in the total group of diabetic patients.

We were unable to demonstrate the previously suggested association between plasma levels of these platelet specific proteins and severity of diabetic retinopathy.\textsuperscript{35} However, our findings do not rule out such an association. Indeed, based upon 95\% confidence interval calculations,\textsuperscript{14} our data are consistent with \( \beta \)-TG values among patients with moderate-severe retinopathy exceeding those of no retinopathy patients by as much as 11 ng/ml. Conversely, our data are consistent with \( \beta \)-TG values in the no retinopathy group exceeding those in the moderate-severe retinopathy group by as much as 9 ng/ml. Similar calculations for PF\(_4\) indicate that the true difference between patients with moderate-severe retinopathy and those with no retinopathy is likely to be between \(-2 \) and \(+4\) ng/ml. In our patients the significant association between plasma levels of FPA and \( \beta \)-TG suggests that platelet activity may have resulted in part from increased thrombin activity.\textsuperscript{37}

Like others,\textsuperscript{24,38,39} we did not confirm early reports\textsuperscript{40,41} of increased von Willebrand factor in diabetic patients with severe retinopathy. Differences between studies could be methodological\textsuperscript{42} or reflect less severe endothelial damage in our patients. The latter possibility is also supported in our study by the absence of increase in the largest molecular weight vWF multimers, although increased clearance of these larger forms cannot be excluded.

In summary, our results suggest that activation of the blood coagulation system, as reflected by raised plasma FPA levels, is associated with the development of diabetic retinopathy. Furthermore, our data suggest that this activation increases with increasing severity of retinopathy. Future prospective studies of a larger group of diabetic patients may be helpful in determining whether or not activation of the coagula-
tion system is a cause, or a result, of the microvascular changes seen in diabetes.

Key words: β-thromboglobulin, diabetic retinopathy, factor VIII, fibrinopeptide A, platelet factor 4, von Willebrand factor

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References