Altered fibrin-clot properties are associated with retinopathy in type 2 diabetes mellitus

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Abstract

Aim. – The development and progression of diabetic retinopathy (DR) in type 2 diabetes mellitus (T2DM) have been associated with poor glycaemic control, long disease duration and other clinical features. However, the pathogenesis of the complication is still poorly understood. As the formation of dense fibrin clots resistant to lysis has been described in diabetes patients, this study tested the hypothesis that altered clot structure and function are associated with DR in T2DM patients.

Methods. – The study included 101 T2DM subjects without DR (NDR) and 60 with DR. Plasma fibrin-clot permeation was assessed using a pressure-driven system, and expressed as the permeation coefficient (Ks), indicating pore size, and as the time required for a 50% decrease in clot turbidity (t50%) as a marker of susceptibility to fibrinolysis. All patients underwent ophthalmological examination. Clinical and biochemical co-variables were also measured. Determinants of DR were identified using stepwise, multivariable, logistic-regression analyses.

Results. – Patients with DR had lower clot permeability (Ks: 6.15 ± 1.18 vs. 7.53 ± 1.24 10−9 cm2; P < 0.0001) and slower fibrin-clot lysis (t50%: 10.12 ± 1.24 vs. 9.12 ± 1.4 min; P < 0.0001) than NDR subjects. Logistic analysis revealed associations between DR and Ks, t50%, fasting glucose and diabetes duration, as well as insulin treatment and statin non-use (P < 0.05). After adjusting for these variables as well as for age and gender, associations between Ks and t50% with DR proved to be significant.

Conclusion. – Formation of compact fibrin clots and impaired clot lysis are both associated with DR in T2DM patients. However, it is unclear whether these abnormalities lead to the development of DR or merely constitute a marker of its presence.

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Keywords: Complications; Retinopathy; Coagulation; Fibrin-clot

Résumé

Des anomalies du caillot de fibrine sont associées à la présence d’une rétinopathie dans le diabète de type 2.

Objectif. – Le développement et la progression de la rétinopathie diabétique (RD) dans le diabète de type 2 (DT2) sont associés à un mauvais contrôle glycémique, une longue durée de la maladie et quelques autres paramètres cliniques. Cependant, la physiopathologie de cette complication est encore mal comprise. La formation de caillots denses de fibrine résistant à la lyse a été décrite chez des diabétiques. Nous avons testé l’hypothèse que des altérations de la structure et de la fonction des caillots étaient associées à la RD chez des patients atteints de DT2.

Méthodes. – Nous avons inclus 101 patients atteints de DT2 sans RD (NDR) et 60 avec RD. Nous avons évalué la pénétration du caillot de fibrine à l’aide d’un système permettant de définir un coefficient de perméabilité (Ks) et indiquant la taille des pores et le temps requis pour une diminution de 50 % de la turbidité du caillot (t50%) comme marqueur de susceptibilité à la fibrinolyse. Tous les patients ont subi un examen ophtalmologique. Des co-variables cliniques et biochimiques ont également été mesurées. Les déterminants de la RD ont été identifiés par analyse de régression multiple.

**Résultats.** – Les patients atteints de RD avaient une perméabilité du caillot plus basse ($K_s$ $6.15 \pm 1.18$ vs. $7.53 \pm 1.24 \times 10^{-9}$ cm$^2$; $P < 0.0001$) et un temps de lyse du caillot de fibrine plus long ($t_{50 \%}$ $10.12 \pm 1.24$ vs. $9.12 \pm 1.4$ min, $P < 0.0001$) que les patients indemnes de NR. L’analyse logistique a révélé des associations entre la RD et le $K_s$, le $T_{50 \%}$, la glycémie à jeun, la durée de la maladie, ainsi que le traitement par l’insuline et la non-utilisation de statines ($P < 0.05$). Après l’ajustement pour ces variables, ainsi que pour l’âge et le sexe, les corrélations entre le $K_s$, le $t_{50 \%}$ et la RD se sont avérées significatives.

**Conclusions.** – La formation de caillots compacts de fibrine et l’efficacité réduite de la lyse du caillot sont associées à la RD dans le DT2. Il est difficile de savoir si ces anomalies provoquent le développement de la RD ou en représentent seulement un marqueur.

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*Mots clés :* Complications ; Rétinopathie ; Coagulation ; Caillot de fibrine

1. Introduction

Chronic vascular complications are the main cause of morbidity and mortality in patients with diabetes. Diabetic retinopathy (DR) is one of the leading causes of blindness in adults. Although several clinical factors, such as diabetes duration and metabolic control [1], and genetic variants [2], have been associated with an increased risk of DR, its pathogenesis is still not fully understood. There is strong evidence that type 2 diabetes mellitus (T2DM) is characterized by a procoagulant state [3,4]. Patients with T2DM have been shown to display increased platelet activation and an enhanced tissue-factor-initiated coagulation pathway leading to heightened thrombin generation. Other specific abnormalities described in patients with T2DM include increased circulating tissue-factor, elevated factor VII activity and prothrombin activation markers [5–7]. This is accompanied by suppressed fibrinolytic potential associated with increased plasminogen activator inhibitor-1 (PAI-1) concentrations. The pathogenesis of these abnormalities of the coagulation process has been linked to insulin resistance, dyslipidaemia and glycation of proteins involved in haemostasis [4,8,9].

The formation of fibrin clots that are relatively resistant to lysis represents the final step in blood coagulation. Fibrin networks composed of thin, highly branched fibres are usually less permeable, more rigid and less susceptible to lysis. Clots formed in diabetic patients from plasma or by purified fibrinogen use are less porous than those of non diabetic individuals [10], and have been shown to be resistant to lysis [11]. The main cause of these abnormalities is most likely higher fibrinogen glycation in patients with T2DM than in non diabetic subjects [9]. This interferes with fibrin polymerization, cross-linking by factor XIII, tissue plasminogen activator (tPA) and plasminogen-binding, and conversion of plasminogen to plasmin [12]. However, glucose-lowering agents can improve fibrin-clot properties [13].

Nevertheless, it remains unclear as to whether there is a prothrombotic fibrin-clot phenotype associated with diabetic microvascular complications. For this reason, the present study tested the hypothesis that altered clot structure and function are both associated with DR in T2DM.

2. Methods

A total of 161 consecutive T2DM patients (mean age at examination: $56.1 \pm 6.4$ years; all European Caucasians) were included in the present study. The inclusion criteria have been described elsewhere [2]. These T2DM patients filled out a standard questionnaire and underwent basic physical examination. Excluded were all subjects with active infections, severe and chronic diseases of the respiratory tract, kidney or liver, or cancer. Hypertension was defined as previously described [2]. The study was performed according to the Helsinki Declaration and was approved by the Ethics Committee of the Jagiellonian University Medical College.

Plasma fibrin-clot permeation was determined using a pressure-driven system. Briefly, 20 mM of calcium chloride and 1 U/mL of human thrombin (Sigma-Aldrich Corp., St Louis, MO, USA) were added to 120 UL of citrated plasma. After 2 hours of incubation in a wet chamber, tubes containing the clots were connected via plastic tubing to a reservoir containing buffer (0.01 M Tris, 0.1 M NaCl, pH 7.5), and the volume flowing through the gels was measured over 60 min. A permeation coefficient ($K_s$), which indicates pore size, was calculated as described elsewhere [14]. The inter- and intra-assay coefficients of variation were <9%. To assess the efficiency of clot lysis, 100 µL of citrated plasma were diluted with 100 µL of Tris buffer, containing 20 mM of calcium chloride, 1 U/mL of human thrombin (Sigma-Aldrich) and 1 µg/mL of recombinant tissue-type plasminogen activator (rTPA; Boehringer Ingelheim, Ingelheim, Germany). Assembly kinetics were monitored by spectrophotometry at 405 nm in a microplate reader (Molecular Devices Corp., Sunnyvale, CA, USA). The time required for a 50% decrease in clot turbidity ($t_{50\%}$) was chosen as a marker of susceptibility to fibrinolysis. The inter- and intra-assay coefficients of variation were <9%. Values of $K_s$ and $t_{50\%}$, measured in 100 apparently healthy individuals, were $9.12 \pm 1.12 \times 10^{-9}$ cm$^2$ and $7.46 \pm 0.82$ min, respectively [14].

In addition, basic clinical laboratory analyses were performed in all patients, including determinations of levels of serum fasting glucose, total cholesterol, HbA1c and creatinine. Standard analytical methods were used. The glomerular filtration rate, based on creatinine clearance, was calculated according to the Cockcroft–Gault formula.

The presence of DR was determined by a trained ophthalmologist using ophthalmoscopy after pupillary dilatation using 0.5% tropicamide. The full-colour photographic documentation of two 45° retinal fields was made with a retinal camera (Genesis, Kowa Optimed, Tokyo, Japan), using the same procedure as previously described [2]. Based on this examination, the patients were assigned to one of three groups:
Table 1
Clinical characteristics of type 2 diabetes patients without and with diabetic retinopathy (DR).

<table>
<thead>
<tr>
<th></th>
<th>Without DR (n = 101)</th>
<th>With DR (n = 60)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women, n</td>
<td>48 (47.5%)</td>
<td>32 (53.3%)</td>
<td>0.48</td>
</tr>
<tr>
<td>Age at diagnosis (years)</td>
<td>48 (43–52)</td>
<td>44 (39–50)</td>
<td>0.0049</td>
</tr>
<tr>
<td>Age at examination (years)</td>
<td>56 (51–60)</td>
<td>57 (52–62)</td>
<td>0.15</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>7 (4–11)</td>
<td>13 (8–17)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Body mass index (kg m(^{-2}))</td>
<td>32.1 (28.4–36.9)</td>
<td>30.8 (28.5–36.8)</td>
<td>0.57</td>
</tr>
<tr>
<td>Insulin treatment, n</td>
<td>38 (37.6%)</td>
<td>46 (76.7%)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Metformin treatment, n</td>
<td>52 (53.1%)</td>
<td>25 (42.4%)</td>
<td>0.2</td>
</tr>
<tr>
<td>Aspirin treatment, n</td>
<td>40 (39.6%)</td>
<td>25 (41.7%)</td>
<td>0.8</td>
</tr>
<tr>
<td>Statin treatment, n</td>
<td>57 (56.4%)</td>
<td>25 (42.4%)</td>
<td>0.9</td>
</tr>
<tr>
<td>Arterial hypertension, n</td>
<td>87 (86.1%)</td>
<td>58 (96.7%)</td>
<td>0.047</td>
</tr>
<tr>
<td>Smokers, current or past</td>
<td>62 (62%)</td>
<td>37 (61.7%)</td>
<td>0.97</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>8.4 (7.0–10.3)</td>
<td>10.2 (7.9–13.5)</td>
<td>0.0011</td>
</tr>
<tr>
<td>Total cholesterol (mmol L(^{-1}))</td>
<td>5.1 (4.4–5.9)</td>
<td>5.1 (4.4–5.9)</td>
<td>0.52</td>
</tr>
<tr>
<td>Creatinine clearance (mL/min)</td>
<td>114.2 (96.1–146.5)</td>
<td>105.6 (85.6–143.4)</td>
<td>0.31</td>
</tr>
<tr>
<td>Ks (10(^{-9}) cm(^{2}))</td>
<td>7.9 (6.6–8.5)</td>
<td>6 (5.4–6.85)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>t50% (min)</td>
<td>8.9 (8–10)</td>
<td>10.15 (9.5–10.95)</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

NB: For categorical variables, the number of patients and percentage of the total number in the groups without and with DR are presented here; for continuous variables, median values (25th and 75th percentiles) are used; P values are the results of univariate logistic-regression analyses; creatinine clearance was calculated according to the Cockcroft–Gault formula.

- no DR;
- non proliferative DR;
- proliferative DR.

Univariate logistic-regression was used to study the associations of Ks and t50% with DR. A univariate screen of clinical and biochemical variables (14 features altogether) seen with DR was performed. The variables significantly associated with DR were used to build multivariate logistic-regression models that included Ks and t50%. Variables associated with DR at a P value < 0.05 were considered statistically significant.

3. Results

Non proliferative DR was diagnosed in 55 (34.2%) patients, preproliferative DR in three (1.9%) patients and proliferative DR in two (1.2%). All DR groups were combined for further analyses. The characteristics of T2DM patients are shown in Table 1 along with all variables included in the logistic-regression.

Those with DR had lower clot permeability compared with those free of this complication (Ks: 6.15 ± 1.18 10\(^{-9}\) cm\(^{2}\) vs. 7.53 ± 1.24 10\(^{-9}\) cm\(^{2}\), respectively; P < 0.0001). Fibrin-clot degradation by tPA was slower in DR patients (t50%: 10.12 ± 1.24 min vs. 9.12 ± 1.4 min; P < 0.0001). Univariate logistic-regression analyses revealed that the following clinical and laboratory parameters were associated with DR: diabetes duration; age at diagnosis; fasting glucose; arterial hypertension; and insulin treatment. The results were of borderline significance for statin use (P = 0.08). The patients’ gender, age at examination, body mass index (BMI), smoking status, total cholesterol level and creatinine clearance, as well as aspirin and metformin use, were not associated with DR. In all of the multivariate logistic-regression models constructed through either stepwise selection procedures or with forced inclusion of variables into the model, clot parameters remained significantly associated with DR. This was also true for the final multivariate models of association with DR, which included insulin therapy, diabetes duration and use of statins, as well as Ks and t50% (Table 2). Additional forced inclusion of glucose concentrations into the model had no effect on the associations of Ks and t50% with DR. When HbA1c was used instead of fasting glucose as a marker of glycaemic control in the analysis, the study outcomes remained unchanged, with clot properties remaining strongly and independently associated with DR (data not shown).

4. Discussion

The present study was the first to examine the relationship of plasma fibrin-clot permeability and lysability with DR in T2DM. The main finding of the study was a strong and independent association between clot properties and the examined complication. The results confirmed that T2DM is indeed associated with reduced clot permeability and prolonged lysis [11–13] compared with values obtained in non diabetic individuals [14].

Fibrin clots, in a physiological setting, are formed as a result of interaction between damaged endothelium and platelets and clotting factors, while clot lysis requires an efficient fibrinolysis...
system. However, the putative pathomechanism of altered fibrin properties in the development and progression of DR remains unclear. One of the possibilities is that the more compact clots that are less prone to fibrinolysis may be associated with the impaired blood supply to retinal vessels [1]. The described associations indicate a possible role for new drugs that can influence clot structure and lysis in DR prevention.

The association of DR with diabetes duration and statin non-use was not unexpected. In the case of statins, it may reflect a true causal relationship in the light of evidence of improved clot characteristics with statin therapy in cardiovascular patients [15]. On the other hand, insulin use most probably constitutes only a marker of the patients’ more severe clinical status. The present study used fasting glucose, as it appears to be a better marker of glycaemic control for this analysis, given that the half-life of fibrinogen is only 4 days [16]. Nevertheless, the inclusion of HbA1c in the models did not change the study results.

Some shortcomings of the present study must be acknowledged. The number of patients with DR was limited. In addition, each examined variable was determined at a single time point. Thus, it remains to be established whether altered clot properties cause DR or merely constitute a marker of its presence. Moreover, potential haemostatic factors affecting fibrin-clot properties such as overall thrombin generation and factor VIII/von Willebrand factor complexes, which are elevated in T2DM, were not assessed in the present study.

In summary, our study showed that the formation of compact fibrin-clots and impaired fibrin clot lysis are independent risk factors for the presence of DR in T2DM patients. Also, it is unclear whether these abnormalities are causal factors in DR development, future prospective studies are necessary to clarify this issue. However, despite its limitations, the present research indicates a possible novel mechanism of DR and suggests a new direction in the search for preventative strategies.

Disclosure of interest

The authors declare that they have no conflict of interest concerning this article.

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Contribution information: MWM: researched data, writing the manuscript; PW: data analysis, writing the manuscript; KC - researched data: BMS: researched data; MTM: interpretation of data, revising the article critically for important intellectual content, final approval of the version to be submitted; AU: concept of the study, interpretation of data, revising the article critically for important intellectual content, final approval of the version to be submitted.

References