Generation of thrombin activity in relation to factor VIII:C concentrations and vascular complications in Type 1 (insulin-dependent) diabetes mellitus

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Summary. A possible association between plasma coagulant activity and the presence of vascular complications in patients with diabetes mellitus was studied by measuring the generation of thrombin in plasma of 20 control subjects and 50 diabetic patients classified according to the presence or absence of microvascular complications. Thrombin production was determined in defibrinated plasma using a semi-automated technique with measurement of thrombin activity using chromogenic peptide S2238. Values determined were the lag time to appearance of thrombin activity and time taken to generate 50 % maximal thrombin activity. Thrombin activity was related to concentrations of coagulant factor VIII activity and fibrinopeptide A and these were correlated with HbA_{1c} levels. The median time to generate 50 % maximal thrombin activity was not significantly reduced in diabetic patients compared with control subjects (53 vs 54 s, $p = 0.076$) and there were no significant differences between patients with and without microvascular complications.

There were no differences in median fibrinopeptide A concentrations between the diabetic and control subjects (1.5 vs 2.2 nmol/l, $p = 0.169$). Time to 50 % maximal thrombin activity correlated inversely with factor VIII:C concentrations in diabetic patients ($r = -0.344$, $p = 0.015$, $n = 50$) and both this and lag time correlated with factor VIII:C in diabetic patients and control subjects combined ($r = -0.395$, $p < 0.01$; $r = -0.327$, $p = 0.006$, $n = 70$). Factor VIII:C concentrations increased with age of the subject and with HbA_{1c} concentrations. The results failed to show enhancement of coagulation in contact-activated diabetic plasma compared with control plasma and suggest that a relationship between high levels of factor VIII:C in diabetes and the development of microvascular complications is unlikely to be mediated through procoagulant activity in plasma.

Key words: Blood coagulation, diabetes mellitus, factor VIII:C, thrombin activity.

Diabetes mellitus is a complex chronic disease associated with both metabolic and vascular abnormalities and although a causal link between the two has been suggested, this has not been established. Similarly, there is controversy as to whether a procoagulant state mediated through higher than normal plasma concentrations of coagulation factor VIII might contribute to some aspects of the disease process. von Willebrand factor (vWF), a component of the factor VIII complex, is higher in diabetic patients than normal control subjects. The increase is more pronounced in the presence of microvascular complications and qualitative abnormalities in the vWF protein have been described [1]. vWF is synthesised by endothelial cells and the increased levels seen in diabetic subjects may reflect endothelial cell damage [1–3]. The coagulant component, factor VIII:C (FVIII:C) is synthesised mainly by the liver and although reports suggest that levels are increased in diabetes [4], it is not clear whether this results in an acceleration in the rate of blood coagulation, or affects

the risk of complications. The overall pathological significance of the haemostatic changes which occur in diabetes are not known [5, 6].

Many years ago it was shown that in dogs infused with adrenaline, plasma concentrations of FVIII:C rose accompanied by a reduction of the coagulation time [7]. Similarly, FVIII:C concentrations rise in humans after administration of adrenaline [8]. Increasing concentrations of FVIII:C reduce plasma coagulation times in vitro [9] and clinical studies have demonstrated acute increases in circulating FVIII:C concentrations in response to various physical stresses such as hypoglycaemia, diabetic ketoacidosis and surgery [10–12]. However, it is not clear whether these sudden increases in FVIII:C lead to a corresponding enhancement of coagulation in vivo.

Investigation of procoagulant changes in clinical studies has been hampered by lack of a sensitive and dynamic test system. Standard clotting tests such as the activated partial thromboplastin time (APTT) and prothrom-

bin time (PT) can detect prolongation of coagulation, but in general have not proved to be sufficiently sensitive and reproducible for measurement of reduced coagulation times. Continuing activation of the coagulation mechanism in the circulation can be detected by measuring levels of fibrinopeptide A (FPA) the peptide cleaved from fibrinogen by the action of thrombin to form fibrin [13]. FPA levels have been found to be raised in diabetic patients in some studies [14] though not in others [15].

We have used an *in vitro* technique devised by Hemker and colleagues [16] that measures the rate of production of thrombin activity against time using a chromogenic substrate, S2238, specifically cleaved by thrombin as the end point, to determine coagulation times in plasma from diabetic patients. The aims of the study were to use this sensitive measure of the rate of generation of thrombin activity to determine whether coagulation times were shorter in diabetic patients than in control subjects and whether coagulation times were related to FVIII:C concentrations. In addition, FPA levels were measured so that the results of thrombin generation could be compared with those of an established technique.

Subjects, materials and methods

Subjects

The study was approved by Leeds Western District Ethics Committee and informed consent was obtained from each subject. Twenty healthy volunteers and 50 patients with long-term Type 1 (insulin-dependent) diabetes of similar age and sex were investigated. Blood samples were taken during the course of a larger study into the fibrinolytic system in diabetic patients and its relationship to complications [17]. The diabetic subjects were subdivided into those without complications, those with proliferative retinopathy and those with retinopathy and neuropathy. None of the patients had proteinuria. The exclusion criteria were: age over 65 years, obesity, cigarette smoking, other medication, hypertension (diastolic blood pressure over 90 mm Hg) and abnormalities of liver function tests or serum creatinine. No patient experienced symptomatic hypoglycaemia on the morning of the study.

Type 1 diabetes was defined as occurrence of ketoacidosis or onset of diabetes under 30 years of age, requiring insulin therapy within a few weeks of diagnosis. All patients were assessed by direct and indirect ophthalmoscopy and the absence of microaneurysms, haemorrhages or exudates was required to place patients in the group without complications. Patients with neuropathy were classified as those above the 90th centile of vibration perception threshold for age using a biothesiometer (Biomedical Instrument, Newbury, Ohio, USA) on the hallux of both feet [18]. Autonomic function was assessed by heart rate variation with deep breathing [19]. Glycaemic control was assessed from venous blood glucose and HbA_{1c} (normal range 5.6–7.4%, coefficient of variation [CV] 3.2%) measured by isoelectric focussing.

Materials and methods

Venous blood samples were taken with minimal haemostasis from the antecubital fossa after 20 min rest in the supine position. A 19 gauge butterfly needle was used and blood was collected into 10% citrate anticoagulant. Samples were stored on ice prior to centrifugation at 2,560 *g* for 15 min at 4°C. Aliquots were snap-frozen in liquid nitrogen and stored at -40°C.

Thrombin activity, produced following activation of the intrinsic pathway of coagulation, was measured using a computer-assisted technique described by Hemker et al. [16]. Briefly, plasma was defibrinated by the addition of reptilase solution (1/50 of the plasma volume) (34 mg/ml; Pentapharm Ltd., Basle, Switzerland) incubated at 37°C for 5 min, followed by 10 min at 4°C, and the fibrin clot removed by winding onto a plastic spatula. FVIII:C levels did not significantly change with reptilase treatment. The reagents used were Diagen phospholipid (Bell and Alton, Diagen, Diagnostic Reagents Ltd., Oxford, UK), light kaolin (1.25 g/l) in Owren's buffer pH 7.35 and 0.025 mol/l calcium chloride and chemicals of the highest grade commercially available. Chromogenic substrate specific for thrombin was S2238 (Kabi Vitrum AB, Stockholm, Sweden). The assay employed automated timing of sampling using a microman pipette (Gilson Medical Electronics, Villiers-Le-Bel, France) connected to an input gate of a personal computer (IBM-compatible) programmed to record the moment of sampling.

Defibrinated plasma was diluted 1:2 in 0.145 mol/l sodium chloride solution and 100 µl of this mixture was incubated with equal volumes of buffer A (0.05 mol/l Tris-HCl, 0.1 mol/l NaCl and 0.5% egg albumin [Sigma Chemical Co., St. Louis, Mo., USA] pH 7.35), phospholipid and kaolin. The mixture was incubated at 37°C for 10 min and coagulation was started by the addition of 100 µl of 0.025 mol/l calcium chloride (time 0). At 15 s recorded intervals from time 0, 25 µl aliquots were sub-sampled separately into 465 µl buffer B (buffer A with 20 mmol/l EDTA) and 25 µl S2238 at 37°C and mixed. After a recorded time of 120 s, the reaction was stopped by the addition of 300 µl of 98% glacial acetic acid. Following centrifugation at 1,400 *g* at room temperature for 5 min, the absorbances of 400 µl volumes of the supernatant were read at 405 nm in the computer-linked spectrophotometer [16]. The generation of *p*-nitroaniline was linear in time up to an optical density of 1.00. The data were processed by a computer program [16] and displayed graphically. The rate of generation of thrombin activity was expressed in two ways: (i) the time from the start of the reaction until first appearance of thrombin activity (lag time in seconds) and (ii) the time from the start of the reaction until the appearance of 50% of maximal thrombin activity (*T*₅₀ in seconds). Reduction in both times indicates an acceleration in the rate of coagulation in plasma.

FVIII:C levels were measured by the one-stage method and expressed in IU/ml using depleted plasma obtained from Diagen (Diagnostic Reagents Ltd.) [20]. Fibrinopeptide A (FPA) (CV's: 5% and 11.8%) was measured by radioimmunoassay (IMCO, Stockholm, Sweden) [21]. Figures quoted in parentheses represent the intra- and inter-assay CV respectively.

Statistical analysis

Results were assessed by non-parametric tests and expressed as the median (interquartile range) values. Differences between groups were assessed by the Kruskal-Wallis one-way analysis of variance and if a significant difference was found (*p* < 0.05), a two-tailed Mann Whitney test was used to define where this occurred. Rank correlation was assessed by Spearman's test.

Results

Details of the control subjects and diabetic patients studied are shown in Table 1. The three groups of diabetic patients are well matched, apart from values for mean vibration perception threshold which was, as expected, higher in the patients with neuropathy.

Two typical thrombin generation curves are shown in Figure 1. Lag time and *T*₅₀ were both reduced in plasma A, in which FVIII:C concentration was four times higher

Table 1. Characteristics of the normal subjects and diabetic patients studied

	Control subjects	Type 1 diabetic patients		
		No complications	Retinopathy	Neuropathy and retinopathy
<i>n</i>	20	20	17	13
Sex (M:F)	10:10	11:9	11:6	10:3
Age (years)	47 (37–55)	43 (36–47)	42 (37–54)	51 (36–55)
Duration of diabetes (years)	0	23 (19–27)	28 (24–31)	26 (23–32)
Blood glucose (mmol/l)	5.1 (4.0–5.4)	12.0 (6.8–13.0)	16.2 (12.9–17.7)	14.4 (9.3–17.4)
HbA _{1c} (%)	6.3 (5.9–6.7)	9.2 (8.6–10.3)	10.1 (9.7–10.7)	10.9 (10.4–12.9)
Mean vibration perception threshold (V)	7 (5–10)	8 (6–9)	11 (9–13)	44 (30–45)

Values are given as median (interquartile range)

Table 2. Lag time (lag), time to generation of 50% maximal thrombin activity (T_{50}), fibrinopeptide A (FPA) and factor VIII:C (FVIII:C) levels in the control subjects and Type 1 (insulin-dependent) diabetic patients studied

	Control subjects	Type 1 diabetic patients			
		Total	No complications	Retinopathy	Neuropathy and retinopathy
<i>n</i>	20	50	20	17	13
Lag time (s)	45 (45–60)	45 (30–45)	45 (30–45)	45 (34–45)	45 (30–45)
T_{50} (s)	54 (53–66)	53 (49–64)	53 (48–63)	55 (46–64)	52 (48–63)
FPA (nmol/l)	2.2 (0.7–18.1)	1.5 (0.9–2.9)	1.6 (1.0–2.5)	0.9 (0.4–2.7)	1.9 (1.0–4.6)
FVIII:C (IU/ml)	0.79 (0.50–0.86)	0.85 (0.70–1.02) ($p = 0.036$)	0.89 (0.68–1.15) ($p < 0.025$)	0.76 (0.69–0.85)	0.93 (0.72–0.97) ($p < 0.025$)

Values are given as median (interquartile range)

p values represent statistical significance in comparison to control subjects

than in plasma B. There were no significant differences between normal subjects and the diabetic patients in values for lag time, T_{50} , and FPA concentration, nor were there significant differences in these values between the different groups of diabetic subjects studied (Table 2). FVIII:C levels (Table 2) were significantly higher in the diabetic patients as a group than in control subjects (median 0.85 vs 0.79 IU/ml, $p = 0.036$) but there were no significant differences in FVIII:C concentrations between the three groups of diabetic subjects. FVIII:C concentrations increased progressively with age in the diabetic patients ($r = 0.429$, $p = 0.002$, $n = 50$), and in all subjects, diabetic patients and control subjects combined ($r = 0.413$, $p < 0.001$, $n = 70$). FVIII:C levels similarly showed a significant positive correlation with HbA_{1c} concentrations, both in control subjects ($r = 0.536$, $p = 0.015$, $n = 20$) and in diabetic patients and control subjects combined ($r = 0.315$, $p = 0.008$, $n = 70$) (Fig. 2). A correlation between age and HbA_{1c} was seen in the control subjects ($r = 0.694$, $p < 0.001$, $n = 20$).

There was a significant inverse correlation between the time taken to reach 50% of maximal thrombin activity in vitro (T_{50}) and FVIII:C concentrations in plasma, both in the diabetic patients ($r = -0.344$, $p = 0.015$, $n = 50$) and in the diabetic patients and control subjects combined ($r = -0.395$, $p < 0.01$, $n = 70$) (Fig. 2). Similarly, there was a significant inverse correlation between lag time and FVIII:C concentrations when all diabetic patients and control subjects were combined ($r = -0.327$, $p = 0.006$, $n = 70$). There were no significant differences in T_{50} , lag

time or FPA concentrations between the three groups of patients with diabetes (Table 2), nor was there a significant relationship between FPA concentration and FVIII:C level, T_{50} or lag time. There were no correlations between the duration of diabetes and the HbA_{1c} concentration, FVIII:C level, lag time or T_{50} .

Discussion

The results of this study have failed to show significant differences in generation of thrombin activity in contact-activated plasma between normal subjects and diabetic patients as a group, and between diabetic patients with and without the complications of retinopathy and neuropathy. The method used in this study to measure thrombin generation is sensitive, precise and reproducible [16]. We have shown T_{50} to be sensitive to the addition of increasing concentrations of FVIII:C in vitro [9] and to be reduced progressively with increasing age of the subject and plasma FVIII:C concentration in vivo [22]. It is more sensitive to reduced coagulation times than standard laboratory tests such as the APTT [22]. The results, accordingly, suggest that any differences in generation of thrombin activity in plasma comparing diabetic patients with normal individuals are likely to be insignificant, and that blood coagulation is not enhanced in diabetic patients compared with control subjects. The finding of similar FPA concentrations in patients and control subjects is consistent with these results.

Fig. 1. The rate of production of in vitro thrombin activity measured as amidolytic activity (nmol/l thrombin equivalent) against time (min). Plasma A (—) with factor VIII:C concentration of 1.62 IU/ml has a shorter lag time (lag) and time to generation of 50% maximal thrombin activity (T_{50}) than plasma B (---), with factor VIII:C concentration of 0.44 IU/ml

Fig. 2. Relationships between plasma factor VIII:C level (IU/ml) and HbA_{1c} concentration (%) and time taken to reach 50% maximal thrombin activity (T_{50} /s). Results from diabetic patients (●) and control subjects (○) combined, $n = 70$. Factor VIII:C correlated with HbA_{1c} ($r = 0.315$, $p = 0.008$) and inversely with T_{50} ($r = -0.395$, $p < 0.01$)

This study has shown that FVIII:C levels were significantly increased in the patients with Type 1 diabetes when compared to healthy control subjects. In general, this finding supports the work of others [4–6] though the importance of raised concentrations of FVIII:C in the pathogenesis of diabetic complications is controversial. No relationship between FVIII:C concentrations and the presence of retinopathy alone or retinopathy and neuro-

pathy was seen. An increase in FVIII:C with advancing age and independently with increasing HbA_{1c} level was demonstrated in all subjects. This suggests that the elevation of FVIII:C concentration in diabetic patients is related more to glycaemic control than to the presence of microvascular complications. In the healthy control subjects HbA_{1c} correlated positively with age supporting the hypothesis that increased glycation of proteins may be part of the ageing process [23].

FVIII:C behaves in some respects as an acute-phase protein and concentrations are increased acutely by exercise, surgery, nausea and 1-desamino-8-D-arginine vasopressin infusion (DDAVP) [12, 24–26]. In diabetic patients FVIII:C levels increase acutely in ketoacidosis and during hypoglycaemia [10, 11]. These findings have encouraged speculation that higher than normal FVIII:C levels could contribute to the long-term vascular complications seen in patients with diabetes [4–6, 27]. In the present study, in individual plasma FVIII:C concentrations correlated with indices of accelerated coagulation in vitro, represented by reduction of the lag time to appearance of thrombin activity and the time taken to generate T_{50} . However, despite elevated levels of FVIII:C in the diabetic patients, no evidence of activated coagulation could be detected in the group as a whole compared to control subjects nor between the diabetic patients with and without microvascular complications. These findings are supported by the absence of significant changes in FPA levels in the diabetic subjects in this study, although the lack of a significant correlation between FVIII:C and FPA concentrations suggests that the chromogenic technique of assessment of in vitro thrombin activity may be a more sensitive measure of activated coagulation than that of the FPA assay.

Overall the findings suggest that in individual subjects, higher FVIII:C concentrations directly accelerate the rate of thrombin production which may be one of several factors involved in the pathogenesis of occlusive vascular disorders and in particular macrovascular disease rather than the specific microvascular complications of diabetes. It is of interest that no relationships between the duration of the diabetic disease process and the HbA_{1c}, FVIII:C levels or the indices of in vitro coagulation, were observed in this study.

Recent interest has focussed on the role of glycated proteins in vascular disease [23]. Prolonged glycation of proteins with long half-lives, such as collagen and myelin, leads to the formation of irreversible adducts which cause cross-linking thereby producing advanced glycation end products (AGE) which accumulate in plasma and the subendothelium. AGE proteins increase in prevalence with both ageing and diabetes and are thought to contribute to the vascular disease of ageing, and the vascular and renal complications of diabetes, by increasing endothelial permeability and promoting clot formation [23]. We found a positive correlation between age and HbA_{1c} in the control subjects studied which may have a bearing on the observations of others who have shown that, at sub-diabetic levels, increasing HbA_{1c} concentration corresponded with an increased risk of cardiovascular disease in non-diabetic subjects [28]. In the present study, FVIII:C

correlated independently with HbA_{1c}, age, and measurement of the rate of generation of thrombin activity in plasma. It seems plausible that accelerated AGE protein formation may increase the propensity to the macrovascular disease of ageing and poorly-controlled diabetes and that high plasma concentrations of FVIII:C could be a contributing factor [29]. However, accelerated coagulation does not appear to be associated with the incidence of diabetic microvascular complications which probably develop through mechanisms independent of the coagulation system.

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