Glycosylation & Disease, 1994, **1**, 287–291

Coagulation inhibitors and glycaemic control in non-insulin-dependent diabetes mellitus

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The relationship between long-term glycaemic control and the activity of coagulation inhibitors was investigated in 60 non-insulin-dependent diabetes mellitus (NIDDM) patients not on insulin therapy. Overall, the activities of antithrombin III (AT III) (median 96%, range 65–133%), protein C (127%, 24–190%) and protein S (130%, 54–163%) were not reduced. Patients in poor long-term glycaemic control as verified by increased glycated haemoglobin (HbA1c) demonstrated significantly decreased median AT III activity in comparison with patients in good glycaemic control (92% vs 101%, $P = 0.016$). However, individual values for AT III activity were not below the critical limit of 60%. An inverse correlation between AT III activity and long-term glycaemic control (HbA1c) was calculated ($r = -0.378$, $P = 0.0029$). As AT III concentrations were found to be normal, we propose that non-enzymatic glycation leads to reduced activity of AT III without affecting its concentration.

Keywords: antithrombin III, glycaemic control, non-insulin-dependent diabetes mellitus, protein C, protein S

Introduction

Cardiovascular complications are major determinants of long-term survival in non-insulin-dependent diabetes mellitus (NIDDM) [1]. Macroangiopathy typical of NIDDM reflects premature and accelerated atherosclerosis and cannot be fully attributed to classical risk factors such as hypertension, smoking and disturbed lipid metabolism [2]. In addition to vascular wall lesions and platelet activation, the fluid phase is thought to contribute to atherogenesis [3]. Changes in blood coagulation and fibrinolysis leading to hypercoagulability [4] are known to occur in diabetic patients [5]. Poor glycometabolic control has been suggested to be one major link between diabetes mellitus and its vascular complications. Hyperglycaemia induces non-enzymatic glycation of plasma proteins with subsequent interferences in their physiological functions [6].

The main physiological coagulation inhibitors antithrombin III, protein C and protein S contribute to the maintenance of a normal balance between coagulant and anticoagulant activities, thus preventing persistent thrombinaemia and subsequent clot formation. Previous studies have provided data supporting changes in coagulation inhibitor activities in NIDDM (for review see refs 5 and 7). However, results concerning the association of coagulation inhibitor activities with glycaemic control remain inconclusive. Antithrombin III activity has been reported to be normal [8], increased [9, 10] or decreased [11, 12]. Similar results have been reported for protein C and protein S [13–15]. This prompted us to undertake an

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investigation of the relationship between long-term glycaemic control and coagulation inhibitors in patients suffering from non-insulin-dependent diabetes mellitus (age below 60 years) who had never been treated with insulin.

Preliminary results were presented at the 14th ISTH Congress, New York, 1993, and were published in abstract form [16].

Materials and methods

Sixty patients with the diagnosis NIDDM according to WHO criteria [17] were included in the present investigation. Treatment consisted only of diet and/or oral hypoglycaemic agents (sulphonylurea compounds). Patients who had been or who are currently being treated with insulin were excluded. Further exclusion criteria were age above 60 years, malignancy, pregnancy and current anticoagulant or antiplatelet drug therapy. The investigation was performed when patients attended the outpatients clinic for routine metabolic follow-up examination. Demographic and clinical data are given in Table 1.

Venous blood samples were drawn between 7.30 and 10.00 a.m. to avoid interferences due to circadian rhythm. Atraumatic venepuncture of the antecubital veins was performed using anticoagulant-prefilled Monovette syringes (Sarstedt, Nümbrecht, Germany) with a 21-gauge needle. Citrate-anticoagulated blood samples (9:1, v/v) were collected for plasma separation. In addition, one EDTA-anticoagulated blood sample was taken. Glycated haemoglobin was investigated in EDTA-anticoagulated whole-blood samples by ion-exchange column chromatography (Diamat, Bio-Rad, Munich, Germany). Values above 7.3% glycated haemoglobin (of total haemoglobin) were considered to reflect poor long-term glycaemic control.

Activity of antithrombin III was measured using IL Test Antithrombin III (cat. no. 97574-15) and an ACL 3000 analyser (Instrumentation Laboratory, Kirchheim, Germany). Briefly, the sample is incubated with excessive amounts of thrombin in the presence of heparin followed by analysis of residual thrombin activity via chromogenic substrate. Residual thrombin activity is inversely proportional to antithrombin III activity, which is expressed as a percentage compared with pooled normal plasma. The normal range for antithrombin III in plasma is 80–120%. Quality control was performed using IL Test normal (cat. no 84670-11) and abnormal plasma (cat. no. 84676-00). The concentration of antithrombin III was determined in citrate-anticoagulated plasma using an immunonephelometric method (BN 100, Behringwerke, Marburg, Germany). N antiserum to human antithrombin III raised in rabbits (code no. OSAY) was obtained from the same company. The procedure was essentially performed according to the manufacturer's instructions including calibration using human source N protein standard PY (code no. OUID 12) and quality control with human source N protein control PY (code no. OWSY 12).

Protein C activity was investigated using ACL 3000 and IL Test ProClot assay (cat. no. 84683-10). The functional clotting assay of protein C is based on the prolongation of activated partial thromboplastin time after activation of protein C by Protac (copperhead snake venom) via inhibition of factor V and factor VIII. Protein C activity is expressed as a percentage compared with pooled normal plasma. The normal range for protein C is 70–140%.

Table 1. Demographic and clinical data from pooled diabetic patients and subgroups formed according to glycometabolic control (median, range)

Index (unit)	All patients (n = 60)	Patients in good glycaemic control (n = 25)	Patients in poor glycaemic control (n = 35)
Sex (male/female)	36/24	15/10	21/14
Age (years)	54 (30–60)	54 (36–59)	54 (30–60)
Body mass index (kg/m^2)	28.7 (17.9–38.8)	29.0 (24.2–37.0)	27.6 (17.9–38.8)
Duration of disease (years)	4.0 (0.08–20.0)	0.6* (0.08–17.0)	9.0 (0.08–20.0)
Glycated haemoglobin (% of total Hb)	8.1 (5.2–12.3)	5.8** (5.2–7.3)	9.1 (7.4–12.3)

*P = 0.0002, **P < 0.0001.

Protein S activity was analysed using ACL 3000 and IL Test Protein S (cat. no. 84688-10). Diluted sample plasma is added to a plasma deficient in protein S. Prolongation of prothrombin time after activation of protein C using Protac forms the basis of the functional clotting assay for protein S. Functional protein S activity is expressed as a percentage compared with pooled normal plasma with an established normal range of 60–140%. Quality control for both protein C and protein S assays was performed using normal and abnormal control plasma as stated above.

Data are presented as median values and full ranges because of the non-parametric distribution. Accordingly, comparison between two groups was carried out by non-parametric Mann–Whitney *U*-test. Linear regression analysis was used to analyse relations between data. A probability value of less than 0.05 was considered statistically significant. All calculations were performed using GraphPad InStat statistical software (GraphPad Software, San Diego, CA, USA).

Results

Data pooled for all diabetic patients ($n = 60$) and subgroups according to glycaemic control are presented in Table 2.

Median values for antithrombin III activity and concentration, protein C activity and protein S activity were within normal ranges as given by the manufacturer. Five patients demonstrated either reduced protein C activity (68%, 40% and 24%) or reduced protein S activity (56% and 54%). Slightly reduced values for antithrombin III activity in the range between 60% and 80% were established in 10 patients (17%).

Median protein C and protein S activity was not different between patients in good and poor glycaemic control (Table 2). In contrast, we observed significantly decreased antithrombin III activity in patients with poor glycaemic control compared with patients in good glycaemic control ($P = 0.016$). Subsequent analysis of antithrombin III concentration demonstrated no differences between the two subgroups formed.

A significant inverse correlation ($r = -0.378$, $P = 0.003$) was established between glycated haemoglobin and antithrombin III activity (Figure 1). No significant correlation could be demonstrated between glycated haemoglobin and antithrombin III concentration ($r = -0.123$, $P = 0.351$), protein C activity ($r = 0.014$, $P = 0.918$) and protein S activity ($r = -0.111$, $P = 0.397$).

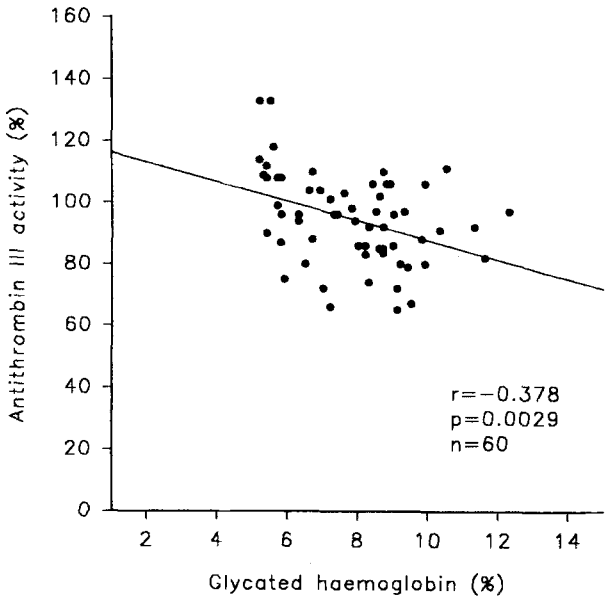

Figure 1. Correlation between antithrombin III activity and glycated haemoglobin.

Table 2. Coagulation inhibitors antithrombin III (AT III, activity and concentration), protein C (PC activity) and protein S (PS activity) in diabetic patients and subgroups formed according to glycaemic control (median, range)

Index (unit)	All patients ($n = 60$)	Patients in good control ($n = 25$)	Patients in poor control ($n = 35$)	*P*-value (good vs poor)
AT III activity (%)	96 (65–133)	101 (66–133)	92 (65–111)	0.016
AT III concentration (g/l)	0.305 (0.220–0.398)	0.309 (0.220–0.398)	0.304 (0.231–0.383)	0.410
PC activity (%)	127 (24–190)	119 (68–190)	128 (24–173)	0.538
PS activity (%)	130 (54–163)	136 (67–142)	128 (54–163)	0.775

Discussion

Long-term glycometabolic control contributes to the manifestation and severity of micro- and macrovascular diabetic complications as documented by The Diabetes Control and Complications Trial Research Group [18]. In case of hyperglycaemia, non-enzymatic glycation of protein structures is believed to be one major mechanism responsible for loss of integrity and function of homeostatic systems in diabetes mellitus [19].

Various changes in the systems of haemostasis and fibrinolysis including deficiency of antithrombin III, protein C and protein S are generally accepted to be factors predisposing to the occurrence of thrombotic disease [4].

We have assessed the association between natural coagulation inhibitors and glycaemic control in patients suffering from NIDDM and have found no significant alterations in the mean inhibitor activity of pooled data from 60 patients. Our data do not support previous reports demonstrating decreased antithrombin III activity [11, 12], reduced protein C activity [14] and diminished protein S activity [13] in type II diabetic patients.

As inhibitor activities below 60% of normal are considered critical for the maintenance of effective antithrombotic defence, the significance of reduced coagulation inhibitor activities in individual patients (four patients with protein C or protein S below 60%) deserves follow-up determinations. It is proposed that pronounced procoagulant activity in diabetes mellitus cannot be effectively counteracted if the activities of naturally occurring coagulation inhibitors are reduced. Activation of the coagulation system has been demonstrated in diabetes mellitus by increased thrombin–antithrombin III complexes, circulating prothrombin fragments 1 + 2 and fibrinopeptide A [10].

Examining the effect of long-term glycaemic control on coagulation inhibitor activity, we noted an inverse correlation between antithrombin III activity and glycated haemoglobin. A similar relation was observed using fructosamine concentrations for the assessment of intermediate-term glycaemic control (data not shown). Antithrombin III antigen concentration was not significantly different in patients in good and poor glycaemic control. Non-enzymatic glycation probably causes the reduction of antithrombin III activity, as has been proposed from *in vivo* and *in vitro* data [11, 20, 21]. Although induced short-term hyperglycaemia has been demonstrated to reduce the activity of antithrombin III [21], we did not include blood glucose analysis in the current investigation because our patients were recruited when attending the outpatients clinic for routine metabolic follow-up. Glycated haemoglobin was used for monitoring long-term glycaemic control. Blood glucose concentration varies widely in diabetic patients and is more useful for the detailed follow-up of glycaemia and in the detection of critical situations.

The effect of poor long-term glycaemic control on antithrombin III activity is small in absolute terms, and activities below 60% have not been demonstrated in our patients. However, the significance of borderline antithrombin III activity for the development of cardiovascular diseases is still an unresolved issue. Considering the prominent role played by thrombin in the progression of atherosclerotic lesions, it seems that effective control of thrombin functions (procoagulant, mitogenic, etc.) by sufficient amounts of antithrombin III is of paramount importance in maintaining vascular integrity and function [22]. The implication of borderline or slightly reduced antithrombin III activity in the progression of atherosclerosis in diabetic patients should be investigated in future, long-term investigations.

Our results on antithrombin III activity and concentration confirm previous reports on the association between antithrombin III and glycaemic control in diabetes mellitus [11, 12, 20, 21]. However, other studies failed to find a relationship between antithrombin III activity and glycaemic control [8–10, 23]. Although an exact explanation cannot be given for these apparently different findings, we propose that sample size, patients' selection criteria, current treatment modalities, etc., could have contributed. Since many studies included mixed populations of diabetes patients, we carefully selected NIDDM patients younger than 60 years of age who had never used insulin.

In contrast to antithrombin III, protein C and protein S activities appear to be independent of glycaemic control in the present investigation. Previous studies have demonstrated the influence of glycaemic control on activity of protein C [24] and decreased free protein S in type I diabetes [25]. Considering the complexity of the thrombomodulin–protein C–protein S anticoagulation pathway, it would appear to be necessary to perform more detailed studies on the various components, in order to obtain a true picture of this system in NIDDM, and its relationship to glycaemic control. Decreased protein C and protein S activities can be demonstrated in individual patients irrespective

of the glycaemic control. Long-term evaluation of these patients is strongly recommended, since patients with reduced activity are at risk of developing thromboembolic events.

In conclusion, antithrombin III activity in non-insulin-dependent diabetes mellitus is inversely related to long-term glycaemic control. We suggest that this should be considered in evaluating the pathogenesis of long-term micro- and macrovascular diabetic complications.

Acknowledgements

The authors gratefully acknowledge critical review and helpful comments by Professor R. Hampel, Department of Internal Medicine, University of Rostock. Technical assistance provided by members of the Coagulation Laboratory from the author's institute is appreciated.

References

1. Colwell JA. Vascular thrombosis in type II diabetes mellitus. *Diabetes* 1993: **42**; 8–11.
2. Donahue RP, Orhchard TJ. Diabetes mellitus and macrovascular complications. An epidemiological perspective. *Diabetes Care* 1992: **15**; 1141–55.
3. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 1993: **362**; 801–9.
4. Nachman RL, Silverstein R. Hypercoagulable states. *Ann Intern Med* 1993: **119**; 819–27.
5. Ceriello A. Coagulation activation in diabetes mellitus: the role of hyperglycaemia and therapeutic prospects. *Diabetologia* 1993: **36**; 1119–25.
6. Hatton MWC, Richardson M, Winocour PD. On glucose transport and non-enzymic glycation of proteins in vivo. *J Theor Biol* 1993: **161**; 481–90.
7. Ostermann H, Van de Loo J. Factors of the hemostatic system in diabetic patients. A survey of controlled studies. *Haemostasis* 1986: **16**; 386–416.
8. Christie M, Fritschi J, Lämmle B, *et al*. Fifteen coagulation and fibrinolysis parameters in diabetes mellitus and in patients with vasculopathy. *Thromb Haemost* 1984: **52**; 138–43.
9. Grignani G, Gamba G, Geroldi D, *et al*. Enhanced antithrombotic mechanisms in patients with maturity-onset diabetes mellitus without thromboembolic complications. *Thromb Haemost* 1981: **46**; 648–51.
10. Donders SHJ, Lustermans FAT, Van Wersch JWJ. Glycometaboic control, lipids, and coagulation parameters in patients with non-insulin-dependent diabetes mellitus. *Int J Clin Lab Res* 1993: **23**; 155–9.
11. Ceriello A, Dello Rosso P, Zuccotti C, *et al*. Decreased antithrombin III activity in diabetes may be due to non-enzymatic glycosylation. A preliminary report. *Thromb Haemost* 1983: **50**; 633–4.
12. Hall P, Tryon E, Nikolai TF, Roberts RC. Functional activities and nonenzymatic glycosylation of plasma proteinase inhibitors in diabetes. *Clin Chim Acta* 1986: **160**; 55–62.
13. Schwarz HP, Schernthaler G, Griffin JH. Decreased plasma levels of protein S in well-controlled type I diabetes mellitus. *Thromb Haemost* 1987: **57**; 240.
14. Biondi G, Sorano GG, Conti M, *et al*. The behaviour of protein C in diabetes is still an open question. *Thromb Haemost* 1991: **66**; 267.
15. Knöbl P, Schernthaler G, Schnack C, *et al*. Thrombogenic factors are related to urinary albumin excretion rate in type 1 (insulin-dependent) and type 2 (non-insulin-dependent) diabetic patients. *Diabetologia* 1993: **36**; 1045–50.
16. Steiner M, Reinhardt K, Burstein C, *et al*. Coagulation inhibitors and soluble thrombomodulin in patients suffering from non-insulin dependent diabetes mellitus (NIDDM) (abstract). *Thromb Haemost* 1993: **69**; 1215.
17. WHO Study Group. *Diabetes mellitus*. Technical report series 727. Geneva: WHO, 1985; 1–113.
18. The Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *New Engl J Med* 1993: **329**; 977–86.
19. Van Boeckel MAM. The role of glycation in aging and diabetes. *Mol Biol Rep* 1991: **15**; 57–64.
20. Ceriello A, Giugliano D, Dello Russo P, *et al*. Metabolic control may alter antithrombin III activity but not its plasma concentration in diabetes: a possible role for nonenzymatic glycosylation. *Diabetes Care* 1986: **9**; 32–5.
21. Ceriello A, Giugliano D, Quatraro A, *et al*. Induced hyperglycemia alters antithrombin III activity but not its plasma concentration in healthy normal plasma. *Diabetes* 1987: **36**; 320–3.
22. Hedin U, Frebelius S, Sanchez J. Dryjski M, Swedenborg J. Antithrombin III inhibits thrombin-induced proliferation in human arterial smooth muscle cells. *Arterioscler Thromb* 1994: **14**; 254–60.
23. Grignani G, Gamba G, Geroldi D, *et al*. Enhanced antithrombotic mechanisms in patients with maturity-onset diabetes mellitus without thromboembolic complications. *Thromb Haemost* 1981: **46**; 648–51.
24. Ceriello A, Quatraro A, Dello Rosso P, *et al*. Protein C deficiency in insulin-dependent diabetes: a hyperglycemia-related phenomenon. *Thromb Haemost* 1990: **64**; 104–7.
25. Ceriello A, Giugliano D, Quatraro A, *et al*. Possible role for increased C4b-binding protein level in acquired protein S deficiency in type I diabetes. *Diabetes* 1990: **39**; 447–9.

(Received 24 August 1994; accepted in revised form 2 November 1994)