Originals

Abnormal endothelial release of fibrinolytic activity and fibronectin in diabetic microangiopathy

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Summary. Endothelial cell function in Type 1 (insulin-dependent) diabetic patients, both with and without retinopathy, was assessed by measuring the plasma fibrinolytic activity and fibronectin after 10 min venous stasis induced by a sphygmomanometer cuff. After venous stasis, diabetic subjects with proliferative retinopathy had fibrinolytic responses (median 0.13 increasing to 0.26 U/ml) in the low normal range, which were significantly less ($p < 0.005$) than control subjects (0.17–0.68 U/ml) and diabetic patients with minimal retinopathy (0.16–0.68 U/ml; $p < 0.01$). Plasma fibronectin levels were similar in the different groups, but after venous stasis, rose significantly in the diabetic patients, both in those with proliferative retinopathy (mean 317–399 $\mu\text{g/ml}$; $p < 0.002$) and without retinopathy (312–371 $\mu\text{g/ml}$; $p < 0.05$) but not in

normal subjects (304–333 $\mu\text{g/ml}$). These changes in fibrinolytic activity and fibronectin were independent of blood glucose, glycosylated haemoglobin, or indices of sensory or autonomic nerve function. These disturbances of endothelial function, together with known abnormalities of haemostatic variables and microvascular reflexes, might convert a usually temporary obstruction of capillary blood flow into a pathological capillary closure, and might contribute to the inexorable progression of advanced diabetic microangiopathy in spite of good diabetic control.

Key words: Fibrinolysis, fibronectin, Type 1 diabetes, retinopathy, diabetes control, microangiopathy.

The factors promoting diabetic microangiopathy are uncertain, and may involve platelet and coagulation abnormalities [1], and abnormal microvascular reflexes [2]. Local endothelial responses may be involved, with increased factor VIII related antigen in diabetic retinopathy [3, 4] indicative of endothelial dysfunction.

The endothelium produces plasminogen activator [5] and there are contradictory reports concerning fibrinolytic activity in diabetes in man [6–9]. Decreased fibrinolytic activity in response to venous stasis has been reported in diabetic patients with and without retinopathy, with a trend to those with long duration of diabetes and with retinopathy having a greater impairment [7]. In these studies, the patients have not been closely matched with control subjects, and have included different types of diabetes on different therapies.

Fibronectin is a glycoprotein present in plasma and in tissues where it acts as an adhesive [10]. It is produced by the endothelium [11, 12] and may affect the adhesion of blood cells to the endothelial surface [13]. It is found in increased amounts in the capillary walls and mesangium of diabetic glomeruli [14] and in the coronary arteries of diabetic subjects [15].

Fibrinolytic activity in blood increases after venous stasis [16], but the response of fibronectin has not been studied. We have investigated the plasma fibrinolytic and fibronectin changes after either venous stasis or ischaemia in the forearm of patients with Type 1 diabetes who have developed retinopathy, in a control group of long-standing diabetic patients with minimal evidence of microangiopathy, and in normal subjects.

Subjects and methods

Subjects

Two groups with long-standing Type 1 diabetes were studied (Table 1). Their basal insulin requirement was supplied by once daily ultralente insulin, and the prandial requirement by twice daily soluble insulin [17]. Eleven had proliferative retinopathy documented by colour photographs and fluorescein angiography. A second group of 10 patients had 'nil/minimal' evidence of retinopathy (fewer than five microaneurysms in a 30° colour photographic field) despite a slightly longer duration of diabetes and no proteinuria. Sensory neuropathy of patients was assessed by measurement of vibration sensory threshold using the top scale of a biothesiometer [18] (Biomedical Instruments, Newbury, Ohio, USA), the precision ($\pm 1\text{SD}$) being 1.3 u. The patients with proliferative retinopathy were less sensitive than those with-

Table 1. Clinical details of the normal and diabetic subjects studied

	Normal subjects (n=13)	Diabetic patients	
		'Nil/minimal' retinopathy (n=10)	Proliferative retinopathy (n=11)
Age (years)	47.3 ± 7.6	50.0 ± 9.0	44.5 ± 9.3
Duration of diabetes (years)	—	27.0 ± 4.8	24.3 ± 4.9
Haemoglobin A _{1c} (%)	7.4 ± 0.7	8.9 ± 1.5	8.8 ± 1.3
Plasma glucose (mmol/l)	4.5 ± 1.2	12.4 ± 6.1	13.0 ± 7.1
Body mass ÷ index (kg/m ²)	26.0 ± 3.5	24.4 ± 1.6	25.2 ± 2.5
Vibration sensory threshold (u)		14.5 ± 5.0	22.0 ± 7.9 ^a
Proportional increase in heart rate on deep breathing		1.3 ± 0.33	1.17 ± 0.16
Proportional increase in heart rate on standing		1.08 ± 0.07	1.06 ± 0.06

Results expressed as mean ± SD. ^a $p < 0.05$, the two diabetic groups had significantly different vibration sensory thresholds

out ($p < 0.05$; Table 1). Autonomic neuropathy was assessed by the response to standing by measuring the degree of RR interval shortening at about 15 beats and lengthening at about 30 beats [19]. The RR variability in response to five deep breaths was also measured. The patients with proliferative retinopathy had a slight, but not significant impairment compared with those without ($p < 0.1$).

A group of 13 normal subjects with similar age, sex and body weight was studied during the same period (Table 1). None had a history of angina, myocardial infarction or other major vascular event.

The study was approved by the Ethics Committee of the Oxford Area Health Authority, and all patients gave informed consent.

Blood sampling

Samples were taken in hospital in the mid-morning, after the diabetic patients had their normal insulin and breakfast at home. All were rested for at least 30 min in a room whose ambient temperature was kept between 20.5 °C and 24.4 °C.

At the start of the test, a blood sample was taken with minimal venous compression for basal fibrinolysis and fibronectin, and for glycosylated haemoglobin level, assayed by the thiobarbituric method [20] and plasma glucose level measured by a glucose oxidase method (GOD-PERID, Boehringer, Mannheim, FRG).

Using a sphygmomanometer cuff, forearm venous stasis was achieved with a pressure of 80 mmHg. After 10 min, a further sample was taken after insertion of an indwelling butterfly cannula. The cuff pressure was released, and blood samples were then taken at 10.5, 11, 12 and 15 min.

Blood was anticoagulated with one-tenth volume trisodium citrate (0.1 mol/l) and all samples were kept on melting ice until the last sample was drawn. Blood was centrifuged at 2800 g for 10 min at 4 °C and fibrinolytic activity measured within 30 min. Euglobulin fractions were prepared and fibrinolytic activity measured using the fibrin plate method [21]. Lysis areas were converted to units by reference to a standard curve prepared from dilutions of purified urokinase (0.01–10 Committee of Thrombolytic Assay units/ml, Choay Institute, Paris, France). In view of the uncertain significance of the basal results, the absolute values after venous stasis were examined. Plasma citrate

Fig. 1. Fibrinolytic activity in individual patients with median (—) before and after 10 min sphygmomanometer cuff occlusion at 80 mmHg. The results show samples taken at 10 min before the cuff was released, and at 0.5, 1 and 2 min later

Fig. 2. Plasma fibronectin concentrations in individual patients before and at the end of 10 min sphygmomanometer cuff at 80 mmHg

samples were stored at –20 °C for later spectrophotometric assay of fibronectin, using an immunoturbidimetric method (Boehringer, Ingelheim, FRG).

All samples were corrected for citrate dilution and haemoconcentration using the formulation:

$$\frac{9(1-PCV_2) + 1}{9(1-PCV_1)}$$

where PCV_1 is the initial packed cell volume and PCV_2 is the packed cell volume for the subsequent sample [22].

Subjects also had vascular reactivity measured after 10 min forearm ischaemia induced by a sphygmomanometer cuff at 200 mmHg, using a transcutaneous oxygen monitor (Hellige, Freiburg, FRG) as reported previously [2]. Some subjects also had samples taken for fibrinolytic activity and fibronectin at the end of the ischaemic period.

Statistical analyses

Statistical analysis was by Student's *t*-test (two-tailed) and correlation coefficient, except for non-parametric data which are expressed as medians, when the Mann Whitney U test and Spearman rank correlation [23] were used.

Results

The fibrinolytic activity before and after 10 min of venous stasis in the diabetic patients with and without retinopathy and in the normal subjects is shown in Figure 1. There was no difference in the unstimulated activity in the three groups. However, at 10, 10.5 and 11 min, the group with proliferative retinopathy had a significantly reduced response compared with the normal subjects, the difference being greatest at 10 min (medians of the 0 and 10 min values were, respectively: 0.13 and 0.26 versus 0.17 and 0.68 U/ml; $p < 0.005$). The patients with proliferative retinopathy had values in the low normal range. Compared with the 'nil/minimal' retinopathy group (medians of the 0 and 10 min values respectively: 0.16 and 0.68 U/ml), the proliferative retinopathy group had a significantly reduced response, the difference being greatest at 10 min ($p < 0.01$). There was no difference between the control subjects and the 'nil/minimal' retinopathy group at any time point.

Blood glucose, glycosylated haemoglobin and body mass index are shown in Table 1. There were no significant differences between the diabetic groups with and without retinopathy. The fibrinolytic activity did not correlate significantly with the plasma glucose or glycosylated haemoglobin levels.

The plasma fibronectin levels were similar in all three groups. Figure 2 shows the response of plasma fibronectin to venous stasis in the two diabetic groups, with a significant increase following venous stasis in the 'nil/minimal' retinopathy group (mean $\pm$ SD of the 0 and 10 min values were respectively: 312 ± 65 and 371 ± 61 μ g/ml; $p < 0.05$) and in the proliferative retinopathy group (317 ± 96 and 399 ± 71 μ g/ml; $p < 0.002$) but not in the normal subjects (304 ± 57 and 333 ± 82 μ g/ml; NS). The rise in fibronectin after venous stasis (using a mean of values at 10, 10.5 and 11.5 min) was greater in the proliferative retinopathy group compared with normal subjects ($p < 0.03$) and the 'nil/minimal' group compared with normal subjects ($p < 0.05$). There was no difference between the two diabetic groups. There was no correlation between basal or stimulated fibronectin and plasma glucose or glycosylated haemoglobin.

Fibrinolytic activity did not correlate significantly with the plasma fibronectin, with the hyperaemic response to ischaemia or to glycosylated haemoglobin or ambient blood glucose. Similarly, plasma fibronectin responses did not correlate significantly with the hyperaemic responses, glycosylated haemoglobin or blood glucose.

Fibrinolytic activity was assessed following 10 min forearm ischaemia in 10 control subjects and seven diabetic patients with 'nil/minimal' retinopathy. There was no rise in any group (basal, the mean of the 10 and the mean of the 11 min values were respectively: 0.17 and 0.12 U/ml, 0.26 and 0.30 U/ml and 0.13 and 0.13 U/ml). Fibronectin response to ischaemia was assessed in three control and seven diabetic subjects (two without, five with retinopathy). There was no rise in either group (basal increasing to mean 10 and 11 min, 341 to 350 μ g/ml, and 340 to 343 μ g/ml, respectively).

Discussion

This study shows that diabetic patients with proliferative retinopathy had a reduced fibrinolytic activity in response to venous stasis, in the low normal range, although their unstimulated levels were comparatively normal. In contrast, patients who were specifically selected with a similar or slightly larger age and duration of diabetes, but who had not developed retinopathy, had a normal response. The diabetic state alone is not responsible for the defect. These results could be explained by either (1) microangiopathy resulting in failure of the vessel wall to respond to distension, or (2) by a low-normal release of fibrinolytic activity being a factor in the genesis of microangiopathy. The response is not secondary to ischaemia as neither normal nor diabetic subjects showed any response to 10 min with the sphygmomanometer cuff at 200 mmHg. The mechanism of plasminogen activator increase after venous stasis is poorly understood [5], but is likely to arise from distended capillaries. The low normal responses of diabetic patients with proliferative retinopathy may reflect a reduced number of capillaries in the hand and forearm, similar to that leading to retinal disease. The absence of correlation between fibrinolytic activity and variables of peripheral or autonomic nerve function suggests that the problem is not primarily one of innervation as suggested previously [7, 25], but is at the local endothelial level. The lack of correlation with cutaneous hyperaemia following forearm ischaemia supports other work suggesting that the fibrinolysis release is independent of blood flow responses [24]. These data do not support the view that blood glucose control directly affects fibrinolytic activity [25]. Low fibrinolytic activity in the blood does not necessarily correlate with low histological plasminogen activator in the vessel walls, and it has been suggested that the low blood level is due to defective release of plasminogen activator [26].

Plasma fibronectin has been reported previously to be normal or raised in diabetes, with a suggestion that patients with retinopathy are particularly affected [27]. In this study, basal levels were similar in the normal and diabetic subjects, with sampling done carefully with minimal venous compression. However, after venous stasis, both diabetic groups had a significant rise. Those

with proliferative retinopathy had a slightly greater, but non-significant, rise than those without retinopathy. Although fibronectin levels did rise slightly in the normal subjects this rise did not achieve statistical significance.

The abnormal fibrinolysis and fibronectin responses, regardless of their cause, may be factors promoting and perpetuating diabetic microangiopathy by impairing the ability of the microvasculature to dispose of an obstruction, including red cell rouleaux, a platelet aggregate or fibrin strands. The inter-relationship of abnormal vasoregulatory and haemostatic responses has been proposed in the pathogenesis of other ischaemic diseases [28]. These responses might convert an otherwise temporary obstruction into pathological capillary closure, and might contribute to the inexorable progression of advanced renal microangiopathy [29] or retinopathy [30] in spite of excellent metabolic control. Further studies are needed to determine whether patients with a low fibrinolytic response are a subgroup who are at increased risk of microangiopathy.

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References

1. Bloom AL (1979) Blood and vascular interactions and their possible relevance to diabetic angiopathies. *Medicographia* 1: 3–8
2. Haitas B, Barnes A, Shogry MEC, Weindling M, Rolfe P, Turner RC (1984) Delayed vascular reactivity to ischemia in diabetic microangiopathy. *Diabetes Care* 1: 47–51
3. Bensoussan D, Levy-Toledano S, Passa P, Cane J, Canivet J (1975) Platelet hyperaggregation and increased plasma levels of von Willebrand factor in diabetics with retinopathy. *Diabetologia* 11: 307–312
4. Porta M, Townsend C, Clover GM, Nanson M, Alderson AR, McCraw A, Kohner EM (1981) Evidence for functional endothelial damage in early diabetic retinopathy. *Diabetologia* 20: 597–601
5. Hedner U, Nilsson IM (1981) The role of fibrinolysis. *Clin Haematol* 10: 327–342
6. Tanser AR (1967) Fibrinolytic responses of diabetics to adrenaline. *J Clin Path* 20: 231–233
7. Almer L, Pandolfi M, Nilsson IM (1975) Diabetic retinopathy and the fibrinolytic system. *Diabetes* 24: 529–534
8. Bern MM, Cassani MP, Horton J, Rand L, Davis G (1980) Changes of fibrinolysis and factor VIII coagulant antigen and ristocetin cofactor in diabetes mellitus and atherosclerosis. *Thrombosis Res* 19: 831–839
9. Fuller JH, Keen H, Jarrett RJ, Omer T, Meade TW, Chakrabarti R, North WR, Stirling Y (1979) Haemostatic variables associated with diabetes and its complications. *Br Med J* 2: 964–966
10. Yamada KM, Olden K (1978) Fibronectins – adhesive glycoproteins of cell surface and blood. *Nature* 275: 179–184
11. Jaffe EA, Mosher DF (1978) Synthesis of fibronectin by cultured human endothelial cells. *J Exp Med* 147: 1779–1791
12. Clark RA, Quinn JH, Winn HJ, Lanigan JM, Dellepella P, Colvin RB (1982) Fibronectin is produced by blood vessels in response to injury. *J Exp Med* 156: 646–651

13. Wautier JL, Paton RC, Wautier MP, Pintigny D, Abadie E, Passa P, Caen JP (1981) Increased adhesion of erythrocytes to endothelial cells in diabetes mellitus and its relation to vascular complications. *N Engl J Med* 305: 237–242
14. Weiss MA, Ooi BS, Ooi YM, Engvall E, Ruoslahti E (1979) Immunofluorescent localisation of fibronectin in the human kidney. *Lab Invest* 41: 340–347
15. Ledet T (1976) Diabetic cardiopathy. Quantitative histological studies of the heart from young juvenile diabetics. *Acta Path Microbiol Scand* 26: 798–803
16. Clarke RL, Orandi A, Clifton EE (1960) Induction of fibrinolysis by venous obstruction. *Angiology* 11: 367–370
17. Phillips M, Simpson RW, Turner RC (1979) A simple and logical twice daily subcutaneous insulin regime. *Quart J Med* 48: 191; 493–506
18. Gregg EG (1951) Absolute measurement of the vibratory sensory threshold. *Arch Neurol Psychiatry* 66: 403–411
19. Ewing DJ, Campbell IW, Murray A, Neilson JM, Clarke BF (1978) Immediate heart rate response to standing: simple test for autonomic neuropathy in diabetes. *Br Med J* 1: 145–147
20. Ross IS, Gibson PF (1979) A semi-automated method for the determination of glycosylated haemoglobin. *Clin Chim Acta* 98: 53–59
21. Astrup T, Mullertz S (1952) The fibrin plate method for estimation of fibrinolytic activity. *Arch Biochim Biophys* 40: 346–351
22. Chan TK, Chan V (1979) The effect of venous occlusion on anti-thrombin III, plasminogen activator and fibrinogen degradation product (fragment E) levels. *Thrombosis Research* 14: 525–534
23. Siegel S (1956) Non-parametric statistics for the behavioural sciences. McGraw Hill, New York
24. Chlmielewska J, Ranby M, Wiman B (1983) Evidence for rapid inhibitor to tissue plasminogen activator in plasma. *Thrombosis Res* 31: 427–433
25. Almer L-O, Lilja B, Lindell SE, Nilsson IM (1981) Endothelial factors in relation to peripheral circulation in diabetes. *Thromb Res* 20: 231–238
26. Gunnarsson R, Nyman D, Walinder O, Ostman J (1980) Fibrinolytic activity and diabetes control – evidence for a relationship. *Acta Med Scand (Suppl)* 639: 23–24
27. Davis TME, Moore JC, Turner RC (1984) Plasma fibronectin, factor VIII-RA and fibrinogen concentrations and diabetic retinopathy. *Diabete Metab* (in press)
28. Hellstrom HR (1982) The injury spasm (ischaemia-induced haemostatic vasoconstrictive) and vascular autoregulatory hypothesis of ischaemic disease. *Am J Cardiol* 49: 802–808
29. Viberti GC, Bilous RW, MacKintosh D, Bending JJ, Keen H (1983) Long-term correction of hyperglycaemia and progression of renal failure in insulin-dependent diabetes. *Br Med J* 286: 598–601
30. Lauritzen T, Frost-Larsen K, Larsen H-W, Deckert T and the Steno Study Group (1983) Effect of 1 year of near-normal blood glucose levels on retinopathy in insulin-dependent diabetics. *Lancet* 1: 200–203

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