Polymerisation and crosslinking of fibrin monomers in diabetes mellitus

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Summary. Polymerisation and crosslinking of fibrin monomers was studied in 35 healthy volunteers and in 42 poorly controlled diabetic patients. Polymerisation did not show any difference between control subjects ($n = 10$) and diabetic patients ($n = 11$) ($p > 0.1$), although fibrinogen was 35% more glycated in the diabetic patients ($p < 0.001$). Alpha chain crosslinking in the diabetic patients, however, was impaired as is shown from an increase in intermediate alpha polymers with a concomitant decrease in alpha monomer disappearance. A significant positive correlation was found between the degree of glycation of fibrinogen and the defective alpha chain polymerisation ($r = 0.86$, $p < 0.005$). These results were consistent with the results of thrombin and reptilase experiments. The reaction rate with reptilase did not show any difference between the two groups ($p > 0.1$), whereas the reaction rate with thrombin was significantly slower in the diabetic group compared to the control subjects ($p < 0.001$). Purified fibrin clots obtained from the diabetic patients were more susceptible to plasmin than clots obtained from control subjects. It is concluded that in poorly controlled diabetic patients polymerisation of fibrin monomers is normal, but crosslinking of the alpha chains is impaired, leading to a higher susceptibility of the clots to plasmin degradation.

Key words: Fibrinogen, polymerisation, crosslinking, glycation, diabetes mellitus, thrombin time, reptilase time, plasmin clot lysis.

Diabetes mellitus is associated with early and sometimes rapidly progressing micro- and macroangiopathic lesions. The accompanying anomalies of the haemostatic system may be either the cause or consequence of the vascular damage. As to fibrinogen, a key protein for the coagulation process, various disturbances have been described, such as: fibrin depositions in various tissues [1], altered fibrin(ogen) metabolism [2, 3], increased fibrin complexes in circulating blood [4, 5], reduced fibrinogen survival [6] and increased concentrations of fibrinogen in plasma [7]. Non-enzymatical glycation of proteins results from sustained hyperglycaemia [8]. Fibrinogen turned out to be no exception [9, 10]. Glycation takes place at lysine molecules, which play an important role in the fibrinogen metabolism [11].

We, therefore, raised the question of whether the increased glycation of fibrinogen influences its function.

In this study the polymerisation, subsequent crosslinking of fibrin monomers and plasmin lysis of fibrin clots in control subjects and in poorly controlled diabetic patients are compared.

Subjects and methods

Four protocols were used to assess the influence of the increased glycation: protocol A: polymerisation of fibrin monomers; protocol B: crosslinking of fibrin polymers; C: reaction with thrombin and reptilase (venom of Batrops atrox); and protocol D: clot lysis by plasmin.

Subjects

Thirty-five healthy volunteers and 42 diabetic patients were included in the study. All patients were Type 1 (insulin-dependent) diabetic patients according to the WHO classification [12].

Table 1 states the characteristics of the subjects participating in the three protocols. Six control subjects participated in two protocols. No drugs influencing the clotting process were taken by any of the participants. Clotting factor XIII was normal in all patients.

Methods

Protocol A: Polymerisation of fibrin monomers. Blood was taken from an antecubital vein without stasis. Clotting was prevented by EDTA 0.19 mol/l. Benzamidine 10 mmol/l was added as serine protease inhibitor.

Table 1. Characteristics of diabetic patients (D) and control subjects (C) participating in protocol A (polymerisation of fibrin monomers), protocol B (crosslinking of fibrin polymers), protocol C (thrombin and reptilase reaction) protocol D (clotlysis). (Mean, range)

Protocol	Group	n	Age (years)	Diabetes duration (years)	HbA$_{1c}$ (%)	Fibrinogen (g/l)	Glucose (mmol/l)
A	D	11	58 (36–68)	22 (8–32)	11.5 (5.2–16.5)	4.0 (3.1–5.5)	12.9 (7.3–17.4)
	C	10	30 (23–50)	–	4.3 (3.8– 4.7)	2.6 (1.8–3.5)	5.0 (4.1– 5.4)
B	D	8	59 (16–85)	20 (2–31)	11.6 (7.0–14.2)	4.1 (2.7–6.1)	12.8 (8.1–15.8)
	C	10	40 (21–63)	–	3.8 (3.1– 4.6)	2.9 (2.5–3.4)	4.8 (4.0– 5.1)
C	D	16	60 (22–80)	18 (3–30)	8.0 (5.7–15.0)	3.0 (2.2–3.9)	10.6 (7.0–14.8)
	C	12	49 (19–75)	–	4.0 (3.0– 4.8)	2.8 (2.2–4.1)	4.7 (3.9– 5.1)
D	D	7	42 (29–78)	14 (2–28)	10.5 (7.7–15.3)	3.2 (2.1–5.3)	11.2 (7.1–16.8)
	C	9	39 (28–60)	–	4.2 (3.9– 4.4)	2.8 (2.3–3.8)	4.5 (3.5– 4.9)

Plasma was prepared by two centrifugation steps (4000 g, 10 min). Fibrin monomers were prepared according to Belitser [13]. To plasma 10 U/ml thrombin was added. After incubation for 30 min at 37°C, the clot was collected and washed 3 times in phosphate buffered saline (0.14 mol/l NaCl in 0.1 mol/l phosphate buffer pH 7.2). The clot then was solved in 0.02 mol/l acetic acid (ionic strength 0.12).

To start polymerisation, a standard amount of the clear monomer solution was brought into twenty fold its volume of 0.06 mol/l phosphate buffer, pH 6.8. Polymerisation was followed spectrophotometrically at 350 nm. (Pye Unicam SP8-400 UV/VIS, Philips, Eindhoven, The Netherlands).

As a measure for the polymerisation rate, the maximal incrase in absorbance at 350 nm/min (ΔA_{max}/min, (350 nm)) was calculated from the maximal tangent of the sigmoidal clotting curve [14].

Protocol B: Crosslinking of fibrin polymers. Blood was collected in 0.1 volume of 129 mmol/l trisodium citrate/10 mmol/l benzamidine. Plasma was prepared by two centrifugation steps (4000 g, 10 min). Plasma was clotted by adding 1.2 U thrombin/5 μmol CaCl$_2$ per milligram of fibrinogen. After various times up to 24 h, the clots were collected and extensively washed in 0.15 mol/l NaCl. The clots, then, were treated overnight with 8 mol/l urea/2% sodium dodecylsulfate (SDS)/0.02% dithiotreitol in 0.1 TrisHCl pH 6.8 at 37°C in order to reduce disulphide bridges and to obtain separate fibrin chains. After further dilution with 8 mol/l urea/0.1% sodium dodecylsulfate, 11 μl of the solution, containing 15 μg fibrin chains, was subjected to polyacrylamide gel (PAG) electrophoreses on a 5% gel [15].

Monomers were obtained by adding EDTA 0.020 mmol/l instead of CaCl$_2$ to the clotting mixture. Coomassie brilliant blau, R250 (Merck, Darmstadt, FRG) was used as dye.

The bands were scanned by an Elvi densitometer (Marius Instruments, Utrecht, The Netherlands).

Protocol C: Thrombin and reptilase reactions Citrate plasma was prepared from blood (0.1 volume of 129 mmol/l trisodium citrate and 0.9 vol of blood). Fibrinogen concentration was adjusted to 2.0 g/l using 0.15 mol/l NaCl. The plasma then was further diluted 1:1 with Owrens Veronal buffer [16] pH 7.35.

To 2 ml of the diluted plasma 1 ml of thrombin (2U/ml) was added at time 0 and the reaction was followed spectrophotometrically at 350 nm. In the reptilase assay plasma (2.0 g/l fibrinogen) was diluted 1:3 with Owrens Veronal buffer pH 7.35 and 0.5 reptil-

ase (Reptilase R, Boehringer, Mannheim FRG) was added, after which the clotting was recorded. The reaction rate was expressed as maximum increase in absorbance at 350 nm/min (ΔA_{max}/min, (350 nm)), calculated from the maximal tangent of the absorbance curve.

Protocol D: Clotlysis by plasmin. Blood was collected in 129 mmol/l trisodium citrate/10 mmol/l benzamidine. After two centrifugation steps (4000 g, 10 min) endogenous plasminogen was removed by lysine-sepharose affinity chromatography [17]. Fibrinogen was purified using two glycine precipitations, using 2.1 mol/l glycine at 4°C. The last precipitate was resuspended in sodium phosphate buffer 0.1 mol/l, pH 6.4/10 mmol/l bezamidine and dialysed against 0.3 mol/l NaCl/10 mmol/l benzamidine during 16 h and against 0.3 mol/l NaCl during another 8 h. To 3 mg/ml of purified fibrinogen 225 μl phosphate buffered saline (0.14 mol/l NaCl in 0.1 mol/l phosphatebuffer pH 7.4), 37.5 μl poolserum as factor XIII donor, 225 μl ^{125}I-fibrinogen (specific activity 3.7 MBq/mg fibrinogen, Amersham, Amersham, Bucksh, UK), 1.2 CU plasminogen (KabiVitrum, Stockholm, Sweden) and 3.6 U thrombin/15 μmol CaCl$_2$ were added. After 20 h, the clots were collected on a glass rod and extensively washed in 0.15 mol/l NaCl. The clots then were immersed in 1200 U streptokinase (Streptase, Hoechst Behringwerke, Marburg/Lahn, FRG) in phosphate buffered saline (0.14 mol/l NaCl in 0.1 mol/l phosphate buffer pH 7.4). After 0.5, 1, 2, 3 and 4 h, an aliquot of the supernatant was taken. Radioactivity was counted in a multiwell gamma counter (Nuclear Enterprises, Beenham, UK). Lysis was expressed as % radioactivity in the supernatant after correction for spontaneous lysis.

General methods

HbA$_{1c}$ was estimated by affinity chromatography (Pierce Chemical Company, Rockford, Ill, USA, Ref. value 3.6–6.8%). Fibrinogen was estimated by the method of Strengers [18]. (Ref. value 2.0–4.0 g/l). Factor XIII was estimated either by a clotting method, using dilutions of deficient plasma (Ref. value 50–150%), or by the clotting FXIII[a] inhibition test (Hoechst Behringwerke AG, Marburg/Lahn, FRG, Ref. value 70–150%). Glycation of fibrin(ogen) was estimated by a modification of the hydroxymethylfurfural method as described previously [10].
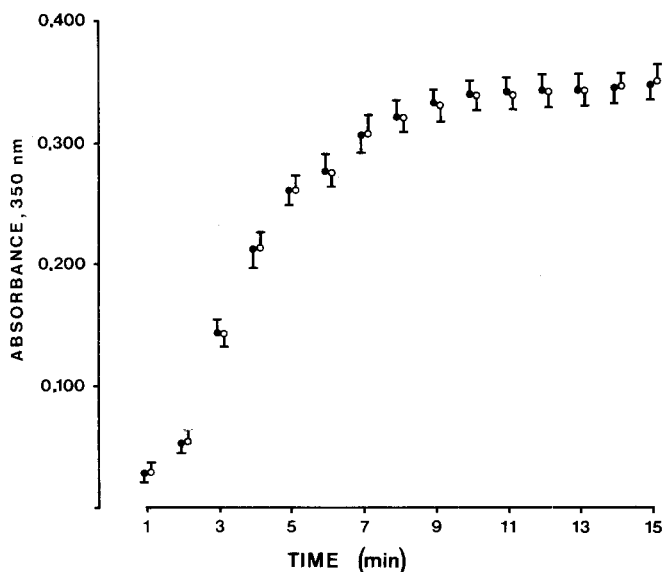
Fig. 1. Polymerisation curve of fibrin monomers. 100 µl (0.020 mg/ml) monomer solution was brought into 2 ml of 0.06 mol/l phosphate buffer pH 6.8. Absorbance at 350 nm was recorded (mean ± SD). ○ diabetic patients; ● control subjects

Statistical analysis

Results are expressed as mean ± SEM or SD when indicated. For comparison of the two groups, Student's t-test for unpaired data was used. Correlation was assessed using the Kendall rank test [19] and orthogonal regression analysis as indicated.

Results

Polymerisation

The results of the polymerisation studies are shown in Figure 1. No difference in lagtime nor in increase in absorbance was found between the two groups. The degree of glycation of fibrinogen was significantly higher in the diabetic group (1.30 ± 0.08 mol glucose/mol fibrinogen) compared to the control subjects (0.83 ± 0.06 mol glucose/mol fibrinogen), whereas $\Delta A_{max}/min$, (350 nm) did not show any difference (0.030 ± 0.001 in the diabetic group against 0.029 ± 0.002 in the control group.)
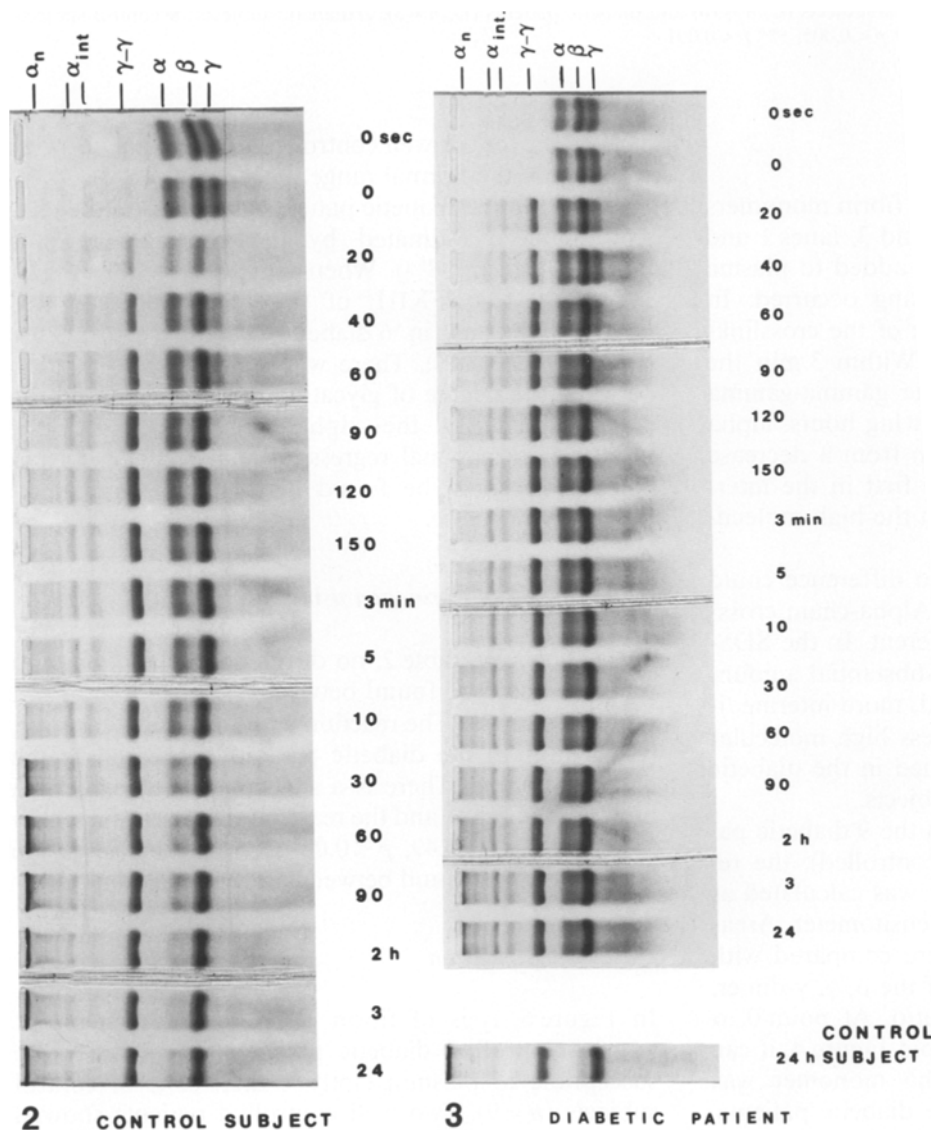
Fig. 2. SDS-PAG electrophoresis on a 5% gel of reduced fibrin monomers (lanes 1 and 2) and fibrin polymers (lanes 3–17) of a control subject. ($\alpha_n = \alpha_n$ polymer; α_{int} = intermediate α-polymers)

Fig. 3. SDS-PAG electrophoresis on a 5% gel of reduced fibrin monomers (lanes 1 and 2) and fibrin polymers (lanes 3–17) of a diabetic patient. Lane 18: 24 h, control subject. ($\alpha_n = \alpha_n$ polymer; α_{int} = intermediate α-polymers)
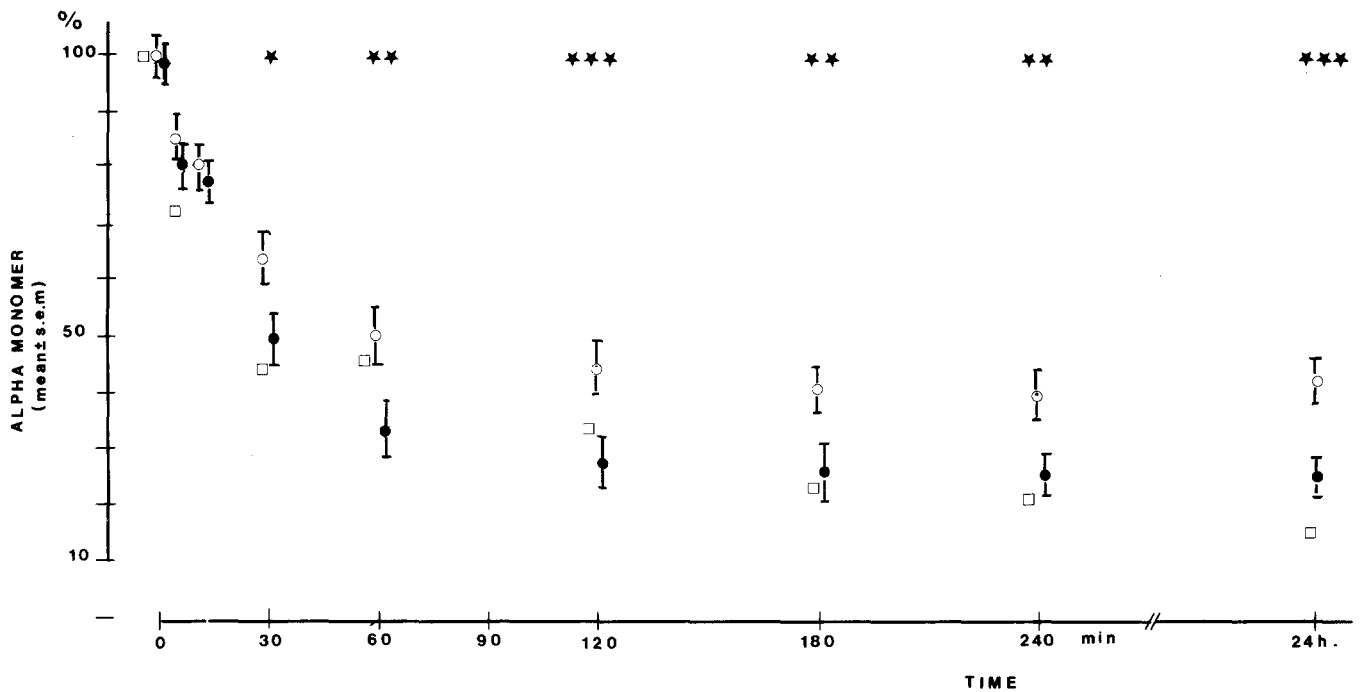
Fig. 4. Decrease of alpha monomers in control subjects (C, $n = 10$) and diabetic patients (D, $n = 8$). ○ diabetic subjects; ● control subjects; □ well controlled diabetic patient. $* \, p < 0.01$, $** \, p < 0.005$, $*** \, p < 0.001$

Crosslinking

SDS-PAG electrophoresis of reduced fibrin monomers showed three distinct bands (Fig. 2 and 3, lanes 1 and 2). When thrombin and $CaCl_2$ were added to plasma polymerisation subsequent crosslinking occurred. In Figure 2 and 3 lanes 3-16 the course of the crosslinking process during 24 h is shown. Within 3 min the gamma band disappeared, while the gamma-gamma dimer was formed. During the following hours, alpha chain polymerisation occurs, as seen from a decrease in alpha monomer and an increase first in the intermediate alpha-polymers and later in the high molecular weight alpha-n polymer.

In gamma-chain crosslinking, no difference could be shown between the two groups. Alpha-chain crosslinking, however, seemed to be different. In the SDS-PAGE slabs, it can be seen that a substantial amount of alpha chain remained unchanged, more intermediate alpha-polymers occurred, and less high molecular weight alpha-n polymers were formed in the diabetic patients compared to the control subjects.

In the 10 control subjects and in the 9 diabetic patients (8 poorly controlled, 1 well controlled), the remaining amount of alpha monomer was calculated as follows: the gels were scanned by densitometer. Areas under scan of the alpha chains were compared with the sums of the areas under scan of the β, γ, γ-dimer, α_{int} and α_n chains to give an α ratio. At point 0, α chain area was postulated 100%. From Figure 4, it can be seen that the decrease in alpha monomer was significantly slower ($p < 0.01$) in the diabetic patients.

The values for the well controlled diabetic patient were well within the normal range.

FXIII in the diabetic patients as well as in the control subjects, estimated by the clotting test, was 80-110% (mean 98%). When estimated by the FXIII[a] inhibition test FXIII of 6 control subjects was $107.5 \pm 8.3\%$ and in 6 diabetic patients $104.2 \pm 9.5\%$ (mean $\pm$ SEM, NS). There was a negative correlation between the degree of glycation of fibrinogen and the disappearance of the alpha monomer ($r = -0.88$, $p < 0.005$, orthogonal regression analysis) (Fig. 5). No correlation could be found between age and alpha monomer decrease.

## Clotting rate of fibrinogen using thrombin and reptilase

As is shown in Table 2, no difference in reaction with reptilase could be found between control subjects and diabetic patients. The reaction with thrombin, however, was slower in the diabetic patients compared to the control subjects. There is a slight negative correlation between the HbA_1 and the reaction-rate in the thrombin experiment. ($\tau = 0.49$, $p < 0.02$, Kendall rank test). No correlation was found between age and reaction rate.

Clotlysis by plasmin

In Figure 6, lysis of fibrin clots shows that clots of poorly controlled diabetic patients ($n = 5$) are more susceptible to plasmin clotlysis than clots of control subjects ($n = 9$). Two well controlled patients showed
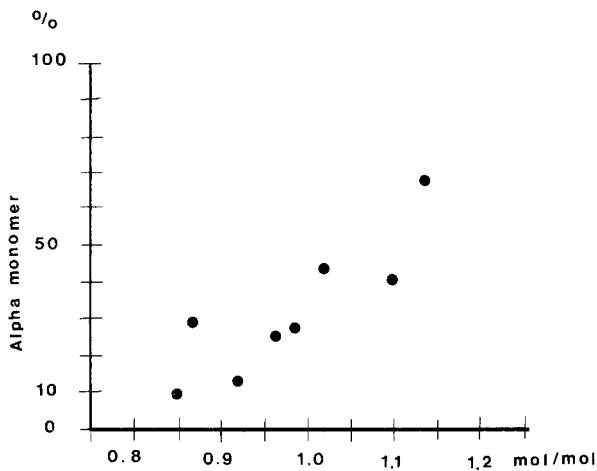
Fig. 5. Correlation between degree of glycation of fibrinogen (mol glucose/mol fibrinogen) and alpha monomer disappearance. ($r = 0.86$, $p < 0.005$)
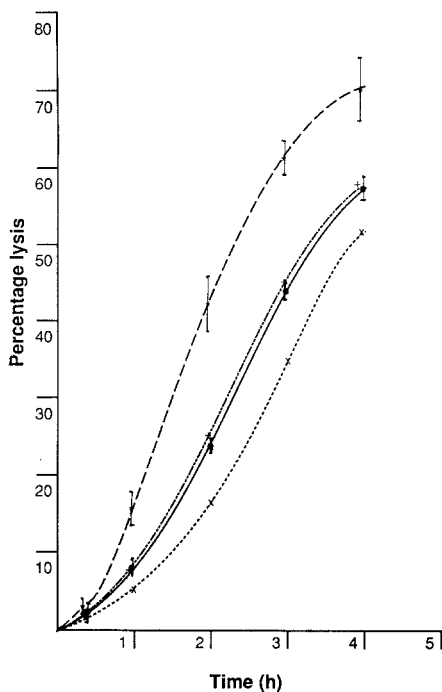
Fig. 6. Lysis rate of fibrin clots from *-----* diabetic subjects $n = 5$, 1.31 ± 0.06 mol glucose/mol fibrinogen; ×-----× diabetic patient, 1.08 mol glucose/mol fibrinogen; +-----+ diabetic patient, 1.11 mol glucose/mol fibrinogen; ●——● control subjects $n = 9$, 1.03 ± 0.03 mol glucose/mol fibrinogen. (mean ± SEM). Difference between diabetic patients and control subjects: at 30 min: NS; at 1 h: $p < 0.01$; at 2 h, 3 h, 4 h: $p < 0.005$

Table 2. Reaction rate, expressed as maximal increase in absorbance at 350 nm (ΔA_{max}, 350 nm/min) after addition of 1U/g fibrinogen of thrombin or reptilase to plasma of 12 control subjects (C) and 16 diabetic patients (D)

	ΔA max, 350 nm/min. (mean ± SEM)		p
	Control subjects $n = 12$	Diabetic patients $n = 16$	
Reptilase	507 ± 22	503 ± 20	NS
Thrombin	784 ± 51	580 ± 21	< 0.001

values comparable to the control group. In the 5 poorly controlled diabetic subjects, 1.31 ± 0.06 mol glucose was bound per mol fibrinogen versus 1.11 mol/mol and 1.08 mol/mol for the 2 well controlled patients. (Nine control subjects: 1.05 ± 0.02 mol glucose/mol fibrinogen, $p < 0.01$ in comparison to the poorly controlled diabetic patients, mean ± SEM). Correlation between mol glucose bound per mol fibrinogen and fibrin lysis was $\tau = 1.0$, $p < 0.005$ (Kendall rank test).

Discussion

When thrombin is added to fibrinogen, fibrinopeptides A and B are released and fibrin monomers are formed. Polymerisation then starts with an end to end alignment of the fibrin monomers, leading to a growing protofibril. Mainly electrostatic forces are involved. When Ca ions and thrombin activated clotting factor XIII are present, covalent end to side isopeptide bonds are formed between adjacent ε-aminogroups of lysine and γ-carboxyamidogroups of glutamine. This process leads to a stable crosslinked clot.

Fibrinopeptide release also is catalysed by reptilase, but no activation of clotting factor XIII occurs [20]. The finding that no difference in polymerisation is shown between the diabetic patients and the control subjects, therefore, seems to be consistent with the results of the reptilase experiment. End to end growth of the fibrin monomers was normal as can be seen from the identical lag times in the polymerisation curves. Polymerisation, therefore, does not seem to be inhibited by the increased glycation of the fibrin molecule. These results also suggest that the fpA release does not differ between the two groups. This has to be confirmed by direct measurement of the fpA release after thrombin addition. Lysine molecules are actively involved in the crosslinking process. Gamma chain dimerisation did not seem to be affected by the higher degree of glycation in the diabetic group. Alpha chain crosslinking, however, showed incomplete disappearance of the alpha monomer and a persistent occurrence of intermediate alpha-polymers. The incomplete alpha polymerisation was positively correlated to the degree of glycation of fibrinogen (Fig. 5). In accordance with this finding, a positive correlation between the slower thrombin reaction and HbA₁ was also observed. These results are compatible with a causal relationship between abnormal clotting behaviour and poor metabolic control.

The finding of normal gamma-chain crosslinking in the presence of decreased alpha crosslinking can be explained by the fact that only one pair of crosslinking sites is present per gamma monomer, versus 6 per alpha monomer. Also, it was suggested by McVerry [9] that in vitro the alpha chain of fibrinogen is glycated to a higher degree than the beta or gamma chains. The diminished crosslinking could not be explained by a low factor XIII concentration in the diabetic patients, as no differences in FXIII concentration are found be-

tween the two groups, when estimated by two methods. This is in accordance with the findings of Kloczko et al. [21], in diabetic patients without angiopathy. In the lysis experiments purified fibrinogen of diabetic patients and control subjects was clotted by the addition of thrombin, Ca^{++} and a standard amount of FXIII. Control SDS-PAG electrophoresis of the clots after 20 h showed the same findings of an impaired disappearance of alpha chains and appearance of α_{int} in the diabetic patients, comparable to Figures 2 and 3, band 24 h (results not shown).

Although the diabetic group was significantly older than the control group, no correlation was found between age and abnormal alpha-chain crosslinking. The well controlled patient, aged 83, showed a normal alpha chain crosslinking pattern. We, therefore, suggest that in poorly controlled diabetic patients crosslinking of the alpha chains is impaired.

As a consequence of impaired crosslinking, a higher susceptibility to plasmin degradation could be expected, as has been shown in several studies not concerning diabetic patients [22, 23]. From Figure 5, it can be seen that there is a higher susceptibility to plasmin degradation in the clots from purified fibrin obtained from poorly controlled diabetic patients. There is a positive relationship between degree of glycosylation and plasmin lysis rate. In these experiments nondiabetic plasminogen was added after removal of the endogenous plasminogen, because of the finding of Geiger et al. [24] who showed an increased glycosylation of plasminogen in poorly controlled diabetic patients, leading to impaired kinetics of plasminogen activation.

In contrast to our findings, Brownlee et al. [25] reported a reduced susceptibility of glycosylated fibrin to plasmin degradation. They, however, used un-physiological, extremely high glucose or glucose-6-phosphate concentrations up to 500 mmol/l over a three week incubation period. No data on the amount of glucose bound per mol fibrinogen are given in their article.

We, therefore, suggest that in poorly controlled diabetic patients impaired alpha-chain crosslinking of fibrin monomers occurs, leading to a less stabilized clot, which is more susceptible to plasmin degradation.

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