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Glycosylation of human fibrinogen in vivo

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Summary. Fibrinogen was purified from plasma from 22 non-diabetic and 26 poorly controlled Type 1 (insulin-dependent) diabetic subjects. In non-diabetic subjects, 0.95 ± 0.17 mol glucose was bound per mol fibrinogen, whereas in the diabetic subjects 1.33 ± 0.21 mol glucose was bound per mol fibrinogen (mean ± SD, $p < 0.001$). Comparison of the amount of bound glucose, when estimated by two different methods, suggested that lysine is the site of glycosylation. It is currently unknown whether this increased glycosylation of fibrinogen alters its function.

Key words: Fibrinogen, glycosylation, Type 1 diabetes.

Glucose has been shown to bind non-enzymatically and irreversibly to all types of protein through Schiff-base formation and Amadori re-arrangement to a ketoamine [1]. This glycosylation appears to be dependent on the duration of exposure and glucose concentration in the surrounding medium. Glycosylation occurs either on the amino terminal end of the protein, as with haemoglobin [2], or on the free amino group of lysine. Because of this glycosylation, the tertiairy or quaternary structure, function and/or degradation of the protein molecule may be altered, as has been demonstrated with glycosylated haemoglobin (HbA_1) [2], collagen [3], lens proteins [4] and many other proteins [5].

Fibrinogen is a protein with a half-life of 3–4 days, which occupies a central position in blood clotting. It is known that the ε-amino groups of lysine in the fibrinogen molecule play an important role in cross-linking fibrin monomers and in fibrinolysis. We have therefore investigated whether glycosylation of fibrinogen is increased in the blood of diabetic patients.

Subjects and methods

Subjects

Twenty-two healthy volunteers (aged 21–59 years, mean 33 years; mean blood glucose level at the time of sampling, 5.1 ± 1.0 mmol/l) and 26 Type 1 (insulin-dependent) diabetic patients (aged 19–57 years, mean 36 years; taking no medication other than insulin) participated in the study.

Methods

Blood was collected in 3.2% sodium citrate (1:10, wt/vol) for purification of fibrinogen and estimation of glycosylation. After centrifugation at 5000 *g* for 20 min, plasma was either processed immediately or stored at −26 °C. Blood was also taken for estimation of glucose and HbA_1. HbA_1 was estimated by the microcolumn method (Isolab In-

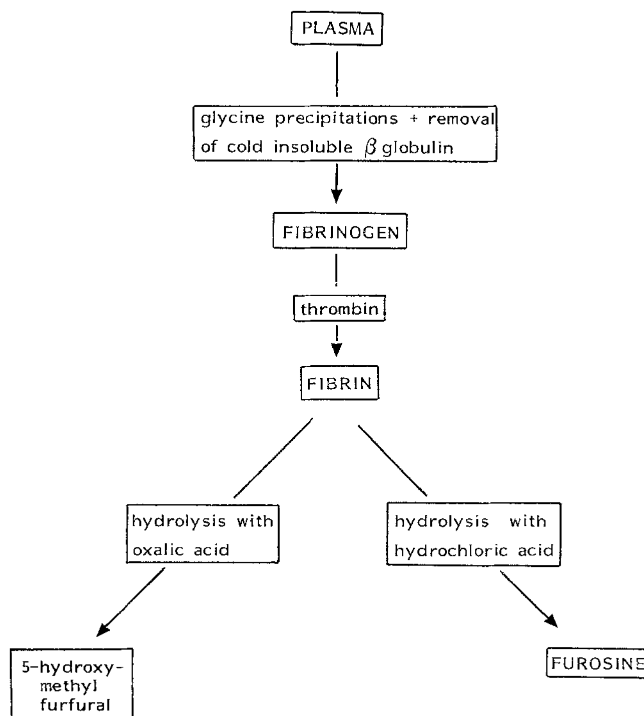

Fig. 1. Purification of fibrin(ogen) and estimation of glycosylation

corporated, Akron, Ohio, USA) after removal of the labile fraction by means of dialysis against NaCl (154 mmol/l). Fibrinogen was estimated by the method of Strengers and Asberg [6] and purified by a modification of the method of Mosesson and Sherry [7] (Fig. 1).

Fibrinogen was twice precipitated using glycine (2.1 mol/l) at 4 °C. The last precipitate was resuspended in sodium phosphate buffer (0.1 mol/l, pH 6.4). After addition of an equal volume of water, the solution was left overnight at 4 °C to remove cold insoluble β-globulin. After centrifugation at 3500 *g* for 10 min, the fibrinogen-containing supernatant was treated by either: (a) addition of ethanol to a final concentration of 8% (vol/vol) at a temperature of −2 to −3 °C; fibrinogen was centrifuged at 4000 *g* for 10 min and the supernatant discarded; or (b) addition of thrombin solution (0.4 ml, 500 U/ml, Topostasin, Hoffman-Laroche, Basel, Switzerland). The fibrin clot formed was extensively washed in NaCl (154 mmol/l). The degree of fibrinogen purification was estimated by the addition of ^{125}I-albumin (0.1 ml, 1.0 µCi/ml, Amersham International, Amersham, Bucks, UK) to 250 ml of plasma, and by isoelectric focussing (1.0% agarose, 12% sorbitol, pH 4–9 for a period of 90 min) [8].

To estimate glycosylation, (a) purified fibrinogen or (b) fibrin (40–50 nmol) were hydrolysed in two ways: (1) for 18 h at 95 °C in oxalic acid (2.0 ml, 0.6 N); or (2) for 18 h at 95 °C in hydrochloric acid (1.5 ml, 6 N).

Hydrolysation under weak acidic conditions caused 5-hydroxymethylfurfural (HMF) to be formed, which was estimated by the thiobarbituric acid method [9]. Hydrolysation in 6 N hydrochloric acid caused further destruction and various products including ε-N-(-2-furoylmethyl)-L-lysine (furosine) were formed, which were estimated by high performance liquid chromatography according to the method of Schleicher and Wieland [10]. A 30 cm µ-Bondapack reversed phase column (Waters Associates, Milford, Mass, USA) was used with 0.7% H_3PO_4 as eluent. Phenylalanine was used as the internal standard, furosine as the external standard.

Statistical analysis

Results are given as mean ± SD. The differences were calculated using Student's t-test for unpaired data. Correlation coefficients were calculated by the method of least squares.

Results

Purification of fibrinogen by glycine precipitations, removal of cold insoluble globulin and ethanol precipitation results in fibrinogen of high purity (albumin contamination of the purified fraction 0.01%; Table 1). However, the yield was low (10–15%).

After clot formation with thrombin, instead of ethanol precipitation, fibrin was isolated of equal purity, but with a higher yield (30–50%; Table 1). Isoelectric focussing of the supernatant after removal of the cold insoluble β-globulin (fraction S_{III}) before and after clot removal showed only trace amounts of albumin and globulins (results not shown). Isoelectric focussing of the purified fibrinogen preparation also showed only trace amounts of other proteins.

When glycosylation of ethanol precipitated fibrinogen and of fibrin formed from this fibrinogen were compared, no significant difference was shown (fibrinogen: 1.20 ± 0.13 mol glucose/mol fibrinogen, $n = 6$; fibrin 1.26 ± 0.14 mol glucose/mol fibrin, $n = 7$).

Using the clot formation method, fibrin was purified from plasma from 22 healthy volunteers and 26 diabetic

Table 1. Purification of fibrinogen/fibrin from blood obtained from ten healthy volunteers

	Yield (%)	^{125}I-albumin (%)
Plasma	100	100
Second glycine precipitate	50–70	0.6
Supernatant after removal of cold insoluble β-globulin	30–50	0.5
Fibrinogen (ethanol precipitation from supernatant after removal of cold insoluble β-globulin)	10–15	<0.01
Fibrin (clot formation from supernatant after removal of cold insoluble β-globulin)	30–50	<0.01

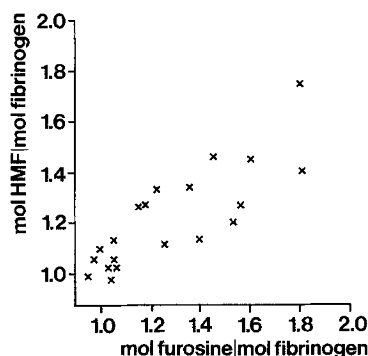

Fig. 2. Correlation between the hydroxymethylfurfural (HMF) and the furosine methods for the estimation of glycosylation of fibrinogen in 8 non-diabetic and 12 diabetic subjects

Table 2. Glycosylation of fibrin(ogen) in normal and diabetic subjects

	Glucose (mmol/l)	HbA_1 (%)	Fibrin(ogen) (g/l)	Mol glucose/mol fibrin(ogen)	
				Hydroxymethyl-furfuralmethod	Furosine method
Normal subjects ($n = 22$)	5.1 ± 1.0	6.6 ± 1.9	2.3 ± 0.5	1.06 ± 0.08	0.95 ± 0.17
Diabetic subjects ($n = 26$)	13.7 ± 5.3	13.3 ± 2.1	4.1 ± 1.4	1.30 ± 0.19	1.33 ± 0.21
p	<0.001	<0.001	<0.001	<0.001	<0.001

Results expressed as mean ± SD

subjects on insulin therapy. Glucose and HbA_1 levels at the time of sampling, as well as the amount of glucose bound per mol of fibrin(ogen), estimated by both the HMF and furosine methods, are shown in Table 2. No significant correlation was found between HbA_1 and glycosylation of fibrinogen or between blood glucose and glycosylation of fibrinogen. However, a highly significant correlation between HMF and the furosine method was found ($r = 0.74$, $p < 0.001$; Fig. 2).

From Table 2, it can be seen that 0.95 ± 0.17 mol glucose was bound per mol fibrin(ogen) in non-diabetic subjects. In poorly controlled diabetic patients 1.33 ± 0.21 mol/mol was bound, the difference being highly significant ($p < 0.001$).

Discussion

Fibrinogen and fibrin have a very similar primary structure. Fibrinogen contains the fibrinopeptides A and B, which are split off by the action of thrombin. These fibrinopeptides do not, at least in man, contain lysine. Fibrin and fibrinogen are glycosylated to a similar extent, from which it can be concluded that glycosylation is not confined to either fibrinopeptide A or B. The clot formation method, therefore, can be chosen for purification.

HMF is a product of hydrolysis of glucose interaction with any amino-acid, whereas furosine is highly specific for glucoselysine bonds. As no difference was found between the amount of HMF and the amount of furosine formed by the hydrolysis of fibrin(ogen), we assume that lysine is the site of glycosylation in fibrinogen. As was shown by Lorand et al. [11], lysine is the amino donor in fibrin cross-linking. They demonstrated that 2.7–4.5 mol lysine/mol fibrin are involved in this process. It is conceivable that these lysine molecules are easily attainable, which also makes them available for glucose molecules. It is tempting to assume that glycosylation of lysine is important for fibrin(ogen) function and degradation. This concept is in agreement with work by Brownlee et al. [12], who showed a reduced susceptibility of glycosylated fibrin to degradation by plasmin. In their study, fibrin was glycosylated by incubation with glucose concentrations far higher than those found in diabetic patients.

In the present study, we have shown that glucose is also bound irreversibly in vivo. In poorly controlled diabetic subjects, a small, but significantly higher, amount of glucose is bound. These results cannot be attributed to the minor impurity of the fibrinogen solution caused by other plasma proteins. From data on plasma protein glycosylation obtained from the literature [13], it can be calculated that the contribution of furosine derived from this plasma protein impurity is less than 0.1% of the total amount of furosine formed.

We therefore conclude that fibrinogen in poorly controlled diabetic subjects contains more glucose, which we suggest is bound to lysine.

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