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NON ENZYMATIC GLYCOSYLATION INCREASES PLATELET AGGREGATING POTENCY OF COLLAGEN FROM PLACENTA OF DIABETIC HUMAN BEINGS

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Pepsin extracted collagen and an acid soluble glycoprotein were purified from placentas of normal and diabetic human beings. Diabetic samples exhibit a significant increase in ketoamine-linked glucose whereas both amino acid and carbohydrate composition were unaffected. This excess non enzymatic condensation of glucose on free amino-groups was found to increase platelet aggregating potency of these proteins independantly of any modification in fiber morphology.

Glucose has been demonstrated to bind covalently several proteins in vitro and in vivo via a non enzymatic process. Ageing and even more diabetes mellitus are propicious situations for the occuring phenomenon. (for review see 1). The so called non enzymatic glycosylation implies terminal amino-group of proteins or the ε-amino-group of lysine and occurs through the initial formation of a Schiff base. The resulting aldimine then undergoes an Amadori rearrangement leading to a stable ketoamine identified as N-1-(1-deoxyfructosyl) amino acid (2) which has been shown to exist in a ring conformation (3).

Non enzymatic glycosylation of both interstitial (4, 5) and basement membrane collagens (6, 7) have been extensively investigated. It is now conceivable that some of the diabetic complications may be a consequence of the over-glycosylation of this protein. However, the incidence of non enzymatic glycosylation on the biological properties of collagens remains poorly documented. A recent study has shown that N-glycosylation of rat

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acid soluble type I collagen occuring either in vitro or as a consequence of experimental diabetes in vivo results in an enhancement of collagen aggregating potency towards normal human platelet (8). However, glycosylation has been reported to slow down the rate of fibril formation and subsequently reduce stabilization via intermolecular cross links (9). This point is important in regards with the fact that the polymerization of collagen is a prerequisite for the induction of platelet aggregation (10, 11).

The purpose of the present study was to establish if the platelet aggregation was directly related to the fixation of glucose on collagen or a consequence of modified molecular associations. Both acid soluble collagen and an acid soluble non collagenous protein were therefore extracted from placentas of normal and diabetic human beings. Results concerning the aggregation of normal platelets by these materials are reported.

MATERIALS AND METHODS

Extraction and characterization of collagenous and non collagenous materials.

Full term placentas from normal pregnancies and from gestations complicated by insulin treated diabetes of the mothers were used. Each step of the preparation was performed at + 4°C . The placental villi were dissected,rinsed in distilled water and homogenized with a Turrax apparatus. After two centrifugations at 350 x g for 10 minutes in distilled water, the precipitate was extracted with 0.5 M acetic acid for 2 days under continuous stirring. The material was centrifuged at 10,000 g for 30 minutes and the supernatant dialyzed against 1 M NaCl for 2 days. The precipitated material was dissolved in 0.5 M acetic acid and purified by ion exchange chromatography on carboxymethylcellulose according to (12) ; a fraction called "acid soluble placenta protein" (ASP protein) was isolated.

The pellet resulting from the first acetic acid extraction was incubated with pepsin added to a 1/100 weight ratio, in 0.5 M acetic acid at + 4°C for 48 hours. The solubilized fractions was then separated by centrifugation at 14,000 x g for 1 hour and dialyzed against 0.9 M NaCl to precipitate collagenous material which was further purified by successive acetic acid redissolutions and NaCl precipitations (13).

Samples of acid soluble and pepsin soluble fractions were submitted to electrophoresis in a 6.6 % polyacrylamide sodium dodecyl sulfate gel as described in (14). Amino-acid analysis was performed on a Beckman 119 CL analyzer packed with W-3 H resin according to (15), the samples being hydrolyzed in 6 N HCl at 110°C under vacuo for 24 hours. The carbohydrate composition was established by gas-chromatography in a Hewlett Packard 5830 equipement, after methanolysis of the samples according to (16).

The amount of glucose bound via ketoamine linkage was estimated as 5-hydroxymethylfurfural (5-H.M.F.),liberated after oxalic hydrolysis, by a modification of the thiobarbituric assay of Fluckiger and Winterhalter (17), pure 5-HMF being used as a standard.

Electron microscope observation of fibrils was performed as follows: a drop of incubated solution was placed on a carbon film supported on a 400-mesh copper grid ; using filter paper, excess solution was drained and the preparation stained with 1 % phosphotungstic acid, pH 7.2, and 1 %

uranyl acetate solutions for 1 minute . Preparations were examined with a Jeol JEM 100 B electron microscope operated at 80 kV.

Aggregometric studies of platelets.

Blood was collected from normal adult volunters who had not taken any drug for at least a week, the anticoagulant being 3.8 % trisodium citrate (1 volume of solution per 9 volumes of blood). A platelet suspension was obtained by centrifugation of the blood at 90 x g for 20 minutes ; it was then adjusted to 300 000 cells/µl by dilution with a platelet poor plasma prepared by centrifugation of the blood at 2 000 x g for 10 minutes. Prior to be tested, collagen preparations were polymerized at 35°C for 30 minutes either in 0.1 M Tris-HCl buffer, pH 7.5, or in 0.10 M phosphate buffered solution, pH 7.0. Platelet aggregation was then assayed at 37°C according to (18) by using a Labor Gmbh aggregometer connected to a potentiometric recorder.7.5 to 20 µl of the incubated preparation (1 mg/ml) were added to 400 µl of the platelet suspension, so that materials from controls and diabetics were tested at the same dose corresponding to the threshold of response.

RESULTS AND DISCUSSION

Table 1 shows the amino-acid composition of the placenta extracted materials. It can be noted that the overall amino-acid composition of pepsin extracted collagen and more precisely the amount of hydroxylysine and hydroxyproline had not been significantly modified by the diabetic state. In this regard, human type I collagen behaves differently from glomerular basement membrane collagen of streptozotocin diabetic rats (6, 19) in which an enhanced level of lysyl and prolyl hydroxylation was previously found.

TABLE 1 - Amino acid composition of fractions isolated from normals and diabetics. Determination are given in residues/1000 residues.

Amino Acid	Collagen from normals	Collagen from diabetics	ASP Protein from normals	ASP Protein from diabetics
4-Hydroxyproline	65.4	65.0	2.9	3.1
Aspartic acid	55.8	54.1	135.2	131.9
Tyrosine	22.7	21.8	51.7	53.4
Serine	33.0	33.1	61.2	57.5
Glutamic acid	74.2	73.2	137.2	128.6
Proline	115.6	108.8	45.1	49.0
Glycine	327.1	328.0	88.5	88.0
Alanine	103.0	113.9	60.8	67.3
Cystein	3.3	3.0	31.1	27.2
Valine	28.7	27.3	54.7	53.5
Methionine	5.8	4.8	17.1	17.2
Isoleucine	21.0	21.2	42.7	41.3
Leucine	36.4	36.5	80.4	79.9
Tyrosine	6.2	6.1	32.2	32.1
Phenylalanine	15.2	16.9	26.3	30.6
Hydroxylysine	10.0	10.0	1.1	1.7
Histidine	6.6	6.2	20.0	20.2
Lysine	22.5	21.1	60.7	62.3
Arginine	47.5	49.0	51.1	55.2

TABLE 2 : Carbohydrate composition and non enzymatically bound glucose content of collagen and ASP protein isolated from placenta of normals and diabetics.

hexose	collagen from normals	collagen from diabetics	ASP protein from normals	ASP protein from diabetics
Mannose (%)	0.6	0.4	0.8	0.8
Galactose (%)	1.3	0.8	1.0	0.8
Glucose (%)	1.0	0.8	0.3	0.3
Nmoles 5 HMF/mg	4.3	6.5	2.2	4.0

The ASP protein, due to its content in hydroxylysine, hydroxyproline and glycine is shown to be non collagenous. This cysteine rich material is strongly acidic with an acidic to basic ratio equal to 2. S.D.S. polyacrylamide gel electrophoresis (not presented) revealed one major band with an apparent molecular weight of about 100,000 and the carbohydrate composition (table 2) demonstrates its glycoproteic nature. The diabetic state does not modify the carbohydrate composition of both collagen and ASP protein. However, the level of non enzymatically bound glucose is markedly increased in the materials (collagen and ASP protein) extracted from diabetics (table 2).

Platelet aggregation was studied in definite conditions of collagen polymerization. In phosphate buffer , collagen yields characteristic striated fibers whereas tris buffer only results in the obtention of hanks of non cross banded fibrils as shown on electron micrographs (figure 1). Glycosylation does not modify the ultrastructural organization of these fibers formed either in phosphate or in tris buffer.

Figure 2A presents the kinetics of platelet aggregation recorded after incubation of collagen in tris buffer ; 3 parameters ; the latency (L) ; the velocity (V) and the relative intensity (I) can be defined as described in the cartridge of fig. 2.

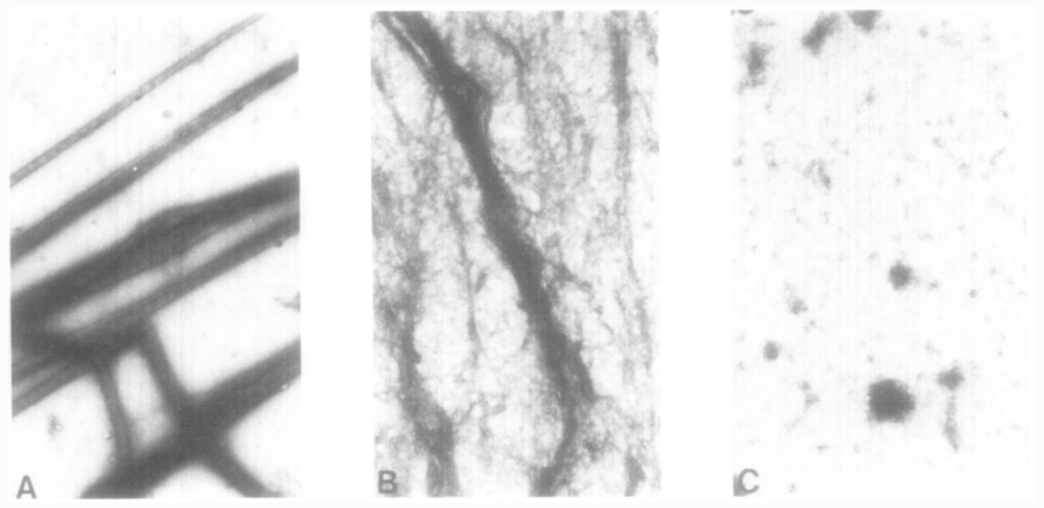

Figure 1 : Electron micrograph of polymerized collagen in (a) : phosphate buffer, (b) tris buffer and of ASP protein in tris buffer (c). Magnification x 28,000.

The shorter latency observed for preparations in phosphate as compared to those in tris buffer (table 3) may be related to the presence of fibers since it has been established that platelet aggregation is more influenced by the quaternary structure of collagen than by the molecular composition (10). However, samples from diabetics lead to a reduced latency and a higher velocity both in phosphate and in tris buffer, the intensity being slightly affected (table 3).

The aggregation patterns obtained with ASP protein show that 10 µg of control sample incubated in phosphate buffer are ineffective to promote aggregation ; however, the same amount of ASP protein isolated from diabetics are quite able to aggregate platelets (fig. 2B and table 4). After incubation in tris buffer, such a hit-or-miss effect is not obtained but a significant increase in aggregating potency is noticable for diabetic ASP protein. This peculiar behavior is not related to variations in its polymerization state due to non enzymatic glycosylation ; indeed when incubated either in tris or in phosphate buffer, samples failed to present any aggregates or fibrils both by turbidity and electron microscopy (figure 1).

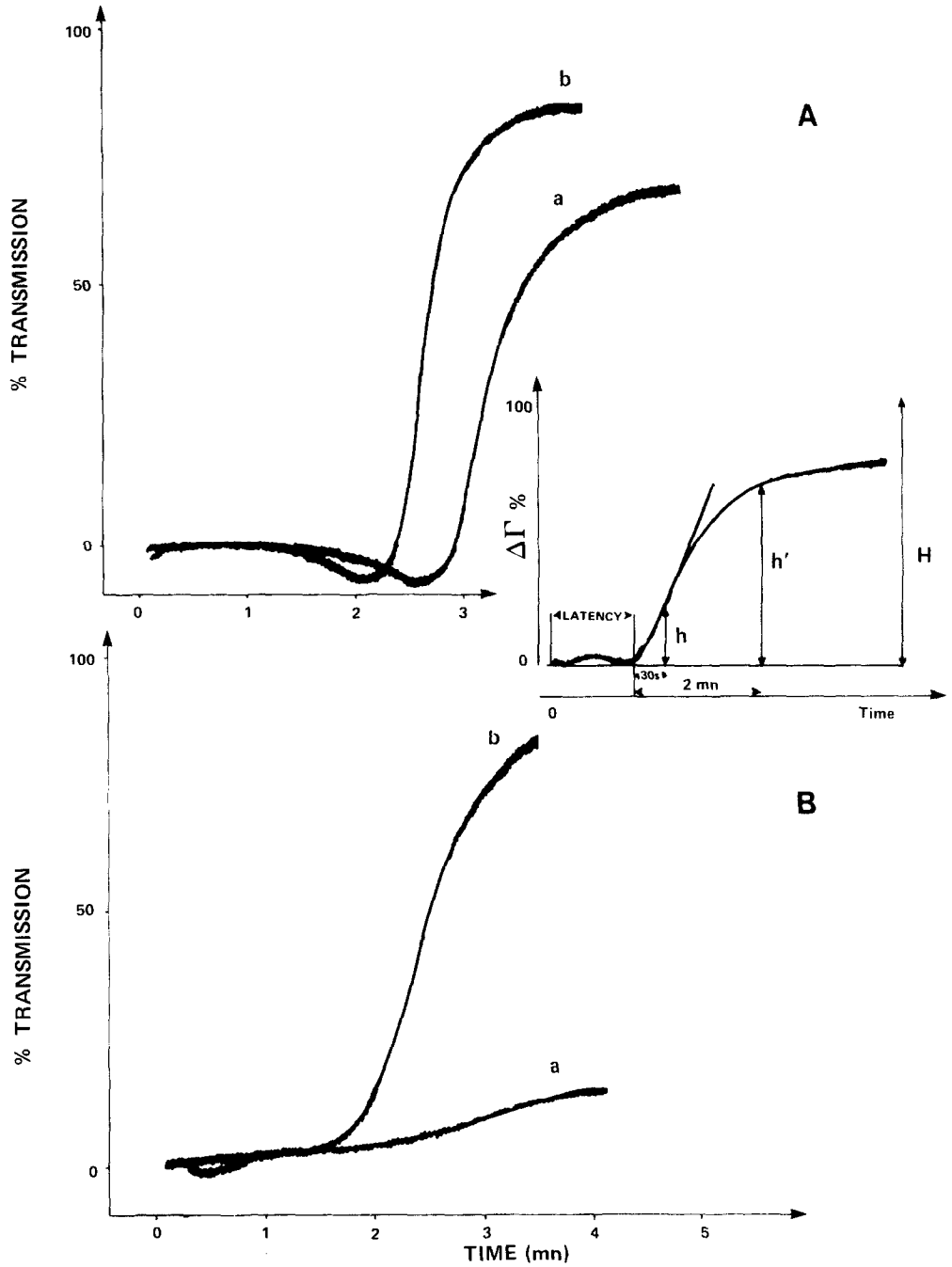

Figure 2 : Platelet aggregation curves obtained from :
A : pepsin extracted collagen from nomals (a) and diabetics (b) incubated in Tris-HCl-buffer.

B : ASP protein from normals (a) and diabetics (b) incubated in phosphate buffer.

Parameters used for comparison of aggregating potencies are defined from the curve presented in the cartridge.

L : latency ; I : relative intensity = $\frac{h'}{H}$ x 100,

V = velocity = $\frac{h}{H}$.

TABLE 3 : Platelet aggregation parameters obtained with pepsine extracted collagen of placenta from normals and diabetics (10 µg). Values are the mean of 5 determinations ± S.D.

	Phosphate buffer		Tris buffer	
	Normal	Diabetic	Normal	Diabetic
Latency (min)	1.10 ± 0.03	0.73 ± 0.02	2.66 ± 0.08	2.22 ± 0.07
Intensity (Δ T %)	75 ± 4	76 ± 5	70 ± 4	81 ± 5
Velocity	0.59 ± 0.05	0.52 ± 0.04	0.41 ± 0.03	0.60 ± 0.05

These results demonstrate that the aggregation of normal platelets by both placental collagenous and non collagenous materials is significantly increased when these proteins have been ε-N-glycosylated in vivo during diabetes.

Non enzymatic glycosylation corresponds to the only compositional modification observed since amino-acid and carbohydrate contents are unchanged in samples from diabetics as compared to controls. An important point is the occurence of this enhanced aggregating potency, independently of both the presence of fibrous material and the size of fibrils. In this regard, the interaction between platelets and a non fibrillar glycoproteic material like the ASP protein seems to be unreported.

TABLE 4 : Platelet aggregation parameters obtained with acid soluble protein of placenta from normals and diabetics. Values are the mean of 5 determinations ± S.D.

	Tris buffer		Phosphate buffer	
	normal (7.5 µg)	diabetic (7.5 µg)	normal (10 µg)	diabetic (10 µg)
Latency (min)	1.18 ± 0.03	0.95 ± 0.02	> 10	1.80 ± 0.06
Intensity (min)	63 ± 5	88 ± 7	0	70 ± 5
Velocity	0.16 ± 0.01	0.22 ± 0.01	0	0.26 ± 0.02

Different authors have pointed out that blocking free amino-groups or deaminating collagen resulted in a loss of its adhesion (20) and aggregation (21) inducing potency. Particular attention must be attached to the Amadori rearrangement which is subsequent to the condensation of glucose on free amino-groups of proteins. As previously shown for glycosylated hemoglobin, this process leads to the formation of a ketoamine linkage, so that the amino-group previously blocked in the Schiff base is unmasked and furthermore protonated at physiologic pH (2).

It must be underlined that platelet aggregation is an overall phenomenon; so, it cannot been discriminated if N-glycosylation bears upon the adhesion of platelets to collagen (or ASP protein), or upon the subsequent cellular events (shape change, activation, secretion) leading to the formation of an aggregate (22) . Does ketoamines which has been shown in a ring configuration (3) combine with some platelet membrane sites during the adhesion phase or with some material released from platelets ? This point-should be elucidated for a better understanding of the phenomenon.

Whatever this mechanism be, it can be acquired that, in diabetes, humam placentas content collagenous and non collagenous materials with increased platelet aggregating potency as compared to normals. This property, observed at threshold concentrations of ASP and collagen, should be however confirmed by other studies performed directly on intact organs as subendothelium where the collagen fibers,mixed with other substances in the matrix,form a surface of infinite concentration. This observation could be most important in the comprehension of the thrombogenic tendency of diabetes in relation to such a postranslational modification of proteins by glucose.

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