Modification of Glycosylated Haemoglobin Concentration during Artificial Endocrine Pancreas Treatment of Diabetics

Evidence for a Short-term Effect on HbA₁ (a+b+c) Levels

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Summary. In order to verify whether or not insulin-induced blood glucose control can acutely lower glycosylated haemoglobin levels, HbA₁ (a+b+c) (HbAI) was measured in 11 diabetics before, during and after 3 days of treatment with an “artificial endocrine pancreas” (Biostator). Initially 5 patients were in fair glycaemic control (group A), while the other 6 showed poor control (group B). HbAI levels decreased significantly after 3 days in both groups A (from $9.6 \pm 0.2\%$ to $8.5 \pm 0.3\%$, mean $\pm$ SEM, $p < 0.05$) and B (from $13.7 \pm 0.2\%$ to $12.6 \pm 0.3\%$, $p < 0.05$). A further HbAI decrease was observed until day 60 following Biostator treatment, during which period glycaemic control improved, as assessed by fasting and post-lunch plasma glucose values and daily glycosuria determined every 10 days.

These results suggest that increased HbAI levels may be reversed early by strict blood glucose control during a 3 day period. It is concluded that HbAI levels not only reflect long-term glycaemic control, but also recent acute variations in mean blood glucose values.

Key words: Glycosylated haemoglobin, artificial endocrine pancreas, continuous blood glucose monitoring, subcutaneous insulin treatment.

The total glycosylated haemoglobin (HbAI) level in poorly controlled diabetics is markedly increased in comparison with normal and diabetic subjects in good control [1, 2]. It shows good correlation with various indices of glycaemic control [3]. It is generally accepted that HbAI constitutes a useful guide to diabetes management [4, 5] and routine simultaneous determination of HbAI and blood glucose in diabetics has been proposed [6].

At present, it is well known that the HbAI levels decrease slowly after several weeks of improved glycaemic control in diabetics [3, 7, 8, 9, 10]. Few results are, however, available concerning changes in HbAI in the course of short-term blood glucose variations in diabetic patients [11, 12], although many studies relating the rapidity of the Hb glycosylation process to the ambient glucose concentration have been reported in vitro [13, 14, 15]. Thus, it is not presently known whether acute therapeutic manipulation of blood glucose levels significantly affects HbAI concentrations.

We have therefore investigated HbAI levels in eleven diabetics before, during and after three days of glucose controlled insulin infusion using a so-called “artificial endocrine pancreas” (GCIIS).

Materials and Methods

Patients and Protocol

Eleven diabetics were recruited for the study after full explanation of its methods and purposes. Their clinical features are shown in Table 1. The patients were not taking any therapy other than insulin, except for case 2, who was given only oral hypoglycaemic agents. In each patient plasma glucose (fasting and 90 min post-lunch) was determined at least twice in the month before admission at fortnightly intervals. A glycaemic profile (plasma glucose concentration measured fasting, post-breakfast, pre- and post-lunch, pre- and post-dinner, and at bed-time), and daily glycosuria were determined in all patients soon after hospitalization. The mean daily plasma glucose level was also calculated. Since therapy was not changed when patients entered the hospital, the results were taken to reflect the degree of control before admission.

Patients were divided in two groups according to the degree of their glycaemic control. Group A consisted of 5 diabetics with fair control (plasma glucose: fasting less than 150 mg/100 ml, post-lunch less than 200 mg/100 ml, mean daily less than 170 mg/100 ml; glycosuria less than 15 g/24 h). Group B consisted of 6 diabetics in poor control (plasma glucose: fasting more than

Table 1. Clinical features of patients

Case no.	Age years	Sex	Ideal body weight %	Diabetes		Glucose control		On admission		Daily insulin dose (Units)			
				Known duration years	Type	Pre-admission		MDPG ^c mg/100 ml	Glycosuria (g/24 h)	Pre-GCIIS		Post-GCIIS	
						FPG ^a mg/100 ml	PLPG ^b mg/100 ml			A.M.	P.M.	A.M.	P.M.
1	51	M	105	16	2	136±14 ^d (2)	172±10(2)	155±8	12	Rapitard 24		Actrapid 7 Monotard 14	Actrapid 2 Monotard 8
2	43	M	107	6	2	142±3 (5)	175±9 (5)	164±10	8	Oral hypoglycaemic agents		Actrapid 6 Monotard 18	Actrapid 4 Monotard 12
Group A 3	34	F	93	25	1	143±4 (4)	158±3 (3)	145±10	7	Actrapid 12 Lente 20	Actrapid 6 Lente 8	Actrapid 10 Monotard 24	Actrapid 5 Monotard 12
4	44	M	108	10	2	139±5 (3)	186±7 (3)	157±7	9	Regular 15 Lente 28		Actrapid 14 Monotard 19	Actrapid 4 Monotard 10
5	47	M	104	9	2	144±6 (5)	195±6 (4)	167±8	8	Rapitard 18	Rapitard 10	Actrapid 7 Monotard 15	Actrapid 4 Monotard 6
Mean±SEM	44±3		103±3			141±2	178±4	157±4	9±1				
6	20	M	87	0.3	1	210±12 (3)	334±19 (3)	231±15	38	Regular 12 Lente 20		Actrapid 10 Monotard 18	Actrapid 8 Monotard 14
7	47	F	105	22	1	240±16 (4)	345±10 (4)	292±23	42	Rapitard 25	Rapitard 15	Actrapid 8 Monotard 20	Actrapid 4 Monotard 10
8	31	M	98	0.08	1	203±6 (2)	266±6 (2)	303±23	45	Regular 10 Lente 18		Actrapid 12 Monotard 27	Actrapid 4 Monotard 15
Group B 9	17	M	90	0.15	1	205±17 (4)	281±9 (3)	236±18	25	Regular 14 Lente 22	Lente 16	Actrapid 10 Monotard 25	Actrapid 7 Monotard 12
10	26	M	95	2	1	193±13 (4)	255±4 (4)	265±24	33	Rapitard 34		Actrapid 6 Monotard 16	Actrapid 2 Monotard 12
11	18	M	98	0.08	1	198±5 (3)	360±20 (2)	255±19	27	Regular 9 Lente 25		Actrapid 8 Monotard 18	Actrapid 6 Monotard 8
Mean±SEM	27±5		96±3			209±6	300±11	264±9	35±3				

^a Fasting plasma glucose; ^b Post-lunch plasma glucose; ^c Mean daily plasma glucose; ^d mean±SEM; number of determinations shown in parentheses

After 3 days treatment patients were given appropriate subcutaneous insulin treatment (two daily injections of "Actrapid" and "Monotard" Novo m. c. insulin mixture) based on the information obtained during GCIS control. Blood samples for glucose measurement and 24 h urine collections were obtained on days 10, 20, 40, 60, 90, 150 after Biostatator treatment.

Glycosylated Haemoglobin Measurement

Venous blood for HbAI determination was collected on admission, one blood before and at 0, 24, 48 and 72 h after the start of GCIIS control; thereafter on days 10, 20, 40, 60, 90, 150 after the end of Biostator treatment. HbAI ($\text{HbA}_{\text{I}(\text{a+b+c})}$) was determined chromatographically by a modification [16] of the Kynoch and Lehmann method [17]. The red blood cells of each sample were washed, haemolyzed with distilled water soon after blood withdrawal, and immediately stored at +4° C for at least four days before chromatographic isolation. Storage of haemolysate for up to seven days at +4° C gave results identical to those of freshly prepared haemolysate (coefficient of variation in normal subjects, 1.5%), confirming previous reports [18]. Samples obtained from each patient during GCIIS treatment were determined together in duplicate. Several glass chromatographic columns (internal diameter 2 cm) were filled with Bio Rex 70 (5 cm high) after careful equilibration in 41 mmol/l phosphate buffer, 10 mmol/l KCN, pH 6.75 (Developer No. 6) [1]. Variable amounts of haemolysate (0.3–0.5 ml) were added to the top of the resin and eluted with the same buffer at 18–20° C. One hundred ml containing glycosylated fractions A_{Ia} , A_{Ib} , A_{Ic} were collected, while the A_0 fraction was eluted with 100 ml of buffer phosphate 0.15 mol/l, pH 6.40. The purity of the HbA_{Ic} chromatographic peak was tested by electrophoresis of the globin chains, as previously reported by Cirotto et al [19, 20]. HbAI concentration was expressed as % total haemoglobin eluted. The interassay coefficient of variation of this method in our laboratory is 1.2% for the assay at high HbAI level (15–16%) and 1.8% at low HbAI level (6–7%). By this method we have found total glycosylated haemoglobin ($\text{HbA}_{\text{I}(\text{a+b+c})}$) to correlate closely with its principal glycosylated A_{Ic} fraction ($r = 0.97$, $p < 0.001$). Therefore, as the $\text{HbA}_{\text{I}(\text{a+b+c})}$ determination is more rapid, all results of the study are expressed as total glycosylated haemoglobin. Normal $\text{HbA}_{\text{I}(\text{a+b+c})}$ values were determined in duplicate in 40 healthy subjects with normal plasma glucose OGTT response [21] aged from 15 to 58 years (42 ± 2 years, mean $\pm$ SEM).

Plasma and urinary glucose levels were determined with a Beckman Glucose Analyzer.

Analysis of variance (ANOVA) was used to assess differences of HbA1c levels before, during and after GCIIS therapy and during

Table 2. Plasma level and urinary excretion of glucose of patients. Pre-GCIIS plasma glucose levels are the mean ($\pm$ SEM) of pre-admission and admission values

Case no.	Pre-GCIIS		Post-GCIIS		UG ^e g/24 h	day 20	day 40	day 60	day 90	day 150											
	FPG ^a mg/100 ml	PLPG ^b mg/100 ml	FPG mg/100 ml day 10	PLPG mg/100 ml day 10																	
Group A	1	140±9 (3)	178±8 (3)	138	158	5	124	147	8	140	189	3	137	194	7	128	195	9	159	214	13
	2	141±3 (6)	177±8 (6)	132	155	2	137	152	abs	115	138	2	98	143	4	113	141	3	124	163	4
	3	134±9 (5)	161±4 (4)	107	144	abs	109	175	6	121	165	1	142	169	2	147	193	6	135	188	9
	4	142±4 (4)	183±6 (4)	104	153	3	130	138	4	143	173	5	123	158	abs	110	151	abs	122	170	abs
	5	144±5 (6)	193±5 (5)	150	136	abs	143	164	abs	127	141	5	131	181	6	107	184	3	137	165	abs
Mean±SEM		140±3	179±3	126±9	149±4	2±1	129±6	155±6	4±2	129±5	161±103±1		126±8	169±9	4±1	121±7	173±11	4±2	135±7	180±10	5±3
Group B	6	204±10 (4)	325±16 (4)	145	177	3	118	151	abs	127	157	abs	134	163	abs	116	171	3	125	169	abs
	7	236±13 (5)	353±12 (5)	137	184	6	106	160	abs	110	148	2	117	161	4	125	152	abs	128	173	abs
	8	198±6 (3)	304±38 (3)	158	210	8	144	189	3	138	181	8	129	170	6	143	194	10	137	185	8
	9	198±15 (5)	288±9 (4)	163	192	14	139	172	4	120	172	3	132	152	2	136	181	5	145	190	10
	10	194±10 (5)	258±4 (5)	170	204	17	131	166	abs	135	159	abs	104	139	2	112	145	abs	97	160	abs
Mean±SEM	11	191±7 (4)	349±16 (3)	168	221	11	115	158	2	112	145	abs	137	164	abs	94	162	abs	118	174	abs
	204±5	311±9	157±5	198±7	10±2	126±6	166±5	2±1	124±5	160±6	2±1	126±5	158±5	2±1	121±7	168±7	3±2	125±7	175±4	3±2	

^a Fasting plasma glucose; ^b Post-lunch plasma glucose; ^c Urinary glucose; ^d number of determinations shown in parentheses

Fig. 1. Significant correlation between HbA₁ and plasma glucose (PG) levels in 11 diabetics investigated in the study. Fasting and 90' post-lunch PG are mean values of at least 2 preadmission determinations. Mean daily PG was calculated based on plasma glucose profile on admission (fasting, 90' post-breakfast, 30' pre- and 90' post-lunch, 30' pre- and 90' post-dinner and at 12 p. m.). Statistical significance estimated by Kendall's rank method: fasting PG $p < 0.01$; post-lunch PG $p < 0.05$; mean daily PG $p < 0.05$

Table 3. Glycosylated haemoglobin levels in diabetics with poor (group B) and fair (Group A) glycaemic control on admission and before, during and after 72 h of GCIIS control

	Case no.	On admission HbA ₁ %	Days before during and after GCIIS control										
			-2 HbA ₁ %	0	1	2	3	10	20	40	60	90	150
Group A	1	9.8	10	10.2	10	10	9.7	9.6	9.2	9.6	9.8	9.5	10.5
	2	9.7	9.8	10	9.7	9.3	8.6	8.7	8.4	8.3	8.1	7.5	7.5
	3	9.3	9.2	9	8.2	8.1	8.2	8.1	8.1	8.2	8.3	8.1	8.2
	4	9.3	9.4	9.2	8.8	8	7.6	7.4	7.4	7.5	7.3	7.6	7
	5	9.6	9.5	9.4	9.5	8.7	8.5	8.3	8.2	7.9	7.7	7.4	7.5
Mean±SEM		9.5±0.1	9.6±0.1	9.6±0.2	9.2±0.3	8.8±0.4	8.5± ^a 0.3	8.4±0.4	8.3±0.3	8.3±0.4	8.2±0.4	8.0±0.4	8.1±0.6
Group B	6	14.4	14.3	14.3	14	13	12.9	12.2	11.2	9.3	8.3	8	8.3
	7	14	14.2	14.3	14.3	14.3	13.9	12.8	11.6	9.4	8.2	8.1	8
	8	13.5	13.4	13.6	13.6	13	12.9	12.3	11.6	11.2	10.8	10.7	11.2
	9	13.2	13.4	13.3	13.1	12.7	12.1	11.4	10.8	10	9.4	9.8	10.3
	10	13.5	13.3	13.3	12.7	12.2	12	11.5	11.1	9.1	7.8	8	7.9
	11	13.4	13.2	13.2	13.2	12.4	12.1	12.2	11.4	8.8	7.9	7.8	7.5
Mean±SEM		13.7±0.2	13.6±0.2	13.7±0.2	13.5±0.2	12.9±0.3	12.6± ^a 0.3	12.1±0.2	11.3± ^b 0.1	9.6±0.4	8.7±0.5	8.7±0.5	8.9±0.6

^a $p < 0.05$ (vs day 0); ^b $p < 0.01$ (vs day 3) by analysis of variance

SC insulin treatment up to day 150 after GCIIS control. ANOVA was applied also to the regression between HbA₁ and time during GCIIS treatment. Paired and unpaired Student's *t*-tests were used where appropriate. Correlations between HbA₁ and plasma glucose level were calculated using Kendall's rank method.

Results

Blood glucose decreased to the normal range in all patients after a few hours [1 to 4] of GCIIS treatment and remained continuously controlled both during fasting (80–110 mg/100 ml) and post-lunch (120–140 mg/100 ml). No significant changes in haematocrit, haemoglobin, red blood cell and reticulocyte count were observed after 72 h blood withdrawal by GCIIS for continuous glucose monitoring. Glycaemic control improved in each

patient during appropriate subcutaneous insulin therapy following GCIIS treatment as assessed by fasting and post-lunch plasma glucose values and daily glycosuria (Table 2).

On admission HbA₁ levels were markedly increased in all patients in comparison with normal subjects ($11.8 \pm 0.7\%$ vs $6.4 \pm 0.1\%$, mean $\pm$ SEM, $p < 0.001$) and showed significant correlations with fasting ($r = 0.65$, $p < 0.01$), post-lunch ($r = 0.57$, $p < 0.05$) and mean daily ($r = 0.61$, $p < 0.05$) plasma glucose values (Fig. 1). HbA₁ levels were higher in diabetics with poor glycaemic control than in diabetics in fair control ($13.7 \pm 0.2\%$ vs $9.6 \pm 0.2\%$ of groups B and A, respectively; $p < 0.001$).

Changes in HbA₁ levels in the course of three days normoglycaemia achieved with the GCIIS and thereafter up to day 150 after Biostator treatment

are summarized in Table 3. After 24 and 48 h of GCIIS treatment no statistically significant difference was present in HbAI levels. In contrast, HbAI decreased significantly after 72 h from $9.6 \pm 0.2\%$ to $8.5 \pm 0.3\%$ in group A and from $13.7 \pm 0.2\%$ to $12.6 \pm 0.3\%$ in group B ($p < 0.05$ in both groups). The mean HbAI level decrease was about 1% in the two groups (11% decrease in group A and 8% in B, HbAI basal value being taken as 100%). The decrease of glycosylated haemoglobin (dependent variable) during 72 h of GCIIS control (independent variable) was confirmed by the significant linear regression slope (group A: $p < 0.025$; group B: $p < 0.01$). The HbAI improvement continued after stopping GCIIS and changing to appropriate subcutaneous insulin therapy (Fig. 2). In group B HbAI levels showed a significant decrease vs day 3 of GCIIS control on day 20 of conventional treatment ($p < 0.01$). A further HbAI decrease was observed until day 60.

Discussion

It is now well established that increased HbAI levels represent a post-synthetic effect of chronic hyperglycaemia [22]. HbAI shows a significantly lower level in poorly controlled diabetics after 3–4 weeks of subcutaneous appropriate insulin treatment [3, 8, 9] or 2 weeks treatment with a portable insulin-infusion pump [10]. Recently we observed a significant decrease in HbAI in five untreated diabetics on the 10th day of conventional insulin treatment with a more marked fall in the following weeks [7]. It, therefore, seems likely that the HbAI level decreases only after several days or weeks of improved glycaemic control and that it represents an index of mean blood glucose value over a period of many weeks before examination [5].

Extensive results of short-term effects of appropriate insulin treatment on the Hb glycosylation process have not been described in diabetics. However, a careful investigation of this problem necessarily implies preliminary methodological considerations.

Firstly, diabetics must be carefully selected so that fixed HbAI levels are demonstrated for several days before the start of investigation. Secondly, an accurate and highly reproducible method for determination of glycosylated haemoglobin is mandatory. Thirdly, and perhaps more difficult to realize, a suitable index of glycaemic control following appropriate insulin therapy must be considered.

In our study the first and second criteria were met, while the third was attempted using a glucose controlled insulin infusion system.

Fig. 2. HbAI levels (mean $\pm$ SEM) in 11 diabetics ($\bullet$ group A, $n = 5$; $\circ$ group B, $n = 6$) before (A), during GCIIS control (B) and on carefully planned SC insulin treatment (C). Analysis of variance: * $p < 0.05$ vs day 0; ** $p < 0.01$ vs day 3

Under these conditions we observed a slight, but progressive decrease in HbAI levels in diabetics, previously in either poor or fair glycaemic control, in the course of three days of GCIIS therapy. The results suggest that some metabolic consequences of diabetic hyperglycaemia, such as enhanced HbAI levels, may be reversed early by strict control of blood glucose values during a three day period and they confirm our recent observation of a significant HbAI decrease in four untreated diabetics after 48 h of GCIIS therapy [11].

The mean HbAI decrease during GCIIS treatment was about 1% in both groups. This rapid change requires explanation. The mean red blood cell renewal in the course of three days accounts for about 2.5% of the total circulating erythrocytes in normal subjects and thus only 2.5% of total haemoglobin will be synthesized in this period. In the hypothetical condition of absent or markedly reduced glycosylation rate, a HbAI decrease should be observed equal to 9.6% (group A) or 13.7% (group B) of the renewed haemoglobin, that is 0.3–0.4% of total glycosylated haemoglobin after 3 days. On the other hand it has been recently demonstrated that erythrocyte half-life as measured by ^{51}Cr labelling is reduced in diabetics with poor glycaemic control [23]. Thus the Hb renewal rate should be more rapid in these patients than in normals and might explain, at least in part, the difference between hypothetically expected and observed HbAI decrease during GCIIS treatment. Acute blood withdrawal was probably of little importance in this regard, based on the reticulocyte count and other haematological parameters, which did not significantly change before and after GCIIS treatment. Moreover, although the iron kinetic data of Bunn et al. [22] show that the HbAI non-enzymatic glycosylation process is slightly reversible in vivo, the 3 days HbAI de-glycosylation percentage is very small and

it would be hard to calculate a quantitative estimate of the HbAI de-glycosylated fraction over this period [24]. Therefore it does not seem possible to consider the HbAI decrease during GCIIS treatment as a consequence of fast de-glycosylation process, as recently proposed by Svendsen et al. [12], whose hypothesis is in sharp contrast with many in vitro and in vivo results [22, 11, 25, 26].

In our patients the HbAI decrease continued until day 60 after GCIIS treatment during conventional but carefully designed insulin therapy. This result agrees with the idea that haemoglobin is glycosylated very slowly, continuously, and nearly irreversibly during the entire life span of the red blood cell [22]. The HbAI decrease in poorly controlled diabetics confirms previous reports [3, 7, 8, 9, 10] and is a tangible proof of reversibility of a biological abnormality following chronic hyperglycaemia. A significant HbAI decrease, however, was observed even in diabetics initially in fair control, confirming that in these patients also HbAI is a reliable indicator of the improved control achieved with more appropriate insulin therapy.

We conclude that the HbAI level not only reflects the long-term effects of previous glycaemic control in diabetics, but is also sensitive to recent acute variations in mean blood glucose levels, as those realized during GCIIS treatment.

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