Originals

Non-enzymatic glycosylation of skin collagen in patients with Type 1 (insulin-dependent) diabetes mellitus and limited joint mobility

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Summary. Forearm skin biopsies were obtained from diabetic subjects with and without limited joint mobility, and from non-diabetic control subjects. Collagen purified from these samples was assayed for non-enzymatic glycosylation. The level in all diabetic patients was significantly greater than that in control subjects ($p < 0.001$), but those diabetic patients with limited joint mobility had a level of collagen glycosylation similar to that in those with normal joints (15.3 ± 1.3 and 16.5 ± 1.3 nmol fructose/10 mg protein, respectively; mean ± SEM). Glycosylation of collagen in the diabetic patients correlated with glycosylated haemoglobin measured at the time of skin biopsy ($r = 0.60$). These results do not support the hypothesis that non-enzymatic glycosylation of collagen, as reflected by the ketoamine link, plays an important role in the development of limited joint mobility in diabetes.

Key words: Collagen, non-enzymatic glycosylation, limited joint mobility, haemoglobin A_1, ketoamine link.

The non-enzymatic adduction of glucose to receptive amino groups – non-enzymatic glycosylation – is an essentially irreversible process which affects many circulating and structural body proteins. The extent of such glycosylation of a protein in vivo depends on two main factors – the rate of turnover of the protein and the average glucose level to which it is exposed. For this reason the level of non-enzymatic glycosylation of most proteins is approximately two to four times higher in diabetic than non-diabetic subjects, and there is speculation that the process may have pathophysiological effects relevant to the long-term complications of diabetes [1–4].

Limited joint mobility, mainly affecting the small joints of the hands, has been reported to occur in 8.6–36.5% of patients with Type 1 (insulin-dependent) diabetes [5–8], and may also be seen in Type 2 (non-insulin-dependent) diabetes [9]. In several of these studies there is a clear correlation between the presence of limited joint mobility and retinopathy, especially the proliferative type [5, 8, 9]. The exact cause of limited joint mobility in diabetes is not known but it has been suggested that a structural alteration in collagen, perhaps related to glycosylation, may play a part [2, 10, 11]. In view of the association between limited joint mobility and retinopathy, elucidation of the processes involved in the evolution of the former may also shed light on the pathogenesis of chronic diabetic complications in general.

In this study we have investigated the glycosylation of skin collagen from diabetic patients with and without limited joint mobility.

Subjects and methods

Subjects

Patients attending the diabetes clinics of the Royal Victoria Hospital, Belfast were examined for limited joint mobility by the method outlined by Rosenbloom et al. [5]. Only patients with clearly normal joint mobility and those with either moderate limitation (involvement of three or more interphalangeal joints) or severe limitation (moderate limitation combined with bilateral large joint or cervical spine involvement) were included in the study. Patients with equivocal changes or only mild limitation were excluded. Examination findings were confirmed by at least two observers. Thirty-six patients with Type 1 diabetes are included in the study. After the experimental nature of the study had been explained and consent obtained, an elliptical full thickness skin biopsy (approximately 1.0×0.5 cm) was taken, under 2% lignocaine local anaesthetia, from the medial aspect of the forearm. Biopsies were also obtained from three healthy non-diabetic volunteers and at autopsy from seven patients who had died of acute illnesses. These seven were not known to be diabetic and had one or more normal plasma glucose measurements during the terminal illness. The study was approved by the Ethical Committee of the Royal Victoria Hospital, Belfast.

Methods

Each skin biopsy was immediately frozen and kept at $-70\,^{\circ}\text{C}$ before preparation for assay. After thawing, the skin was scraped to remove keratin and subcutaneous fat. It was then washed in distilled water

Table 1. Characteristics of diabetic patients and normal subjects

	Diabetic patients with:		Control subjects ($n=10$)
	Normal joints ($n=24$)	Limited joint mobility ($n=12$)	
Sex (M:F)	10:14	9:3	3:7
Age (years)	41.6 ± 3.2	49.1 ± 3.9	46.9 ± 6.2
Age at onset of diabetes (years)	24.6 ± 2.9	22.4 ± 3.9	
Duration of diabetes (years)	17.0 ± 2.7	26.7 ± 2.7[a]	
HbA₁ (%)	10.9 ± 0.6	10.8 ± 0.5	

Results are mean $\pm$ SEM

[a] $p < 0.05$, compared with diabetic patients with normal joints
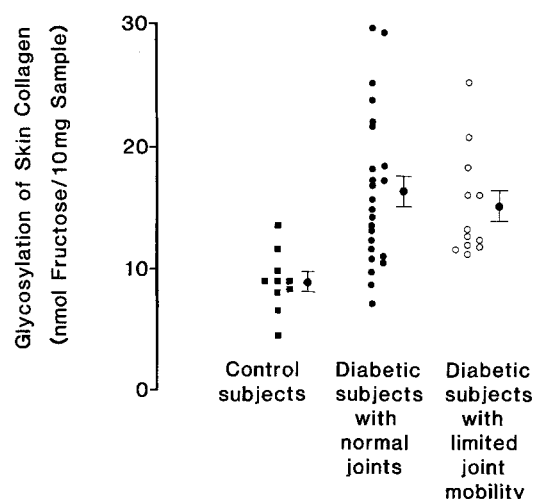
Fig. 1. Levels of non-enzymatically glycosylated skin collagen. Bars indicate mean $\pm$ SEM for each group
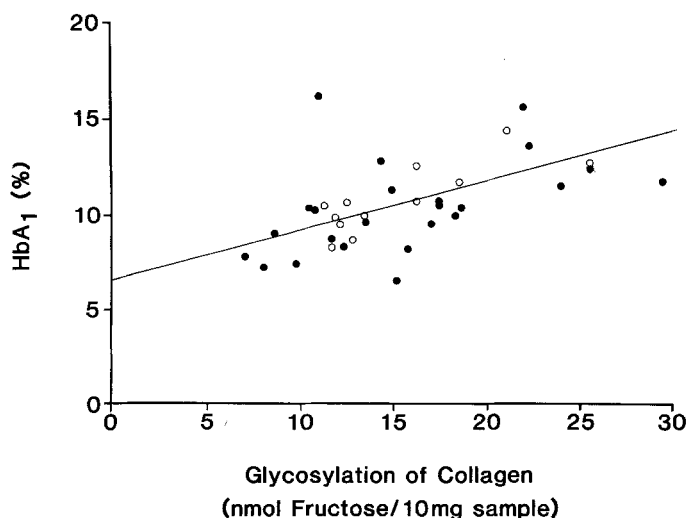
Fig. 2. Correlation between glycosylated collagen and HbA₁ in diabetic patients with (O) and without (●) limited joint mobility, data combined ($r=60$). See text for details of regressions

and extracted by agitating sequentially at 4 °C in 1.0 mol/l NaCl, hexane and 0.5 mol/l acetic acid, for 24 h each, to remove soluble proteins and remaining fat [12, 13]. Further washing in distilled water was followed by lyophilisation. This resulted in a yield of 20–30 mg of insoluble protein from each biopsy and hydroxyproline estimation [14] indicated that samples contained approximately 80% collagen (range in 12 samples, 76–88%). Measurement of non-enzymatic glycosylation was by weak acid hydrolysis, adapted from the method of Fluckiger and Winterhalter [15]. Approximate 10–12 mg pieces of lyophilised material (accurately weighed) were added to 650 µl distilled water and 750 µl 1.0 mol/l oxalic acid. Hydrolysis was carried out for 1 h in an autoclave (model ST19, Arnold & Son, Basildon, Essex, UK) at 121 °C and 1.05 kg/cm² [16,17]. The amount of 5 hydroxymethylfurfural (5-HMF) yielded was quantitated by reaction with thiobarbituric acid and measurement of absorption at 443 nm. For every sample a blank was run, in which a similar weight of lyophilised material was incubated with 400 µl 0.1 mol/l NaBH₄ and 100 µl distilled water for 15 min and then quenched with 150 µl reagent grade acetone before hydrolysis. This reduces the ketoamine link between glucose and protein to a non-reacting form [18]. During each assay run, a standard curve was constructed from fructose solutions of varying concentrations which were hydrolysed and reacted with thiobarbituric acid in the same fashion as the tissue samples. Fructose is hydrolysed to 5-HMF and hence this allows for small differences in the efficiency of the hydrolysis between assay runs [19]. Specific glycosylation of collagen was expressed as nmol fructose/10 mg protein. Using collagen, prepared as outlined above, from skin samples obtained from a below-knee amputation in a diabetic patient with peripheral vascular disease, an intra-assay coefficient of variation of 10.3% ($n=12$) and a between-assay coefficient of variation of 17.7% ($n=11$) were obtained for the complete procedure.

Glycosylated haemoglobin (HbA₁) was measured in blood samples (drawn into EDTA) obtained from the diabetic patients at the time of skin biopsy. Red cells were incubated in 0.154 mol/l NaCl for 18 h to remove labile HbA₁ [20], and stable HbA₁ was measured by agar gel electroendosmosis [21] (Corning, Halstead, Essex, UK), the method routinely used at our clinics. The normal range in non-diabetic subjects in our laboratory is 3.6–7.2%, and the coefficient of variation is 1.6% within and 6.4% between assay.

Statistical analysis

Results are expressed as mean $\pm$ SEM. Comparisons were made with the unpaired Student's t-test and correlations by linear regression analysis.

Results

Twenty-four diabetic patients had normal movement in their joints and 12 had limited joint mobility – in nine this was moderate and in three severe. Age, sex, age at onset, duration of diabetes and HbA₁ levels are shown in Table 1. Only duration of diabetes differed significantly between the two diabetic groups ($p < 0.005$).

The levels of skin collagen glycosylation are shown in Figure 1. Compared to control subjects the diabetic patients, considered either in two groups or collectively, had a significantly increased level of collagen glycosylation ($p < 0.001$). There was no significant difference in collagen glycosylation between diabetic patients with and without limited joint mobility (15.3 ± 1.3 versus 16.5 ± 1.3 nmol fructose/10 mg protein, respectively). When diabetic patients with normal joint movement and duration of diabetes ≥ 10 years ($n=16$) only were

considered, to give a duration similar to that in the group with limited joint mobility (23.4 ± 3.0 years), the collagen glycosylation was also similar (16.0 ± 1.5 nmol fructose/10 mg protein).

The clear correlation between collagen glycosylation and HbA$_1$ in the 36 diabetic patients is shown in Figure 2 ($r = 0.60$, $p < 0.001$). The correlation between these two parameters was similar in those with ($r = 0.64$) and without ($r = 0.60$) limited joint mobility. In the diabetic patients collagen glycosylation did not correlate with either duration of diabetes or age.

Discussion

Investigation of possible structural or biochemical abnormalities involved in chronic diabetic complications is hampered in man, not least because it is difficult or ethically unjustifiable to obtain tissue from those organs directly involved – notably the eyes and the kidneys. Even when tissue may be made available in patients with severe complications – for example, at renal transplant, vitrectomy or autopsy – there remains the problem of adequate control tissue from the diabetic subject free of complications. It now seems that limited joint mobility is another chronic complication of diabetes evolving alongside retinopathy [5, 8, 9], nephropathy [5] and probably also neuropathy [22]. Many of the patients with limited joint mobility have thickening and contracture of palmar tendons and also thickening of skin; it thus seems reasonable to suspect that an abnormality of collagen may be present. We therefore believe that our study may be unique in that it examines, in patients with and without a particular chronic diabetic complication, an abnormality of a protein which may be intimately involved in the development and clinical manifestations of that complication.

The hypothesis that non-enzymatic glycosylation may underlie the evolution of chronic diabetic complications is attractive, as it would fit in with the considerable evidence from animal studies, and the widely-held belief among clinicians, that there is some link between the degree of long-term glycaemia and complications. Increased glycosylation of collagen in vivo has been correlated with decreased solubility, elasticity and sensitivity to protease digestion [13], and increased thermal stability [23]. Incubation of collagen with glucose in vitro also inhibits its degradation by proteases [24]. These observations are consistent with an increase in collagen cross-linking in diabetes. Furthermore in three patients with severe joint limitation, Buckingham et al., using a technique similar to ours, observed a considerable increase in hexose bound to skin protein in ketoamine linkage as compared with non-diabetic controls, though there was no comparison with diabetic patients free of joint limitation [10].

Our data do not support the concept that non-enzymatic glycosylation, at least as reflected by the keto-amine link, is central to the development of limited joint mobility. Patients with clinically normal joints had just as high levels of collagen glycosylation as those with quite advanced degrees of joint limitation. We chose to examine collagen from forearm skin as this was acceptable to patients and we did not consider it practical, or ethical, to attempt to obtain biopsies from fingers or joints. We cannot know whether collagen from those sites, where the clinical abnormality is most obvious, would have yielded completely similar results, but we have no real reason to believe the conclusions would be different. The ketoamine link between glucose and amino groups, which was measured in our collagen glycosylation assay, is the first stable product of the non-enzymatic glycosylation reaction. It is known to undergo further dehydration, rearrangement and cleavage reactions resulting in final products that are highly cross-linked, insoluble, pigmented polymers called melanoidins [25, 26]. This may help to explain the good correlation observed between HbA$_1$ and glycosylated collagen (Fig. 2). Since collagens are among the longest lived proteins in the body one would expect collagen glycosylation to reflect glycaemia over a period of many months. However, if the ketoamine link undergoes further rearrangement in vivo after a period of time not too dissimilar from the life span of the average erythrocyte, then the measured glycosylation of collagen might well reflect glycaemia for a similar period of time as HbA$_1$, which is known to respond to improved blood glucose control within 6–10 weeks [27, 28]. The corollary of that hypothesis is that if the ketoamine link is relatively short lived it is less likely to be involved in the development of chronic complications than the subsequent products (melanoidins), about which much less is known in vivo.

In conclusion, our data highlight the importance of including adequate and appropriate controls in clinical studies, before attributing undue pathophysiological significance to any biochemical parameter which is known to be altered or increased in diabetic as compared with non-diabetic subjects. Although our results do not support the concept that the early products of non-enzymatic glycosylation play an important part in the development of limited joint mobility seen in diabetes, the possibility that subsequent degradation of the ketoamine link may play a role remains to be investigated.

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