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Review articles

Non-enzymatic glycosylation and the chronic complications of diabetes: an overview

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Even before the introduction of insulin therapy in 1922, retinopathy, neuropathy and proteinuria had all been described in association with diabetes mellitus. However, as the use of insulin has permitted long-term survival of young diabetic subjects, the full extent of the problems caused by these and other chronic complications (nephropathy, generalized microangiopathy and atherosclerosis) has become more apparent. Diabetes is one of the major causes of visual loss in the developed world; and premature death, now usually due to atherosclerosis or renal failure, is still all too common in the diabetic subject.

In the continuing debate about the pathogenesis of diabetic complications, much speculation and controversy has focussed on the possible role of chronic hyperglycaemia. The long-term, evolutionary nature of the complications, the lack of reliable objective indices of glycaemic control, and the inability to achieve normal physiological glycaemic control with subcutaneous insulin therapy are among the factors that have prevented resolution of this basic controversy. Nevertheless, there is an increasing body of evidence, based on both clinical experience with patients [1, 2] and animal model studies [3], which indicates that chronic hyperglycaemia may be the major factor contributing to the long-term complications of diabetes.

Detection of the minor haemoglobins, HbA_{1c} and $HbA_{1(a+b+c)}$ [4] and their chemical characterization as non-enzymatically glycosylated derivatives of haemoglobin [5–7], has added new dimensions to the continuing debate on the role of hyperglycaemia in the complications of diabetes. Thus, measurement of glycosylated haemoglobin provides an objective, retrospective index of glycaemic control [8–12]. Serial measurements should provide a truer reflection than previously possible of long-term glycaemic control in prospective studies of the development of diabetic complications. In addition, it has become clear that haemoglobin is not unique, and that non-enzymatic glycosylation is in fact a common post-translational modification of many body proteins. Thus glycosylation results from a direct chemical reaction between glucose and amino groups on protein. The first stable product of the reaction is termed the ketoamine adduct to protein, but, from studies of protein-reducing sugar systems in vitro, the ketoamine is known to undergo further dehydration, rearrangement and cleavage reactions. The final products in vitro are highly crosslinked, insoluble, pigmented and fluorescent carbohydrate-protein polymers, called melanoidins (hence this is termed the browning reaction). Whether these products of the browning reaction are also formed in vivo is not yet known. However, since non-enzymatic glycosylation is enhanced during hyperglycaemia, it is not surprising that there has been much speculation concerning its role, and the role of the subsequent browning reactions in the pathogenesis of the complications of diabetes [13, 14]. The purpose of this article is to review and evaluate the existing evidence in support of this speculation. To this end, it is convenient to consider individual proteins or groups of proteins in turn.

Glycosylation of specific proteins

Erythrocyte proteins

Haemoglobin is glycosylated at both intra-chain lysine and amino-terminal valine residues [15, 16]. Overall, there are three to four times more lysine than valine modifications in the protein but the lysine modifications are not known to have any effect on the protein's overall structure and ligand binding properties. The primary site of glycosylation of HbA_{1c} is the valine residue at the terminus of the β-chain, which is in the binding site for 2,3-diphosphoglycerate, the physiological regulator of haemoglobin's oxygen affinity. In vitro, HbA_{1c} has been shown to have altered oxygen saturation curves and/or decreased sensitivity to the allosteric effects of organic phosphate [17]. However, in vivo, the higher oxygen affinity of haemoglobin in diabetic patients appears to be independent of the state of glycosylation of haemoglobin [18], and whole blood oxygen saturation curves for normal and diabetic populations are essentially identical [19, 20]. It is unlikely, therefore, that glycosylation of haemoglobin has any pathological effects.

In addition to haemoglobin on the inside of the erythrocyte, red cell membrane proteins are also subject to increased non-enzymatic glycosylation in diabetes [21–23]. These proteins are probably the longest lived plasma membrane proteins in the body, and, as such, are likely to show the effects of cumulative damage resulting from glycosylation. It is clear that various physical properties of the erythrocyte membrane, such as deformability [24], doublet formation [25] and microviscosity [26] may be altered in poorly controlled diabetes. There is, however, no evidence which directly links non-enzymatic glycosylation with these abnormalities, and other factors such as pH, changes in the lipid composition of the membrane [27], lipid-protein interactions [28] or a direct insulin effect [29] may be important.

Plasma proteins

Non-enzymatic glycosylation of albumin and other plasma proteins appears to provide an index of glycaemic control in the preceding 1–2 weeks, and may be clinically useful in the same way as glycosylated haemoglobin measurements [30–32]. Alterations in drug and bilirubin binding capacity in vitro have been reported for glycosylated albumin [33, 34]. However, relevant studies have not been performed in vivo to establish that, even in severe hyperglycaemia, glycosylation of albumin would be sufficient to compromise its physiological functions; for example, in the binding, transport and delivery of fatty acids. Endothelial cells are reported to ingest and degrade albumin in vitro in proportion to its degree of glycosylation [35]. The physiological significance of this observation is equally uncertain, however, since, in experimental animals, the fractional catabolic rate of albumin is decreased in diabetes [36] despite enhanced glycosylation. Also its half-life in plasma does not appear to be affected by glycosylation of the degree observed in diabetes [37, 38]. Repeated intravenous injections of non-enzymatically glycosylated plasma proteins into normal mice for 12 weeks have caused glomerular basement membrane thickening and mesangial changes similar to those observed in human and experimental diabetes [39]. These results suggest that glycosylation of plasma proteins may be involved in the development of diabetic nephropathy. Although deposition of albumin and immunoglobalins along basement membranes in the kidney [40] and throughout the body [41] is a common finding in diabetes, there was no evidence in these studies, and a similar study with glycosylated albumin [42], that the glycosylated proteins bound preferentially to renal basement membranes. Thus, the link between the glycosylation of plasma proteins and their deposition along basement membranes is still tentative.

Lipoproteins

Glycosylation of low density lipoprotein (LDL) was shown to alter its rate of uptake and degradation by cultured human fibroblasts [43, 44], and also to decrease its fractional catabolic rate in the guinea pig circulation [44]. These effects resulted from inhibition of LDL binding to the high-affinity receptor involved in regulation of cholesterol metabolism, suggesting a possible defect in receptor-dependent catabolism of LDL and an increased requirement for LDL catabolism by endothelial cells and macrophages in diabetes. However, even though the level of glycosylation of apoprotein B is increased in diabetic patients [45], when LDL from patients with Type 2 (non-insulin dependent) diabetes was compared with that from normal subjects, binding and degradation by human fibroblasts and uptake by mouse peritoneal macrophages was similar in the two groups [46]. The explanation may lie in the observation that in diabetic patients the level of glycosylation of LDL appears to increase only to about 2 mol glucose/mol apoprotein B [45], which is the lower limit at which effects on binding and catabolism were observable [44]. In contrast to LDL, catabolism of high density lipoprotein (HDL) appears to be accelerated by glycosylation [47], but the effect was clearly demonstrable only after extensive modification of the protein (>15% of lysine residues glycosylated). With both LDL and HDL, effects of glycosylation on protein catabolism were most apparent at a high level of glycosylation, but were proportional to the degree of substitution. It is possible that even the slight increase in glycosylation of these proteins in diabetes could have a marginal effect on their catabolism, sufficient to induce the gradual development of vascular disease.

Nerve proteins

Increased glycosylation of myelin and myelin-associated proteins has been noted in both the peripheral and central nervous system in diabetes [48, 49]. The two- to fivefold increase in glycosylation of these proteins is comparable to increases measured for plasma proteins. The significance of enhanced glycosylation in the development of diabetic neuropathy was suggested by some earlier work on cyanate induced neuropathy, which had similar clinical findings and was also associated with modification of lysine residues in myelin [50]. One hypothesis is that glycosylation of myelin may yield products which act as signals for recognition and degradation by macrophages, thus inducing excessive myelin turnover and demyelination in diabetes. Increased glycosylation of intracellular tubulins was also observed in diabetic rat brain [51]. Tubulin from diabetic animals exhibited a defect in guanosine triphosphate-induced polymerization, which could be mimicked by glycosylation of normal tubulin in vitro. Thus, glycosylation could also affect microtubule-dependent processes in neural tissues. A number of other biochemical abnormalities besides enhanced glycosylation also occur in the diabetic nerve. Alterations in the sorbitol pathway and resultant accumulation of sorbitol may exert osmotic stress and affect the redox potential and metabolic pathways in cells.

Lens proteins

Non-enzymatic glycosylation of lens crystallin proteins has been proposed to have a role in the development of diabetic cataracts. Lens crystallins were shown to aggregate and precipitate more rapidly in solutions containing glucose than in a sugar-free medium [52]. Inhibition of this aggregation by reducing agents, coupled with the increased disulphide content of cataractous lens, led to the hypothesis that glycosylation increased the susceptibility of sulphydryl groups to oxidation, inducing formation of disulphide-linked protein aggregates [52, 53]. Subsequent studies have shown an increase in protein glycosylation in cataractous lenses in diabetic rats [53] and man [54, 55], and have also established that increased galactosylation of crystallins is associated with the development of cataracts in galactosemia [53]. However, in one study, no correlation was observed between the extent of glycosylation and total disulphide content of lens proteins [55].

As with peripheral nerves, sorbitol accumulates in the lens as a consequence of poor diabetic control. Treatment of diabetic rats with an aldose reductase inhibitor prevented formation of cataracts despite a level of crystallin glycosylation similar to that in untreated rats which developed cataracts [56]. Aspirin, which can acetylate and inhibit glycosylation of albumin and plasma proteins [37, 57] may retard the development of cataracts in diabetes [58]. This drug equilibrates rapidly from plasma into ocular fluids, but, in addition to its possible role in protecting lens proteins by acetylation, it is now recognized as a potent inhibitor of aldose reductase. Surprisingly, salicylate, an aspirin analogue which cannot acetylate protein, is equally effective in inhibiting sugar cataracts in the rat, and also inhibits the aggregation of lens proteins in the presence of reducing sugar in vitro. Thus, aspirin's mechanism of action appears to be independent of acetylation, although its effect on glycosylation in the lens has not been evaluated. Overall, the evidence seems to indicate that alterations in the sorbitol pathway are more significant than increased non-enzymatic glycosylation of protein in the development of both neuropathy and cataracts in diabetes.

Extracellular matrix proteins

Collagens are among the longest lived proteins in the body, and are continually exposed to glucose in vascular and extravascular fluids. Increased non-enzymatic glycosylation of diaphragmatic tendon and skin collagens is observed with both age and diabetes in man [59, 60]; and, in rats similar effects were noted with aortic and glomerular basement membrane collagens [61, 62]. The increased glycosylation of collagen in vivo has been correlated with its decreased solubility, elasticity and sensitivity to protease digestion [60], and increased thermal stability [63], all of which suggest increased cross-linking of collagen in diabetes. Incubation of collagen (and other proteins) with glucose in vitro also inhibits their degradation by proteases [64], possibly because of cross-linking, but perhaps also because glycosylation of lysine residues renders them resistant to trypsin enzymes. Enhanced glycosylation and cross-linking of collagen by glucose provides an attractive explanation for many of the long-recognized alterations in the extracellular matrix in diabetes, including basement membrane thickening and stiffening of connective tissue.

Limited joint mobility was recently described as a common clinical finding in childhood and adult Type I (insulin-dependent) diabetes [65, 66]. This appears to be correlated with the presence of thickened waxy skin, principally on the dorsum of the hands, and thickening and contracture of tendon. Increased glycosylation of collagen in skin biopsies from three young diabetic patients with joint contractures was reported recently [67]. Increased enzymatic cross-linking of collagen may also be involved in some of these changes, since the enzyme, lysyl oxidase, which catalyzes the formation of natural cross-links in collagen, may be increased in some tissues in diabetes [68]. Further, inhibitors of lysyl oxidase can prevent the insolubilization of collagens in diabetes [69], also suggesting that the increase in cross-linking may be due to induction of enzymatic pathways. Non-enzymatic glycosylation may also be involved, however, since some recent studies have shown that solubilized dermal collagen from juvenile-onset diabetic patients is browner and more fluorescent than collagen from age-matched control subjects [70]. The absorbance and fluorescence spectra of the diabetic collagens were different from those of normal collagen, and strikingly similar to those produced by melanoidins formed in browning reactions between protein and glucose in vitro.

Significance of protein glycosylation

It is clear from these diverse studies that many proteins, some intimately involved in the complications of diabetes, may be affected by non-enzymatic glycosylation. However, the mere finding of increased glycosylation in association with complications does not prove a causal relationship. An understandable, but important omission in many studies, for example glycosylation of lens crystallins or collagen from diabetic patients, is the lack of comparable data on diabetic subjects with and without the relevant complication but with similar duration of disease. While this is hardly practicable in the case of lens crystallins, it should be possible with, for example, collagen from skin biopsies. For those proteins in which a potential pathological effect of glycosylation can be demonstrated in vitro, e.g., lipoproteins and haemoglobin, the observations may not be relevant to the situation in vivo – in other words, the in vitro effect is hardly detectable in vivo and the body seems to be able to compensate for any loss of function which may be caused by glycosylation. Finally, at least in the case of neuropathy and cataracts, the evidence supporting an alternative hypothesis is as convincing, or more so, than

that suggesting a role for non-enzymatic glycosylation. However, it would probably be naive to think that any or all chronic diabetic complications could be explained by one single mechanism. Probably several factors, non-enzymatic glycosylation among them, are involved.

A strong case exists for glycosylation as a potential source of collagen abnormalities in diabetes, based on the recent observation of non-enzymatic type browning and fluorescence of diabetic collagen [70]; but evidence for the occurrence of the browning reaction in vivo is still circumstantial. One of the serious problems with research in this area is that browning, fluorescence, insolubility and indigestibility of collagen are relatively imprecise terms. These types of changes could be caused by alterations in normal enzymatic pathways of collagen metabolism or by numerous compounds besides glucose, perhaps including products of lipid degradation (peroxides, malonaldehyde, ketone bodies) or other by-products which may accumulate in diabetes as a result of metabolic alterations or renal insufficiency. Ideally, it would be desirable to measure the concentration of true melanoidins in tissue as an index of cumulative, long-term damage to collagens by non-enzymatic glycosylation; but what is needed first is a better chemical characterization of these products in model browning reactions so that they can be investigated in more complex physiological systems. While literally hundreds of reaction products have been identified already in browning reactions, much of this work has been done under significantly non-physiological conditions, e.g., at high temperatures and extremes of pH and in non-aqueous solvents. Unfortunately, when physiologically relevant conditions are maintained, the browning and cross-linking reactions proceed slowly [71], not unlike the situation in vivo. In addition, the protein-bound products are difficult to isolate because of their instability to conditions normally used for hydrolysis of proteins, and more gentle techniques, such as proteolytic digestion of the protein, may be required for their isolation. It seems, however, that characterization of melanoidin structures or their precursors formed in vitro will be a necessary step before they can possibly be identified and quantitated in tissue protein. Only then can the role of the browning reaction in connective tissue pathology in diabetes be rigorously evaluated.

Current assay procedures

Some caution should be noted concerning the methods used for detecting and quantitating non-enzymatic glycosylation in many studies, in that most methods provide only comparative and qualitative rather than accurate, quantitative measures on non-enzymatic glycosylation. Thus, for human albumin, different sources report a range of 10%–30% glycosylation, depending on the assay procedures and conditions used [72, 73]. There is no established reference method for determining the extent of non-enzymatic glycosylation of a protein. The most widely used chemical procedure is the thiobarbituric acid assay, originally developed for quantitation of haemoglobin glycosylation [74]. Careful standardization of the assay procedure is essential to obtain reproducible results [75]. Many substances, including free glucose [76] and sugars bound in glycosidic linkage to glycoproteins [73] will interfere in the reaction so that a blank determination is essential [72]. When applied to haemoglobin, the thiobarbituric acid assay can be standardized with purified HbA_{1c}. For other proteins, however, because of variable release of glucose during hydrolysis the assay yields only a semi-quantitative, but useful estimate of the actual extent of protein glycosylation. Comparisons between different proteins may not be valid, and results with insoluble matrix proteins may also be affected by losses of sugar during extraction and preparation of the protein.

A fluorimetric assay for haemoglobin glycosylation was described recently [77], based on measurement of formaldehyde released on periodate oxidation of the ketoamine adduct. This assay is simpler than the thiobarbituric acid procedure, and appears to have both the precision and accuracy required of a reference chemical procedure for measuring haemoglobin glycosylation. However, it will be limited in its application to membrane proteins and other glycoproteins because of interference by sialic acid. Another method, which determines only glucose bound to lysine residues, is based on analysis of furosine recovered after strong acid hydrolysis of a protein [73]. This assay seems to work well with many proteins, including haemoglobin, lipoproteins and erythrocyte membrane proteins [27, 73]. Its accuracy has not been rigorously established, but, like the thiobarbituric acid test, it provides good discrimination between normal and diabetic populations, and test results correlate well with the degree of hyperglycaemia.

Accurate quantitation of glycosylated amino acids in protein is theoretically possible by amino acid analysis. In this case, the protein must be reduced with sodium borohydride to convert the ketoamine adduct to an acid stable hexitol derivative of the amino acid. The analyses are difficult, however, because the glycosylated derivatives are not always resolved from the natural amino acids during chromatography and because they normally represent only about 1%–2% of the total of each residue in a protein. Radiochemical procedures based on reduction of glycosylated proteins with tritiated sodium borohydride provide much greater sensitivity and accuracy, if properly standardized, but technically are more complicated. Direct measurement of total radioactivity incorporated from tritiated sodium borohydride into a protein has been used for comparative purposes, but is not an adequate measure of glycosylation, since other identified radioactive products are obtained in addition to hexitollysine [16, 55]. Quantitation of radioactivity actually recovered in hexitollysine is the appropriate method for comparing the extent of glycosylation of proteins. In this case, after hydrolysis of the protein, radioactivity in hexitollysine is measured after isolation by ion exchange or affinity chromatography [15, 16]. However, absolute quantitation of glycosylation

requires an internal or external standard to calibrate the effective specific radioactivity of the tritiated sodium borohydride, and control experiments to establish that the reduction reaction has proceeded to completion. This is in fact rarely done, and most investigators rely on comparative data from normal and diabetic samples treated identically.

None of the current chemical procedures for measuring non-enzymatic glycosylation of protein can be described as convenient or straightforward, and there are legitimate concerns about their absolute accuracy, especially with insoluble proteins from the extracellular matrix. These reservations may be countered by the fact that mean levels of glycosylation are consistently two to four times higher in diabetic than in non-diabetic samples from all tissues regardless of the technique employed. There is still a need, however, for fresh approaches to the assay problem, based on new chemical, and perhaps enzymatic, reactions of the protein-bound glucose. A common limitation of the present assays is that they focus on measurement of the ketoamine adduct. In fact this is just the first step in the series of browning reactions, and may be of little direct consequence in the pathology of diabetes. Development of procedures for detecting the later products will be essential for evaluating their relevance to the complications of diabetes.

In the 1980 Claude Bernard lecture, Unger suggested that "post-translational modification of proteins by glucose does provide both a unifying biochemical link between hyperglycaemia and diabetic tissue damage, and an attractive explanation for nature's rigorous efforts to keep the glucose concentration below 200 mg/dl" [78]. The evidence in support of this hypothesis is still inconclusive, but it will continue to provide a valuable stimulus for research into the chemical basis of the complications of diabetes.

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