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HYPERGLYCAEMIA IN THE DEVELOPMENT OF CHRONIC COMPLICATIONS OF DIABETES MELLITUS: NO LONGER A QUESTION OF WHETHER, BUT HOW★

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Hyperglycaemia is the hallmark of diabetes mellitus. Indeed it is by measurement of blood glucose that we diagnose and define the condition. In practice raised blood glucose is the biochemical aberration in diabetes which, either by modification of diet or administration of oral hypoglycaemic drugs or insulin injection, is most responsive to therapeutic intervention. Because of the consistency of hyperglycaemia as a finding in untreated diabetes it is natural to speculate that excessive levels of glucose or related sugars are in some way responsible for most, if not all, of the ills which may subsequently befall diabetic patients. Our ability to manipulate the blood glucose towards normal adds attractiveness to this hypothesis, as both the patient and the attending physician are encouraged to believe that their successful therapeutic endeavours, easily demonstrated by simple blood tests, will yield handsome long-term health dividends. Also our realisation that it is almost impossible, in all but a very few patients, to achieve complete normalisation of the blood glucose provides the rationale, and, for the physician, the intellectual consolation, if our best efforts fail to prevent the relentless onslaught of vascular complications.

The question of whether there is a link between hyperglycaemia and the development of complications has, in my opinion, rightly been the burning issue in clinical diabetes since shortly after the discovery of insulin. My contention is that whether the link exists is no longer the appropriate question. Rather, we should be asking how? How does hyperglycaemia lead to the development of complications, and how are we going to prevent it?

Glycated haemoglobin

Why do I feel so confident that the argument about a link between control and complications is over? They key to this, I believe, is glycated haemoglobin. The chemical reaction which we now refer to as glycation—the non-enzymatic linkage between a reducing sugar and a receptive amino acid—was first described, in relation to food proteins by Maillard in 1912.[1] However, the relevance to clinical diabetes mellitus was not recognised until half a century later following the serendipitous finding by Huisman and Dozy that a minor negatively charged haemoglobin component referred to as HbA1c was increased in diabetic patients.[2] This finding was confirmed in several other studies during the 1960s and early 1970s, but it was not until 1976 that Fluckiger and Winterhalter showed that HbA1c is formed *in vitro* when purified haemoglobin is incubated with glucose.[3] This clearly indicated the non-enzymatic nature of the reaction; HbA1c is formed by the adduction of a glucose molecule to the amino-terminal valine of the beta

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chain of adult haemoglobin. This is achieved through an aldimine linkage which then undergoes an Amadori rearrangement to form a stable, and largely but not completely irreversible, ketoamine product.

It was then a logical hypothesis that in diabetes the level of HbA1c, or 'glycated haemoglobin', should reflect the average blood glucose level over a period equivalent to the half-life of the red blood cell, approximately 7 to 8 weeks. The first convincing clinical proof of this hypothesis appeared also in 1976, when it was shown that elevated HbA1c levels in newly diagnosed diabetic patients stabilised at a lower level some 6 weeks after insulin treatment reduced the prevailing blood glucose.[4] Ditzel and his colleagues reproduced these findings in a larger number of newly diagnosed patients[5] and, even in these early studies of the clinical utility of glycated haemoglobin, two points relevant to the practical management of diabetes were apparent. First, it was clear that, at least in newly diagnosed patients, it is possible to normalise the glycated haemoglobin and achieve an average blood sugar not dissimilar from that of the non-diabetic individual. The second point, however, is that it is difficult or impossible to achieve this in some diabetic patients. All practising diabetologists now know that maintenance of normal glycated haemoglobin levels is notoriously difficult in all but a very few insulin-dependent patients, and particularly so when endogenous insulin secretion is exhausted some years after the onset of clinical diabetes.[6]

Glycated haemoglobin and diabetic complications: early observational studies

Two factors had traditionally hindered attempts to demonstrate a link between glycaemic control and hyperglycaemia. First, microvascular complications usually develop only after many years of insulin-dependent diabetes, so prospective studies are difficult. Second, the known lability of blood glucose in insulin treated patients had always made it difficult to quantify long-term glycaemic control accurately and objectively. The availability of glycated haemoglobin, as HbA1c or as HbA1, a larger haemoglobin fraction which involves HbA1c, as a routine test since around 1980 has solved effectively the second problem, and made the task of facing up to the first rather less daunting.

Davies and colleagues in Wisconsin found that a single measurement of HbA1 was predictive over a four year period of the development and progression of retinopathy.[7] Stereoscopic fundus photographs were performed on 891 patients whose diabetes had been diagnosed before 30 years of age, and at the same time HbA1 was measured. Four years later they were reassessed by the same means and the development or progression of retinopathy was analysed in relation to the baseline HbA1. Retinopathy appeared for the first time in 18.8 per cent of those whose baseline HbA1 was in the lowest quartile compared to 64·4 in the highest quartile. HbA1 was also predictive of those patients whose pre-existing retinopathy would progress to become proliferative, i.e. sight threatening. Those in the highest quartile for HbA1 had a relative risk of 21·8 for the development of proliferation when compared to the lowest quartile. The problem with this study is that the single measurement of glycated haemoglobin was related only to integrated glycaemia for the few weeks prior to the initial eye assessment, not to the entire period of study in each patient.

The obvious next step was to relate individual mean glycated haemoglobin levels over a period of time to the development of complications. As one might expect retrospective studies appeared first. Chase and colleagues in Colorado

studied 230 patients who had had insulin dependent diabetes mellitus for at least 5 years and HbA1c measured regularly for at least 3 years.[8] They expressed the mean HbA1c level in each patient as it related to the upper limit of normal for their assay, arbitrarily dividing patients into groups of 'good', 'intermediate' and 'poor' glycaemic control. The prevalence of advanced retinopathy in the poor control group was 2·5 times that of subjects with good control. No patient with a mean HbA1c less than 1·1 times the upper limit of normal had retinopathy, whereas 37 per cent of those with HbA1c 1·5 times normal had advanced proliferative retinopathy. Remarkably similar were their findings in relation to microalbuminuria, the earliest measurable indication of nephropathy. Nine per cent of patients with good control had microalbuminuria compared to 29 per cent with poor control. By multivariate analysis they showed that the risk of developing either advanced retinopathy or microalbuminuria after 15 years of diabetes was 4–5 times greater in poorly controlled patients than in those with good control.

At our diabetes clinic in the Royal Victoria Hospital in Belfast we examined 216 patients with insulin-dependent diabetes who had had regular HbA1 measurements, approximately every 3–4 months, over a 6 year period.[9] Mean HbA1 levels showed a strong correlation with increasingly severe grades of retinopathy, even when differences in duration of diabetes were taken into account. Proliferative retinopathy was seen only in patients whose mean HbA1 was greater than 10 per cent (range in non-diabetics 3·6–7·2). This raised the intriguing possibility that there could be a threshold level of hyperglycaemia below which patients have protection from the most serious effects of microangiopathy. The existence of a cushion of 'safe' mild hyperglycaemia would be a boon both to patients and to diabetic carers, bearing in mind the difficulty in achieving normoglycaemia in insulin-dependent patients. However, the number of patients with proliferative retinopathy in our study was small, and the Wisconsin group found no evidence of a threshold effect in their patients.[7]

Just under 10 per cent of our cohort of insulin-dependent patients had microalbuminuria, defined as an albumin excretion rate of more than 20 μg/min. Mean HbA1 levels over the previous 6 years were significantly higher in these patients than in those who were microalbuminuria negative, and, conversely, when patients were categorised according to their mean HbA1 levels there was a significantly higher urinary albumin excretion in patients with higher mean HbA1.[10] Similar relationships were observed between urinary albumin and the most recent HbA1 measurement, as opposed to the mean HbA1 over 6 years. This suggests that microalbuminuria, as an early manifestation of diabetic nephropathy, may be amenable to improved glycaemic control over a relatively short period of time. And, since we did not find a relationship between the most recent HbA1 and degree of retinopathy,[9] one might reasonably hypothesise that microalbuminuria represents a lesion unequivalent to the retinal microaneurysm in reflecting early diabetic complications.

Prospective studies and clinical trials

Retrospective analyses, however persuasive they may appear, must be interpreted with caution and are no substitute for studies that are truly prospective. Preliminary results became available in 1989 from the Pittsburgh Prospective IDDM Cohort Study, in which the natural history of insulin-dependent diabetes, includ-

ing glycated haemoglobin levels and the evolution of diabetic complications, were documented from the time of diagnosis, but without randomisation of patients to particular treatment groups.[11] After 5 years of diabetes 7 of the first 62 patients recruited to the study had developed retinal microaneurysms. The 5-year mean HbA1 was significantly higher in these patients than in patients without microaneurysms, and the message from these small numbers was confirmed by the results of the Diabetes Control and Complications Trial (DCCT).

Begun in the early 1980s this trial recruited 1441 patients with insulin-dependent diabetes who had either no retinopathy or early background retinopathy.[12] The aim of the trial was to determine if randomisation to either conventional or intensive insulin therapy would reveal significant differences in glycaemic control, the development of new retinopathy and the progression of already established retinopathy. The trial was spectacularly successful. Patients who received intensive insulin therapy achieved, and maintained throughout the 9 years of the trial, mean HbA1c levels 2 per cent less (i.e. 7 v 9) than those on conventional therapy. The risks of developing retinopathy and early nephropathy were reduced by almost 50 per cent in the intensively treated patients, with an even greater reduction in the risk of neuropathy. Progression of established retinopathy was also significantly reduced in the intensively treated group.

The price to be paid for reducing the risk of microvascular complications by intensive insulin therapy was an increased likelihood of severe hypoglycaemia.[12] However, there were no deaths due to this complication, and the the incidence decreased dramatically after the first 2–3 years of the trial, suggesting that patients learned to adapt to the intensive regimen without compromising improved blood sugar control. The key to the success of the trial may have been the close contact maintained between the patients in the intensively treated group and their professional carers, both medical and nursing—patients attended clinics at least once a month and were phoned once a week throughout. Can we realistically expect to be able to sustain such a labour-intensive and lifestyle-disruptive system of care for more than a small fraction of all insulin-dependent patients outside clinical trial conditions? And even if we could, can we afford it? These are, I believe, the real challenges thrown up by the DCCT.

Further convincing evidence that improved glycaemic control slows the progression of established microvascular complications was provided by the Stockholm Diabetes Intervention Study.[13] One hundred and two insulin-dependent patients with non-proliferative retinopathy and a mean duration of diabetes of 17 years were randomised to conventional and intensified insulin treatment groups. After 7 years the intensively treated patients had significantly lower HbA1c levels; the risk of progression to proliferative retinopathy was approximately half of that in the conventional group; and the protection against nephropathy was even greater with an odds ratio of 0·1 (95% confidence interval, 0 to 0·8).

There is little dispute now that glycaemic control is probably the major determinant of development and progression of microvascular complications in diabetes. A remaining question is whether the findings of these studies support the concept of a threshold effect—a glycated haemoglobin slightly higher than normal, but below which complications are unlikely to occur. In this regard further analysis of the data from the DCCT is awaited.

GLYCATION: A PATHOGENETIC LINK BETWEEN HYPERGLYCAEMIA AND DIABETIC COMPLICATIONS?

It would be naive to think that one single mechanism could account for the diffuse abnormalities which constitute the full gamut of micro- and macro-angiopathy in diabetes. However, I have limited considerations in this review to only one possible pathogenetic process rather than attempting to cover the many hypotheses proposed at one time or another. There can be no biochemical alteration which relates so directly to the degree of hyperglycaemia than the process of glycation. Increasing clinical preoccupation with glycated haemoglobin begged the question as to whether glycation might be a general phenomenon and, if so, is it involved in the development of complications?[14,15]

Some general criteria would have to be satisfied to give credence to a 'glycation theory of diabetic complications'. First, one should be able to demonstrate that proteins relevant to the development of complications are susceptible to glycation and that this happens *in vivo*. In this respect collagen, which is widely distributed in fibrous form in tendons and ligaments and in non-fibrous form in basement membranes and blood vessel walls, is a good candidate protein.[16]

Having established that a particular protein can be glycated it then would be necessary to demonstrate that some pertinent protein property or function is affected. Next, it would be helpful to be able to correlate circulating or, better still, tissue, levels of glycation products with the presence and severity of complications through clinical studies. Finally, and perhaps most crucially, would be the ability, by manipulating the level of glycation independently of hyperglycaemia, to alter the extent or rate of formation of lesions characteristic of a diabetic complication.

Skin, joints and complications

At this stage I take a slight diversion. Diversions may present as irritating impediments to smooth progress along a defined route to a planned destination, but by forcing us to approach from a new direction they can cast familiar landscapes in a fresh perspective and open up new vistas. In 1981 Rosenbloom described what had hitherto been an infrequently observed phenomenon in childhood diabetes—limited mobility of the joints of the hands. Almost 30 per cent of diabetic youngsters attending a summer camp in Florida had this feature, associated sometimes with thickening of the palmar fascia or with skin thickening.[17] Subsequently my colleagues and I in Belfast set about defining this condition in older patients with more long-standing insulin-dependent diabetes.[18] We found that 88 out of 204 patients attending our clinic had limited joint mobility affecting mainly the small joints of the ring and fifth fingers, but, in some cases virtually all the hand joints and even the wrists, elbows and cervical spine. The presence of limited joint mobility correlated with duration of diabetes and, independently, with the presence and severity of retinopathy. Patients with long-standing diabetes were more than twice as likely to have proliferative retinopathy if limited joint mobility was present than if it was absent. We also found a correlation with markers of neurological dysfunction. It is now accepted that limited joint mobility may be regarded as another manifestation of chronic diabetic complications developing in parallel with retinopathy, neuropathy and nephropathy. Shiny and slightly waxy thickened skin is easily observed in many of these patients and provided readily accessible tissue for investigation.

'Early' and 'late' glycation products

To return to my main thesis—a glycation theory of complications—the first of the arbitrary criteria I suggested above is easily satisfied, as it is now known that virtually every circulating or tissue protein so far studied is susceptible to glycation both *in vitro* and *in vivo*.[19–24] Before considering possible functional effects we need to expand a little on the glycation process itself. Although the site of glycation of haemoglobin, the greatest focus of attention in early studies of glycation, is the amino-terminal valine, the epsilon-amino groups of lysine residues are the principal sites of glycation and formation of the Amadori ketoamine product in most proteins.[25] This ketoamine product which I will refer to as 'early glycation' can undergo further modification and degradation to form highly insoluble pigmented complexes. These 'late glycation' products are described variously as browning products, melanoidins, or advanced glycation end-products—AGEs for short. This is particularly the case in long-lived proteins. Therefore one would expect the accumulation of early glycation products in long-lived proteins to be limited, as an equilibrium is established between their synthesis and their subsequent transformation into AGEs. On the other hand AGEs will continue to accumulate throughout the lifetime of a protein. When we are considering the effects of glycation it is essential to distinguish between early and late products, and it would be logical that early products are more likely to affect the function of short-lived circulating proteins and AGEs more likely to be important in long-lived structural proteins, if functional effects can indeed be demonstrated.

Functional and clinical correlates of glycation

In studies of human skin biopsies from diabetic and control patients, short-term incubation of non-diabetic collagen with glucose, leading to an increase in early glycation to levels typically seen in diabetic patients, did not alter its solubility or collagenase digestibility.[27] In diabetic patients we found a good correlation between early glycation of skin collagen and concurrent HbA1 levels.[28] Patients with and without limited joint mobility had similar levels of early glycation of collagen, seeming to rule out a role for early glycation products in the evolution of this particular diabetic complication.

However, if we turn to a shorter lived circulating protein, low-density lipoprotein (LDL), there is indeed evidence of a potential role for the ketoamine product. In 1982 Lopes-Virella and colleagues in South Carolina found that LDL from diabetic patients with poor glycaemic control was internalised and degraded by human fibroblasts much less efficiently than LDL isolated from the same patients once glycaemic control had been improved.[29] The same group demonstrated that glycated LDL from diabetic patients had an enhanced uptake in human macrophages, and that this could also be demonstrated with LDL glycated *in vitro*,[30] Macrophages are the main precursors of the lipoprotein laden foam cells characteristic of the early atheromatous plaque.

To return to collagen: that the connective tissue of patients with long-standing diabetes can be abnormally tough has long been recognised by surgeons, and was readily apparent to us in our work with skin biopsies. Schnider and Kohn found that collagen from diabetic patients had reduced solubility and increased resistance to collagen digestion in comparison to that from age-matched non-diabetic control subjects.[31] While I have shown that this is unlikely to be due to accumulation of early glycation products, what about AGEs? A major problem has been the identification and assay of specific AGEs, but increased fluorescence which provides an indirect measure of AGE formation[32] is seen in diabetic patients, and prolonged incubation with glucose *in vitro* induces similar changes. Using this indirect assay Monnier and his colleagues at Case Western Reserve University showed that AGE formation in skin collagen from diabetic subjects increased in proportion to the extent of retinopathy, nephropathy, arterial stiffness and limitation of joint mobility.[33] This was the first demonstration of a correlation between the glycation process and diabetic complications.

Such studies would be greatly enhanced by the demonstration and assay of specific AGEs, rather than simply relying on an indirect measure. It is generally agreed that an important mechanism in the formation of these end-products is covalent crosslinking, though as yet there is no consensus on exactly how this happens. Cerami's group in New York have presented evidence that condensation of two ketoamine products leads to the formation of a yellow-brown compound called 2-furoyl-452-furanyl-1-H imidazole (FFI).[34] Using an assay for this substance they found a correlation between serum creatinine and circulating AGE levels in diabetic patients with and without nephropathy.[35]

More recently Sell and Monnier have described a fluorescent compound comprising a lysine and an arginine residue cross-linked by a pentose, which they have named pentosidine.[36] From 103 human skin biopsies obtained randomly at autopsy they demonstrated an exponential rise in pentosidine levels with increasing age. There was a three- to ten-fold increase in pentosidine levels in collagen from patients with end-stage diabetic nephropathy requiring renal dialysis.[36] This work has been extended by John Baynes' group in South Carolina who found that pentosidine levels were elevated in diabetic patients with incipient nephropathy, as shown by microalbuminuria, and that the pentosidine levels correlated with severity of retinopathy.[37]

These studies represent a promising progression, and I have no doubt that further revelations concerning the process of cross-linking and AGE formation are just around the corner.

Inhibition of glycation

Thus far I have touched on three of the four criteria I proposed as essential for a glycation theory of diabetic complications—glycation of relevant proteins, effects on protein function, and correlation with clinical disease. I am as yet unaware of any studies in humans that give useful insights into the final criterion—the ability to alter the angiopathic process by manipulating the level of glycation independently of blood sugar. However, I would draw attention to two approaches that seem likely to bear fruit. Aminoguanidine is a nucleophilic hydrazine compound which binds to a number of substances which are, or may be, important in AGE formation.[38] These include the initial ketoamine product, deoxyglucasone, and glyoxal, which is a 2-carbon fragment of glucose autoxidation. Aminoguanidine, therefore, has the potential to inhibit AGE formation regardless of blood glucose levels. A second approach, rather more oblique, derives from the observation that the enzyme aldose reductase, central to the sorbitol pathway by which glucose is metabolised first to sorbitol and then to fructose, may depend on glycation for its activation.[39] If so, one would expect this metabolic pathway, which operates principally in tissues independent of insulin action, to be facilitated and increased

in poorly controlled diabetes, resulting in increased tissue concentrations of fructose. Fructose is known to be many times more reactive than glucose in initiating glycation and cross-linking.[40] So inhibition of the sorbitol pathway may, in theory, limit the potential for AGE formation at a given level of hyperglycaemia.

CONCLUSION

Nowadays it is hard to remember that before the discovery of insulin the diagnosis of insulin-dependent diabetes was equivalent to a death sentence by slow starvation. But we must not forget that it is still a life sentence. Perhaps the time will come, as a result of advances in immunosuppression or transplantation, when even that life sentence can be commuted, but I suspect that is still some way off.

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