Review Article

Below the Radar:
Advanced Glycation End Products that Detour "around the side"

Is HbA_{1c} not an accurate enough predictor of long term progression and glycaemic control in diabetes?

***Josephine M Forbes[1,2], Georgia Soldatos[1], Merlin C. Thomas[1,3]**

[1]Albert Einstein Centre for Diabetes Complications, Baker Heart Research Institute, [2]Department of Immunology, Monash University, AMREP Precinct, [3]Department of Epidemiology, Monash University, AMREP Precinct, Melbourne, VIC 8008, Australia.*For Correspondence: Dr Josephine Forbes e-mail: Josephine.forbes@baker.edu.au

Abstract

Advanced glycation is the irreversible attachment of reducing sugars onto the free amino groups of proteins. Its physiological roles are thought to include the identification of senescent proteins and hence there is a time dependent accumulation of advanced glycation end products (AGEs). AGE labelled proteins are catabolised by cells into low molecular weight peptides and amino acids and excreted primarily via the kidneys. This process appears to be tightly controlled by AGE clearance receptor complexes containing AGE-R1, AGE-R2 and AGE-R3 and scavenger receptors such as CD36, SR-AII and SR-BI.

Conditions such as diabetes, however, which have a metabolic overload of reducing sugars, rapidly accelerate AGE formation. In addition, advanced glycation is facilitated by oxidative stress and renal disease even in the absence of increases in reducing sugar concentrations. As part of our western diet, we also ingest AGEs of which approximately 50-80% are absorbed, catabolised and excreted over a period of two days.

As AGE levels rise during diabetes, interruption of normal function occurs via three distinct mechanisms, namely AGE induced cross-linking of extracellular matrices, stiffening elastic fibres, disturbing cellular adhesion and preventing turnover. The second is by intracellular formation of AGEs, which causes generalised cellular dysfunction. The third is via the chronic activation of specific receptors such as RAGE, the receptor for advanced glycation end products, which produces excesses in inflammatory molecule production.

Due to the range of dysfunction produced by the accumulation of AGEs in diabetes, there is a growing need for early recognition and intervention in this process.

Introduction

Diabetes and its complications are rapidly becoming the world's most significant disease epidemic. Within Australia, some 1 million patients have been diagnosed with diabetes, with approximately 90% of these classified as type 2 diabetes. Unfortunately, these figures are conservative and are predicted to double within the next ten years.[1,2] Patients with type 1 [Diabetes Control and Complications Trial (DCCT)] or type 2 diabetes (UK Prospective Diabetes Study) have a progression rate to microvascular diabetic complications of about 40% even in the setting of acceptable glycaemic control.[3,4] Macrovascular complications including cardiovascular, cerebrovascular and peripheral vascular disease develop in more than 50% of the diabetic population and account for 50-60% of mortality.[5] It is this relentless progression that has highlighted the need to develop more accurate predictors of diabetic complications.

The advent of therapies which block the renin-angiotensin system (RAS) and the lowering of hyperlipidaemia with HMG-CoA reductase inhibitors (statins), has revolutionised the management of patients with diabetic complications. Although some 50% reduction in the progression to end stage renal disease is seen in diabetic patients treated with

either angiotensin converting enzyme-1 inhibitors (ACEi), angiotensin receptor 1 antagonists or with combinations of both, most subjects continue to ongoing decline in renal function, albeit at a slower rate.[6-8] These patients most often die from a cardiovascular event. This relentless progression may be attributed to activation of other pathways of damage by diabetes including advanced glycation. Indeed, the most likely future interventions in diabetic complications will involve combinations of therapies, which block multiple pathways.[9]

Formation of Advanced Glycation End Products in Biological Sytems

Advanced glycation was first discovered as the non-enzymatic Maillard or 'browning' biochemical reaction between reducing sugars and amine residues on proteins.[10,11] As a native protection mechanism against advanced glycation in biological systems, glucose has evolved as the predominant sugar used for fuel, as it is the least reactive of the biological sugars to Maillard chemistry. This acts to limit the intracellular accumulation of deleterious advanced glycation end products (AGEs) and their precursors.[12] As a result, the in vivo Maillard reaction is slow under normal metabolic conditions and predominantly affects long lived molecules, such as collagen and lens crystallins.[13] Physiologically, it has been suggested that the degree of AGE-modification represents one mechanism to judge the age of a molecule, to allow for the recognition of senescent targets for excretion or catabolism.[14] Molecular turnover is also reduced with increasing chronological age, most likely due to deteriorating renal function and therefore, the quantity and variety of AGE-modified proteins increases in parallel.[15]

In both major forms of diabetes, persistent hyperglycaemia and oxidative stress act to hasten the formation of AGEs.[16] This causes long-lived proteins to become more heavily modified, in addition to rendering shorter-lived molecules as targets for advanced glycation.[13,14] In addition, the intracellular formation of AGEs from reactive carbonyl intermediates (such as the glycolytic intermediate, 3-deoxyglucosone and the sugar phosphates, methylglyoxal and glyoxal) occurs at a much faster rate than glucose-derived AGE formation. This appears to be triggered by increased oxidative stress induced in response to intracellular hyperglycaemia, although glucose appears to be fundamental for the generation of these reactive intermediates.[17,18] Reactive oxygen species (ROS) are also formed during the formation of AGEs causing a self-perpetuating cycle of ROS/AGE formation in diseases such as diabetes. The proposed sources of reactive oxygen species in the Maillard reaction are many fold, including the autoxidation of glucose (Wolff pathway), Schiff bases (Namiki pathway) and Amadori adducts (Hodge pathway), as well as AGE proteins themselves.[16] There are a number of antioxidant systems in place to limit tissue damage initiated by the Maillard reaction including reducing agents such as glutathione, antioxidant pathways/enzymes, detoxification systems (e.g. glyoxalase pathway, aldose reductases, aldehyde dehydrogenases and the chelation of metal ions, all of which are known to accelerate the reaction).[19,20] However the incidence of tissue injury ultimately depends on the balance between the rate of formation of AGE modified proteins and protection by these various systems in addition to renal clearance.

Although, great attention has been recently focused on the oxidative chemistry of the Maillard reaction, a number of reactive intermediates are efficiently formed under anaerobic conditions. Three deoxyglucosone, a dicarbonyl intermediate in AGE formation is one such example. It is formed non-oxidatively either by decomposition of Amadori compounds or fructose-3-phosphate, a metabolite of glucose.[18] Methylglyoxal (MGO) is another example of a reactive intermediate formed from the anaerobic decomposition of triose phosphate intermediates during glycolysis.[21] MGO produces a diverse group of AGEs including N-ε-(carboxyethyl)lysine, a homologue of $^{\varepsilon}$N-carboxymethyllysine (CML), redox active di-imine cross links between lysine residues, arginine-imidazolone adducts and methylglyoxal lysine dimer (MOLD).[22] The latter however, as well as other AGEs including N-ε-(carboxyethyl)lysine, can also be formed from oxidative reactions. Each of the AGEs has variable cross-linking and fluorescence capacity, the latter characteristic able to be exploited in the measurement of AGE levels.

Some of the best-characterised AGEs, such as pentosidine, MOLD (methylglyoxal lysine dimer) and GOLD (glyoxal lysine dimer), represent intermolecular cross-links between modified proteins.[18] These cross-links can result in important changes to protein structure and function. A good example is the formation of inter- and intra-molecular cross-links following the glycation of collagen, which lead to structural alterations, including changes in packing density and surface charge, manifested by increased stiffness, reduced thermal stability and resistance to proteolytic digestion.[23-26] The latter quality is expressed as collagen pepsin solubility, and its decrease, due to the increased number of acid-stable cross-links in diabetic collagen is reflected by a marked increase in acid-insoluble collagen in diabetic tissues.[27]

Higher serum AGE levels seen in individuals with renal impairment are a direct consequence of reduced renal clearance of circulating low molecular weight AGEs.[28] Given that atherosclerosis, arteriosclerosis and diastolic dysfunction are more prevalent in both diabetic patients and those with renal impairment, AGEs may provide the common pathophysiological link.

Advanced Lipoxidation End Products

Many products of lipid oxidation are also able to undergo Maillard-type chemistry to form so-called advanced lipoxidation end products or ALEs.[29] The resulting compounds may be indistinguishable from those formed by advanced glycation such as CML and $^{\varepsilon}$N-carboxyethyllysine (CEL). Dyslipidemia is common in subjects with diabetes and with advanced renal disease. It is now recognised that lipids are an important source of modified proteins, formed in an analogous way to AGEs.[30] The oxidation of polyunsaturated fatty acids including linoleate and arachidonate in plasma lipoproteins and cell membranes leads to the formation of reactive carbonyls including malondialdehyde (MDA) and 4-hydroxynonenal, with the capacity for protein modification. AGEs and ALEs can be formed simultaneously in tissue proteins, with both CML and MDA-lysine found together in atherosclerotic plaques.[30] A study by Alderson et al. in chronically hyperlipidemic, obese Zucker rats demonstrated increased formation of CML, N-ε-(carboxyethyl)lysine, as well as the AGE pentosidine and the ALEs, MDA -lysine and 4-hydroxynonenal-lysine in skin collagen in the absence of hyperglycemia.[31] Pyridoxamine, both an AGE and ALE inhibitor, significantly inhibited the increase in these compounds, as well as the development of hyperlipidemia. The absence of hyperglycemia in this model supports an important independent role for lipids in the chemical modification of proteins. Alternatively, there may be common biochemical pathways preceding the development of both AGEs and ALEs, such that carbohydrate or lipid derived dicarbonyl intermediates including glyoxal, glycoaldehyde and MGO can form both groups of compounds.

These AGEs and ALEs potentially represent an important source of glycation products, as their levels may be increased after only days of hyperglycaemia, well before similar changes can be demonstrated in vitro from the incubation of protein and glucose.[13]

AGEs and Clinical Diabetes

A strong correlation in clinical studies in patients with type 1 diabetes has been demonstrated between AGE accumulation and the severity of micro and macrovascular complications.[32] Specifically, serum concentrations of AGEs are significantly increased with the progression to microalbuminuria and subsequently to overt nephropathy.[33] This is also evident when correlating skin collagen-associated levels of AGEs with the severity of complications in patients with long-standing type 1 diabetes and with carotid intimal thickening.[34,35] In type 2 diabetic patients, plasma levels of AGEs have also been correlated with hypertension and ischaemic heart disease, suggesting that they may be potential biomarkers of diabetic cardiovascular risk.[36] Although AGEs occur as a result of hyperglycaemia, their effects can occur independently of glycaemic control with some studies demonstrating AGEs levels may only loosely correlate with glycaemic control in the clinical setting.[34,37] This finding may explain the paradoxical progression of diabetic complications in some patients with comparatively good glycaemic control. However, this does not diminish the importance of glycaemic control in the management of diabetes. Indeed, studies from the DCCT demonstrate a strong link between hyperglycaemia and advanced glycation, with significant reductions in AGE levels and improvements in soluble skin collagen seen with intensive glycaemic control.[34] Interestingly, within the DCCT study, AGE levels were a better predictor of progression to complications than HbA_{1C}, with over a third of the variance in complications attributed to differences in AGE indices. The influence of AGE levels was even more evident in the intensive glycaemic control cohort, suggesting that, while glycaemic control is important, it is not sufficient to prevent progressive complications.[34] This is consistent with the hypothesis that other factors such as oxidative stress may be more important mediators of advanced glycation than hyperglycaemia per se in patients already receiving interventions directed towards improved glycaemic control.[38,39]

So which AGE is the most pathogenic in biological systems? The molecular structure of the AGEs which are involved in development of diabetic complications has not been clearly determined. Many of the AGE cross-linked moieties, such as pentosidine have intrinsic fluorescence, and therefore tissue and plasma fluorescence may be used as markers for the presence of AGE modifications. With the development of diabetes, there is a marked increase in fluorescence within the kidney, the retina, skin and other sites of diabetic microvascular disease.[40-42] Renal and hepatic impairment are also associated with increased tissue fluorescence, reflecting the role of these organs in the catabolism and excretion of AGEs.[43] In addition, circulating levels of fluorescent AGEs correlate with complications in patients with type 1 and type 2 diabetes.[33] Recently, skin AGE fluorescence measurements taken from a device which evaluates skin fluorescence non-invasively has shown correlations with renal disease and diabetic neuropathy.[44, 45]

Other AGEs, such as CML, are neither cross-links nor fluorescent. In clinical studies, Makita has reported increased serum CML-AGE levels in type 1 diabetic patients.[28] Type 2 diabetic patients follow a similar pattern with increases in circulating CML and the precursor dicarbonyl methylglyoxal.[46,47] A number of studies have reported that non-fluorescent CML-AGE levels were also associated with the presence of microvascular complications, including retinopathy and nephropathy.[48]

The low molecular weight (LMW) fraction of serum contains less than 10% of the circulating AGEs, with LMW-AGEs more numerous than intact AGE-modified proteins.[49] Assuming that LMW-AGEs are able to bind in a stable stoichiometric fashion to AGE-receptors, the predominant effector may be fragmented LMW-AGEs rather than AGE-proteins. Advanced glycation leads to a hugely diverse range of a large variety of chemical species including heterocycles, polymers and advanced Maillard products in vivo, and therefore chemical specificity would not seem to be the biological basis of recognition of a senescent protein or peptide. Indeed, AGEs with measurable AGE-receptor binding affinity may be produced in vitro, regardless of the reducing sugar or the nature of the precise chemical modification.[50] To this end, the precise chemical modification is probably of less consequence than its binding affinity and ability to activate pathogenic pathways.

AGE-Receptor Interactions as Mediators of Damage

The effects of AGEs appear in part, to be mediated via interactions with specific receptors and binding proteins. These receptors are present on most renal cell types including endothelial cells, proximal tubular cells, mesangial cells and podocytes.[51,52] The AGE receptors include the receptor for advanced glycation end products (RAGE), AGE-R1 (OST-48, p60), AGE-R2 (80k-H, protein kinase C substrate), AGE-R3 (galectin-3), lysozyme as well as the macrophage scavenger receptors (MSR) ScR-II and CD-36 and the recently identified members of the ezrin-radixin-moesin family.[14,53,54] Other multi-ligand receptors such as megalin may also have the ability to bind AGEs in the proximal tubule.[55] Expression of these receptors appears to be tightly regulated under physiological conditions, however, there is marked up-regulation in response to metabolic states such as diabetes, dyslipidaemia and uraemia, possibly due to high levels of AGEs. In particular, activated cells at sites of diabetes-associated injury show high level expression that co-localises with AGE deposition.[42]

The role of AGE receptor-mediated pathways in the pathogenesis of diabetic complications is illustrated by studies where specific AGE-receptors have been knocked out or over-expressed. Interruption of AGE-R1 dependent uptake of AGEs and subsequent degradation is associated with accelerated glomerular renal pathology in the spontaneously non-obese diabetic (NOD) strain of mice.[56] Similarly, AGE-R3 (galectin-3) deficient mice develop accelerated glomerulopathy, as evidenced by proteinuria, increases in extracellular matrix gene expression and mesangial expansion following the induction of diabetes.[57] Indeed, the galectin-3 knockout mouse has reduced renal expression of AGE-R1, yet shows increased expression of RAGE and AGE-R2.[57] These results suggest that the AGE-R1 and -R3 receptor pathways may protect against AGE-mediated tissue injury and indeed other studies have suggested that these receptors are involved in the clearance of AGEs.[58,59] On the other hand, RAGE-mediated signalling may augment inflammation and tissue damage when chronically activated. This conclusion is further supported by studies in RAGE transgenic mice which have increased glomerulosclerosis following the induction of diabetes whereas RAGE knockout mice have less renal tissue injury when diabetes is induced.[51,60] More recently, long-term administration of a RAGE neutralizing antibody to *db/db* mice has been shown to confer renoprotection.[61] The contribution of these receptors to diabetic macrovascular disease is not well characterised.

Recently, the study of RAGE has been further complicated by the recognition of three functionally distinct splice variants.[62,63] These are the full-length RAGE receptor, the N-terminal variant that does not contain the AGE-binding domain and the C-terminal splice variant, soluble RAGE, which does not contain the trans-membrane and effector domains. The soluble form of the receptor, sRAGE, has been identified as having potential therapeutic value in experimental atherosclerosis and diabetic nephropathy.[51,64] It is conceivable that sRAGE acts as a scavenger for soluble AGEs and other RAGE ligands and competes against binding and activation of cell surface RAGE. Therefore, the balance between synthesis of soluble RAGE and full length RAGE may be an important determinant of AGE-induced dysfunction.[65]

As many of the AGE receptors are multi-specific and therefore able to bind and be activated by a range of molecules including non-AGE moieties, many may be 'accidental' AGE receptors. This is best illustrated by RAGE, since in addition to AGEs, RAGE has the ability to bind amphoterin, and a number of S-100 calgranulins to produce acute inflammatory responses.[66] In addition, AGE-receptors have the ability to bind a plethora of structurally distinct AGEs. A common characteristic of most known RAGE ligands is a net negative charge at physiological pH. Polyanionic molecules such as heparin, fucoidan, and dextran sulfate compete out the interaction between AGEs and RAGE.[67, 68] It remains to be established, however, which AGEs have the greatest affinity and activating potential for the AGE-receptors. While it is possible that one structural moiety has greater pathogenicity, it seems more likely that, given the broad chemical variety of AGEs, the number of modifications per molecule may be more important.[50] Specific receptor blockers may be difficult to develop given the poly-specific affinity of an AGE receptor that recognises a variety of AGE and non-AGE ligands.

AGEs Ingested from Exogenous Sources

It is now thought that exogenous AGEs, acquired from the diet and cigarette smoking contribute to the overall AGE

burden particularly in diabetes. Indeed, long-term storage or prolonged heating of foodstuffs in the presence of sugars generates a number of biologically reactive AGEs, capable of interacting with AGE-receptors involved in the inflammatory response and fibrogenesis.[69] The curing of tobacco also produces a number of AGEs, which are inhaled in cigarette smoke.[70] The importance of exogenous AGEs is especially apparent in the presence of impaired clearance of AGEs, seen in patients with renal disease. There is now evidence to suggest that exposure to high levels of exogenous AGEs may directly contribute to the development of albuminuria and atherosclerosis in otherwise normal animals.[71,72] Further to this, tissue and circulating AGE levels are significantly higher in smokers and in patients on high AGE diets with concurrent increases in inflammatory markers.[73]

Dietary interventions which minimise the absorption of AGEs via the gastrointestinal tract, reduce serum levels of AGEs in conjunction with improvements in levels of inflammatory mediators in patients with diabetes.[74] Such diets may ultimately provide an important adjunct to interventions directed towards the inhibition of endogenous glycation.

Non-Receptor Mediated Actions of AGE Accumulation

AGE modification of proteins has important structural and functional consequences, with proteins of the extracellular matrix such as elastin and collagen being more prone to AGE accumulation due to their slow turnover. AGE mediated inter and intramolecular cross- linking of collagen alters its surface charge and packing density, leads to a decrease in enzymatic proteolysis and degradation rate, ultimately favouring accumulation of extracellular matrix.[75,76] Furthermore, AGE modification disturbs normal protein function, including a reduction in self assembly as well as altered interactions with other matrix proteins, leading to changes in cellular adhesion and cell growth.[77] This could, at least theoretically, explain the reductions in vascular and myocardial compliance characteristic of ageing and diabetes, via a decrease in the elastic properties of both the arterial and ventricular walls.[78]

Inhibition of Advanced Glycation

Interventions that reduce AGE accumulation appear to be protective against the development of the complications of diabetes.[79-81] A number of sites for therapeutic intervention have been developed and their mechanisms of action are discussed below.

AGE Formation Inhibitors

Aminoguanidine

A hydrazine derivative, aminoguanidine reduces the formation of AGEs by binding of early glycation and glycoxidation products such as 3-deoxyglucosone, reactive dicarbonyl (RCO) and aldehyde products and facilitates their clearance by the kidneys.[82] Aminoguanidine reduces tissue levels of AGEs in experimental models of diabetes and retards the development of neuropathy, retinopathy and nephropathy.[49,81] This effect is related to the duration of treatment with aminoguanidine, consistent with the view that the generation of AGEs in the kidney is time-dependent and closely linked to the development of experimental diabetic nephropathy.[83]

Pimagedine (aminoguanidine hydrochloride ACTION I and II) has been used in diabetic patients with moderate renal impairment and demonstrated a reduction in urine protein excretion, and a reduction in the progression of retinopathy.[84] Sadly, aminoguanidine is toxic as the actions of this drug are not confined to inhibition of AGE formation as it is a potent inhibitor of iNOs. In addition, a number of patients showed renal glomerular deposition of immune complexes that may have formed as a response to the aminoguanidine/precursor AGE complexes.

Pyridoxamine

Pyridoxamine as a natural intermediate of vitamin B_6 metabolism, can reduce AGE accumulation in association with improvements in renal and vascular function in experimental diabetes.[80] Pyridoxamine reacts with carbonyl intermediates of the Maillard reaction, blocking the formation of advanced glycation and lipoxidation end products (AGEs and ALEs). In addition, pyridoxamine is able to scavenge reactive carbonyl products of glucose and lipid oxidation.[85] In contrast to AGE inhibitors such as aminoguanidine, pyridoxamine has minimal toxicity and is now in Phase II clinical trials.

OPB-9195

OPB-9195 [(+/-)-2-isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide] is a thiazolidine derivative, originally described as a hyperglycaemic agent. While OPB-9195 does not lower blood glucose levels, it has a powerful action to reduce serum and tissue deposition of AGEs.[86] Inhibition of CML and pentosidine formation by OPB-9195 has been reported to be even more efficient than aminoguanidine.[87] In addition, it is also effective in blocking the carbonyl amine chemical processes involved in the formation of two ALEs, malondialdehyde-lysine and 4-hydroxynonenal-protein adduct. OPB-9195 has now been shown to prevent progression of mesangial expansion and glomerulosclerosis in experimental diabetes.[88]

Benfotiamine and Thiamine

Other novel compounds also have the ability to reduce AGE accumulation in diabetes. Among these agents, the lipid-soluble thiamine derivative benfotiamine and thiamine have been utilised. Benfotiamine appears to be able to block the

major biochemical pathways implicated in the pathogenesis of diabetic complications including the accumulation of AGEs and was initially reported in experimental diabetic retinopathy.[89] Similar end organ protection has also been reported by Thornalley et al. in diabetic nephropathy.[39] It is thought that, as a consequence of hyperglycaemia in diabetes there are increased concentrations of the triosephosphate glycolytic intermediates glyceraldehyde-3-phosphate (GA3P) and dihydroxyacetonephosphate (DHAP). These metabolites are thought to trigger processes such as mitochondrial oxidative stress and methylgloxal formation, which facilitate the production of AGEs.[90] Consequently, an agent such as benfotiamine, that reduces the accumulation of triosephosphate intermediates could reduce these downstream pathways and indeed this has been shown in vivo in experimental diabetic complications.[39,89] The efficacy of these compounds once complications have already developed is currently under investigation.

Inhibitors of the Renin-Angiotensin System

Over-activity of the intra-renal renin-angiotensin system (RAS) has been strongly implicated in the pathogenesis of diabetic complications.[91] Interruption of the RAS with drugs such as angiotensin converting enzyme (ACE) inhibitors and AT1 receptor antagonists have proven to be the most effective clinical intervention for both the prevention and treatment of diabetic complications to date.[6-8] Beneficial effects, beyond those achieved by blood pressure control alone, have been noted in numerous experimental and clinical trials in type 1 diabetes and type 2 diabetes.[92,93] It is clear that these effects are not simply haemodynamic, as blood pressure reduction with other agents does not confer the same benefits to blockade of the RAS.[94] Consequently other mechanisms must be invoked such as effects on advanced glycation.

We have previously described the rapid development of glomerulosclerosis and tubulointerstitial fibrosis in association with overactivity of the RAS.[95] Interestingly, although this process was angiotensin II-dependent, there was amelioration of renal injury following treatment with a selective and potent inhibitor of AGE formation, ALT-946.[96] In addition, our own group have demonstrated concomitant attenuation of AGE accumulation in association with RAS blockade.[97] The major mechanism of action on AGE accumulation by ACE inhibitors is yet to be established although decreases in reactive oxygen species has been suggested. Our group have recently shown that ACE inhibitors can also increase circulating levels of soluble RAGE thereby competing with excess AGEs thereby enhancing their renoprotective capacity. ACE inhibition may also directly reduce AGE formation, as simultaneous incubation of ACE inhibitors or AT1 receptor antagonists with glucose and protein prevents the in vitro formation of AGEs.[98] It is important to establish the mechanisms whereby ACEi and AT1 receptor antagonists reduce AGE accumulation as this may be a limitation of combination therapies which target both the RAS and advanced glycation, by unwittingly 'covering the same areas'. We have however, recently demonstrated that combination therapy between the ACEi perindopril and the AGE inhibitor aminoguanidine provides superior renoprotection in experimental diabetes suggesting that this is not the case.[99]

LR-90

LR-90, methylene bis [4,4'-(2chlorophenylureido phenoxyisobutyric acid)] is a recently recognised AGE inhibitor of similar action to that seen with aminoguanidine and pyridoxamine. Specifically, LR-90 appears to be a powerful metal chelator, which inhibits glycoxidative-AGE formation and also interacts with reactive carbonyl species.[100] LR-90 has been shown to prevent the progression of diabetic nephropathy in experimental diabetes.[100]

Metformin

A number of studies have shown that the insulin sensitiser metformin is beneficial in reducing diabetes-associated vascular risk beyond the benefits expected from its anti-hyperglycaemic effect.[101] Clinical studies have shown that metformin has the ability to reduce the accumulation of toxic dicarbonyls and AGEs.[102] This effect could be related either to the binding of the alpha-dicarbonyls, methylglyoxal (MG) or 3-deoxyglucosone, or to an increase in enzymatic detoxification. Additional studies of the potential cellular effects of metformin on MG production, are required to further elucidate the actions of and role of metformin in long-term diabetic complications.

AGE "Cross-Link" Breakers

Another group of compounds that reduce AGE levels are the so-called "*cross-link breakers".* These agents are reported to cleave pre-formed AGE cross-links, thereby promoting their clearance by the kidney and liver.[103] Whether these agents are actually able to break AGEs cross-links in vivo is controversial, but the prospect of reversing the AGE burden in a diabetic patient with heavily modified proteins is extremely desirable.[104] Nevertheless, the efficacy of these compounds to reduce the accumulation of AGEs, is not challenged. The first cross-link breaker described was the thiazolium compound, PTB, N-phenacylthiazolium bromide, which has been shown to reduce the accumulation of vascular AGEs in experimental diabetes but is unable to be used in the clinical setting because of an extremely short half-life.[105,106]

ALT-711 [4,5-Dimethyl-3-(2-oxo2-phenylethyl)-thiazolium chloride] is a new generation AGE "cross-link breaker".[107]

This agent, now named alagebrium chloride, is also associated with reduced serum and tissue accumulation of AGEs in animals with experimental diabetes.[107,108] In patients with isolated systolic hypertension (but not specifically diabetes), alagebrium was associated with improvements in large artery stiffness.[109] Preliminary results from the DIAMOND (Distensibility Improvement and Remodelling in Diastolic Heart Failure) study also demonstrated reductions in left ventricular mass and improvement in left ventricular diastolic filling following treatment with alagebrium (http://www.alteonpharma.com/cross1.htm). This was manifested clinically by improvements in their NYHA class and quality-of-life. Future studies using this agent in patients with or at the onset of diabetic nephropathy are now warranted.

Other AGE Reducing Strategies

Soluble Receptors

Another approach to intervene in AGE accumulation is via the soluble form of RAGE, sRAGE. Soluble RAGE competes with cellular associated RAGE receptors for AGE binding and thereby reduces endogenous activation. Several studies have shown that soluble RAGE is able to modify AGE-mediated activation of pathways implicated in the development of diabetic complications.[51,110,111] Similarly, lysozyme, another soluble AGE binding protein has been shown to normalise serum levels of AGEs and improve albuminuria in murine models of diabetes although this would be difficult to translate readily to the clinical context.[71]

Laboratory Measurement of AGEs

Clinical studies have shown that serum and plasma measurement of circulating AGEs is both warranted and valid. This can be accurately achieved biochemically with sophisticated mass spectrometry, gas chromatography and high performance liquid chromotography. Diagnostically however, these techniques have limitations with respect to the sample size required and in their labour intensive nature although this is anticipated to change in the near future. In human plasma and serum samples, AGEs were most effectively quantified by their fluorescent properties attributable to double bonds and ring structures in a number of moieties. This was achieved crudely by fluorometry at 370/440nm (Ex/Em). Later, fluorometry evolved into a more sophisticated high throughput assay, which was used to determine circulating AGE peptides by flow injection assay as a biomarker of disease.[112] Owing to the fact that a number of AGEs are not fluorescent, ELISA for CML was developed.[113] Delayed europium lanthanide fluorescence immunoassays for the quantification of AGEs and CML in serum have also been successfully developed and are high throughput.[114] Possibly some of the most important AGEs to measure are those from the family of hydroimadazoles, which ultimately are not represented by patient HbA_{1C} levels as they are derived via other intermediates.[21] Currently this is achievable by tandem mass spectrometry.[49]

As has been discovered recently for a number of routinely measured parameters, blood tests are ultimately difficult to standardise. As such a recent innovation in clinical testing of AGEs has been the development of a skin autofluorescence reader.[45] This device involves the excitation between 300-420nm and the reading of the emission spectra by the skin within a chamber. It has shown extraordinary correlations with AGE measurements taken concurrently by skin punch biopsy and is likely to become an extremely useful non-invasive tool for use in diabetic patients.

Conclusion

While AGEs appear to be capable of individually contributing to diabetic complications, it is evident that advanced glycation is clearly only one pathway by which injury may be induced in diabetes. The interaction of metabolic and haemodynamic factors clearly compounds the deleterious effects of the diabetic milieu and reduces the threshold for injury via common mechanisms.[9] It seems unlikely that the future of preventative therapy in diabetes will involve a single "cure-all" agent. Therefore a better understanding of what pathways current therapies are already addressing, in addition to the successful targeting of other pathways should be our long-term aim in the development of new therapies for diabetes complications. In the interim, it appears that the determination of circulating levels of AGEs or the use of non-invasive skin fluorescence to assess AGE levels in vivo may provide powerful tools to predict progression to complications in the future.

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