PERSPECTIVE

Advanced glycation: an important pathological event in diabetic and age related ocular disease

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The formation of advanced glycation end products (AGEs) is a key pathophysiological event with links to a range of important human diseases. It is now clear that AGEs may act as mediators, not only of diabetic complications[1 2] but also of widespread age related pathology such as Alzheimer's disease,[3] decreased skin elasticity,[4 5] male erectile dysfunction,[6 7] pulmonary fibrosis,[8] and atherosclerosis.[9 10] Since many cells and tissues of the eye are profoundly influenced by both diabetes and ageing, it is fitting that advanced glycation is now receiving considerable attention as a possible modulator in important visual disorders. An increasing number of reports confirm widespread AGE accumulation at sites of known ocular pathology and demonstrate how these products mediate crosslinking of long lived molecules in the eye. Such studies also underscore the putative pathophysiological role of advanced glycation in ocular cell dysfunction in vitro and in vivo.

This article reviews some of the important effects that advanced glycation has on ocular tissues and the role that AGEs, and their specific receptors, have in the initiation and progression of sight threatening disorders such as diabetic retinopathy, glaucoma, cataract formation, and age related macular degeneration (AMD). This review also considers pharmacological strategies to prevent or neutralise the effects of AGEs and the recent development of potential therapies for AGE induced disease processes.

Biochemistry of AGE formation in biological systems

AGEs form via non-enzymatic condensation reactions between reducing sugars and ε-amino groups or N-terminal groups. These glycation modifications occur preferentially on lysine and arginine amino acids, although they can occur on free amine containing lipids and DNA and proceed spontaneously via a complex series of chemical rearrangements to yield reactive products with varying crosslinking, pigmentation, and fluorescence properties.[11] Non-enzymatic glycation reactions were first described around the turn of the century by Louis Camille Maillard who predicted that they could have an important impact on medicine and coined the term "Maillard reaction."[12] Unfortunately Maillard chemistry was not recognised by medical researchers until after its "rediscovery" by food scientists nearly 50 years later, who realised that the products of non-enzymatic glycation were important in food flavour, aroma, and nutritional bioavailability.[13] Only recently has the full pathophysiological significance of this ubiquitous reaction emerged as a field of study in medicine in its own right.

In biological systems, reducing sugars react with free amino groups to form Schiff base adducts and Amadori products such as fructose-lysine. It is worth noting that glucose is among the least reactive sugars within biological systems, while other sugars and dicarbonyls, many of which are located intracellularly, such as glucose-6-phosphate and glyceraldehyde-3-phosphate, are much more reactive and participate in glycation reactions at a proportionally faster rate[14] (Fig 1). In any case, the chemically unstable Schiff bases and Amadori products are freely reversible and therefore exist in an equilibrium which is proportional to the amount of free sugar. An understanding of non-enzymatic glycation kinetics in vivo led to the conceptualisation of glycosylated haemoglobin and to the eventual development of the clinical assays which measure Amadori product formation on the HbA_1 amino terminal valine of the β chain over a 28 day period (HbA_{1c})[15] thereby providing diabetologists with a useful index of glycaemic control.[16] Significantly, the levels of Amadori products in diabetic patients are usually no more than twofold to threefold higher than in their non-diabetic counterparts, which is an indication of the freely reversible nature of these products and the equilibrium which is always reached between modified and non-modified forms of a protein. Therefore, Amadori modifications do not accumulate indefinitely on long lived macromolecules and there is no correlation between the formation of these adducts on tissues and diabetic complications.[17]

Non-enzymatic glycation reactions culminate in the formation of AGEs. The majority of these products are formed from a vast range of precursor molecules, the variable chemical nature of which contributes to AGE heterogeneity. For example, the Amadori intermediate can undergo metal catalysed oxidative reactions and gives rise to irreversible "glycoxidation" products such as N-ε-carboxymethylated lysine (CML) or N-ε-(carboxyethyl)lysine (CEL)[18 19] which can accumulate on the substrate to which they are attached and/or lead to the formation of highly reactive dicarbonyl compounds. Dicarbonyls such as 1-, 3-, or 4-deoxyglucosones, glyoxal, and methylglyoxal are highly reactive intermediates, which will in turn react with proteins and propagate intramolecular or intermolecular crosslink formation[20 21] (Fig 1). These pathways are an equally important source of AGEs within the cell and, because they arise from highly reactive "AGE intermediates," they can occur very rapidly.[20 21] The chemical nature of these biologically important AGEs, as they occur naturally in vivo, is largely unknown owing to their heterogeneous and unstable nature; nevertheless, there is a growing population of structurally defined AGE adducts such as pyrraline,[22] pentosidine,[23] CML,[18] and crossline[24] (Fig 2) which have been found to be elevated in diabetic tissues.[25 26]

While AGEs form in vivo, it is now clear that extrinsically formed moieties can also have a significant role in our advanced glycation burden. Tobacco curing is essentially a Maillard "browning" reaction and combustion of these adducts during smoking can release reactive,
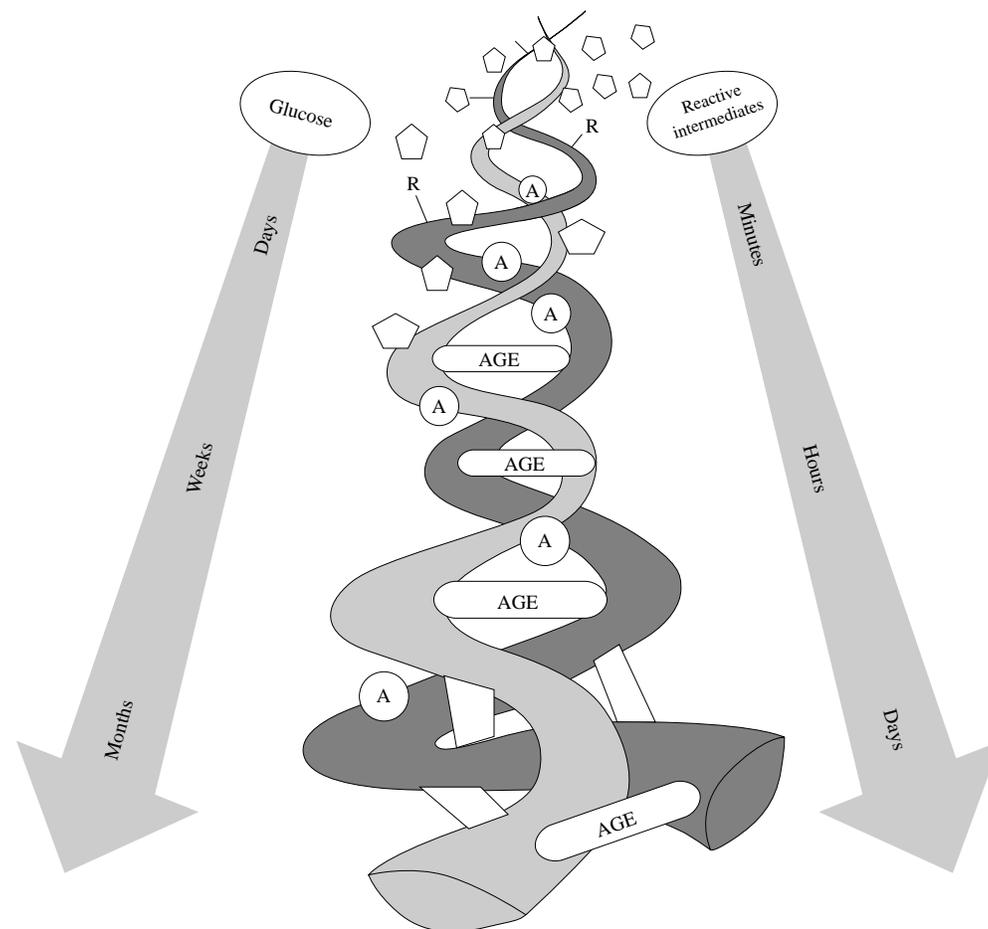
Figure 1 Schematic diagram illustrating the course of AGE formation on a hypothetical fibrilar protein. Open chain sugars or glycolytic intermediates (△) react with amino groups (R) to form Schiff bases and Amadori products (A) and eventually AGEs. Glucose may take several weeks to culminate in AGE formation leading to irreversible crosslink formation between protein fibrils or oxidative products. Reactive glycolytic intermediates such as methylglyoxal or 3-deoxyglucosone take much less time to form AGEs. Such AGE crosslinks can have a serious influence on protein structure and function.

toxic glycation products (or glycotoxins) which enter the blood stream and because of their crosslinking properties become fixed in tissues.[27 28] Recent evidence also suggests that many foods can form very high AGE levels during cooking which, upon digestion, release reactive peptide species into the circulation with an ability to form covalent crosslinks.[29 30] In the presence of normal renal function most of these reactive species are rapidly cleared from the circulation; however, in individuals with compromised renal clearance, these glycotoxins may remain within the circulation for prolonged periods of time[29] with crosslinking potential and resultant pathophysiological consequences.

Role of AGEs in cellular systems
Since AGEs are constantly forming under physiological conditions complex receptor systems have evolved to remove senescent, glycation modified molecules and/or degrade existing AGE crosslinks from tissues thereby limiting their deleterious effects. Such receptors play a critical part in AGE related biology and the pathology associated with diabetes and ageing.[1 2] Several AGE binding molecules have been described and it has been established that many of the adverse effects caused by advanced glycation are mediated via AGE receptors such as RAGE,[31] the AGE receptor complex (AGE-RC),[32 33] and the type I and II scavenger receptor.[34] The precise role of these receptors in instigating pathological events is currently ill defined and it remains controversial if some or all AGE receptors serve to

promote or limit AGE mediated cell and tissue dysfunction. The elucidation of AGE receptor modulatory roles and signal transduction pathways are areas of intensive investigation and recent evidence suggests that AGE receptor binding can initiate important signalling pathways involving activation of protein kinase C,[35 36] tyrosine phosphorylation of Janus kinase (JAK)/signal transducers and activators of transcription (STAT),[37] recruitment of phosphotidylinositol 3' kinase to Ras,[38] and induction of oxidative stress cascades which culminate in NFκB and AP-1 transcription.[39 40]

AGEs can initiate a wide range of abnormal responses in cells and tissues such as inappropriate expression of growth factors, alterations in growth dynamics, accumulation of extracellular matrix, promotion of vasoregulatory dysfunction, and initiation of death pathways.[1 2 9] Many of these responses are mediated through receptor mediated pathways[1] and the pathogenic influence of high AGE levels is well illustrated through several studies by Vlassara *et al* in which normoglycaemic animals were chronically injected with preformed AGE albumin. Such animals developed high concentrations of crosslinked collagen in their vascular walls with accompanying hyperpermeability and defective vasodilatory responses to acetylcholine and nitroglycerin.[41] Predictably, these effects were significantly reversed by the pharmacological AGE inhibitor aminoguanidine.[41] AGE infusion of normoglycaemic rats also upregulated glomerular collagen IV, laminin β1, and transforming growth factor βI mRNA levels leading to renal hypertrophy
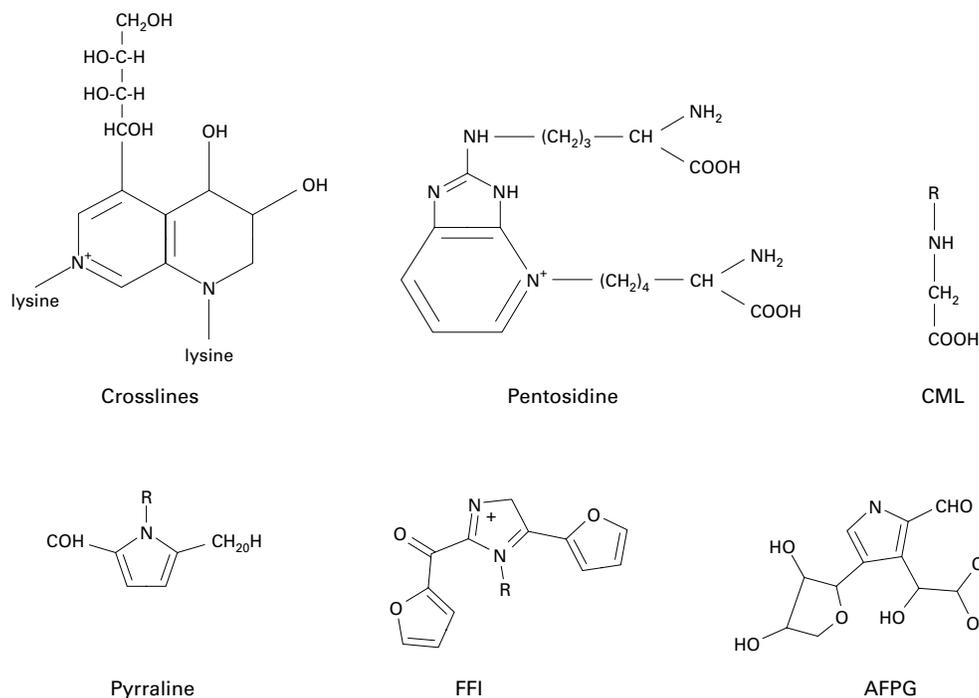
Figure 2 Structures of advanced glycation end products.

after only 4 weeks.[42] Longer infusion (4 months) caused marked glomerulosclerosis and albuminuria[43] correlating with changes observed in long term diabetic rats.

These various AGE modifications have serious consequences for macromolecular function, especially in the case of DNA,[44] important structural proteins,[45][46] enzymes,[47] and growth factors/hormones.[48][49] Indeed, such effects, in combination with inappropriate receptor interactions can initiate a wide range of deleterious cellular responses, especially within the context of diabetes and ageing where accumulative levels of AGE are highest and renal function may be impaired.

AGEs in ocular tissues

LENS

Cataract formation is the leading cause of visual impairment across the world.[50] While there are many causes of lens opacity, ageing is by far the major risk factor[51][52] with excessive ultraviolet light exposure[53] and associated free radical damage of crystallins[54] being the key pathogenic factor. The role of Maillard reactions in cataract formation has also been extensively studied in both the aged and diabetic lens where AGEs of various derivations and molecular structures have been shown to be markedly elevated.[55–58] Glycation generates significant age related alterations in lens fibre membrane integrity and tertiary structure of lens proteins. This leads to aggregation and covalent crosslinking of lens crystallins which, irrespective of cataract formation, can result in reduced deformability with accompanying presbyopia.[59] The action of highly reactive dicarbonyl compounds such as glyoxal and methylglyoxal is enhanced in diabetes and ageing, leading to AGE crosslinks on α crystallins with resultant loss of chaperone activity, increased αβ crystallin content and dense aggregate formation.[60–62]

The action of metal catalysed, Fenton reactions which culminate in hydroxyl radical generation may have major pathogenic significance in cataract formation, especially in diabetics where there is a significant accumulation of copper in the lens cells.[63][64] Recent evidence suggests that a close association exists between advanced glycation, metal ions, and generation of free radicals during age related cataract formation, where AGE formation on crystallins leads to binding of redox active copper which in turn catalyses ascorbate oxidation.[65]

As stated previously, tobacco products may be a rich source of reactive glycation products, capable of promoting AGE formation in vivo.[27] In a study of cataractous lenses there were significantly higher levels of immunoreactive AGEs in those patients with a history of smoking[28] (Fig 3). Smoking releases highly reactive gas phase oxidants into the blood stream[66] and is a clear risk factor for cataract formation.[67] It is now evident that cigarette smoke mediated AGE formation may act in concert with heavy metal deposition and oxidative stress to precipitate cataract formation.

CORNEA

The Maillard reaction has a significant role in altering corneal biochemistry during diabetes and ageing. Diabetic keratopathy[68] is manifested by thickening of the stroma and basement membranes, recurrent erosions, corneal oedema, and morphological alterations in the epithelial and endothelial layers.[68] Such alterations in the human diabetic cornea are accompanied by decreased protein stability in the stroma and basal laminae and increased immunoreactive AGEs which have been partially characterised as pentosidine[69] and CML.[70] Bowman's membrane is heavily glycated in diabetic patients[70] while in vitro AGE modified substrates can significantly reduce corneal epithelial cell adhesion and spreading,[70] possibly by disruption of integrin/non-integrin receptor-matrix interactions which has obvious pathogenic implications for recurrent erosions.

AGEs also accumulate in the ageing cornea[71][72] as they do in extracellular matrix proteins in other tissues.[4][5] Such age related crosslinking occurs largely on the collagen component of the cornea (stroma and lamina) and can be effectively reversed using aspirin-like analgesics which have defined antiglycation properties.[73] Interestingly, it has been proposed that AGE mediated crosslinking could have benefits as a means for stiffening and strengthening the weakened cornea of patients with keratoconus.[74]
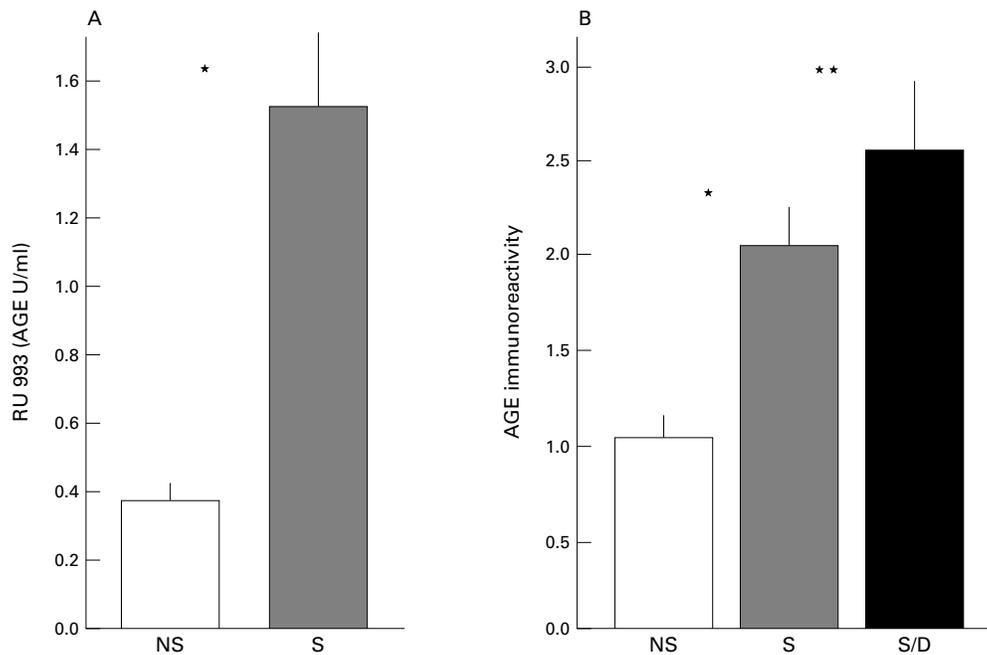
Figure 3 *AGEs accumulate at high levels in the lens and coronary arteries of smokers. (A) The cataractous lenses of smokers (S) and non-smokers (NS) were removed, the protein extracted and quantified for AGE immunoreactivity using a competitive AGE-ELISA. AGE levels were significantly higher in the lenses of smokers (*p<0.0007) (Nicholl et al[28]). (B) AGE immunoreactivity in the vascular walls of coronary arteries from smokers, non-smokers, and smokers with diabetes. AGEs deposited at higher levels in patients with a history of smoking tobacco products. Significantly, patients who had diabetes and also smoked had supraelevated levels in their coronary arteries (*p<0.015; **p<0.001).*

VITREOUS

The vitreous gel is composed largely of a complex network of crosslinked collagen (type II,V/IX, and XI) fibrils and the hydrophilic glycosaminoglycan, hyaluronan.[75] Disorders of the vitreous often manifest themselves as morphological changes to the collagen component within the cortical gel and age related vitreous degenerations are usually a direct result of dissociation of collagen and hyaluronan.[76] Structural changes to the vitreous such as liquefaction and posterior vitreous detachment (PVD) are associated with ageing while in diabetics such changes occur earlier than in non-diabetics in a condition Sebag has called diabetic vitreopathy.[77]

The molecular basis of vitreous degeneration remains somewhat equivocal.[77] In terms of advanced glycation mediated pathology, it has been demonstrated that glycation can induce abnormal crosslinks between vitreal collagen fibrils leading to dissociation from hyaluronan and resultant destabilisation of the gel structure.[76] Moreover, AGEs have been described in human vitreous where they correlate with age and accumulate at an even higher level in diabetic patients.[78] The significance of this has also been shown in bovine vitreous incubated ex vivo in high glucose conditions where immunoreactive AGEs formed on the vitreous collagen component and resulted in enhanced crosslinking of the fibrils—a process which could be significantly inhibited by the AGE inhibitor aminoguanidine.[78]

Sebag has described the pathogenesis of vitreous degeneration in diabetics as a process of "precocious senescence"[79] and while other non-glycational physiological and biochemical processes contribute to vitreous degeneration it would appear that AGEs have an important role in diabetic and ageing vitreous dysfunction.

RETINA (DIABETIC RETINOPATHY)

In diabetes the retinal microvasculature becomes progressively dysfunctional in response to variable hyperglycaemia and in this progressive disease there is widespread loss of retinal pericytes and failure of endothelial cells, leading to capillary closure and retinal ischaemia.[80] In common with other vascular beds in the body, AGEs and/or late Amadori products have been localised to retinal vessels and neuroglia of diabetics.[81–85] The precise part played by these adducts in the pathogenesis of diabetic retinopathy remains ill defined although experimental studies have demonstrated that AGEs may be responsible for retinal vascular lesions[86 87] and that aminoguanidine can prevent this pathology.[88] Interestingly, aminoguanidine does not prevent the initial phase of experimental diabetic retinopathy in rats,[89] although a secondary intervention study with this drug has been shown to retard disease progression.[90] In diabetic rats, AGEs are not only localised to vascular basement membranes (BMs), but also appear to accumulate in the retinal pericytes after 8 months of diabetes[81] (Fig 4). Moreover, when non-diabetic animals are infused with preformed AGE albumin, these adducts accumulate around and within the pericytes, co-localise with AGE receptors, induce BM thickening, and cause breakdown of the inner blood-retinal barrier.[81 87 91] In clinical studies it has been reported that the levels of serum AGEs, and also the glycoxidation product CML, correlate with the degree of diabetic retinopathy.[92 93]

In vitro investigation of retinal vascular cells has provided important insights into the action of these adducts, their receptors, and how they contribute to tissue dysfunction in diabetes. Retinal vascular endothelial cells exposed to AGEs show abnormal endothelial nitric oxide synthase (eNOS) expression, which may account for some of the vasoregulatory abnormalities observed in the diabetic vasculature.[94] Advanced glycation can mediate pathophysiological differentiation events (for example, calcification) in retinal pericytes[95] and initiate abnormal growth responses in retinal vascular cells[96 97] which can be modulated, at least in part, by AGE receptors. In addition, vascular endothelial growth factor (VEGF), which at high levels is important for vascular incompetence and proliferation can be upregulated in many retinal cell types after exposure to AGEs,[98–100] an effect exacerbated by low P_{O_2}
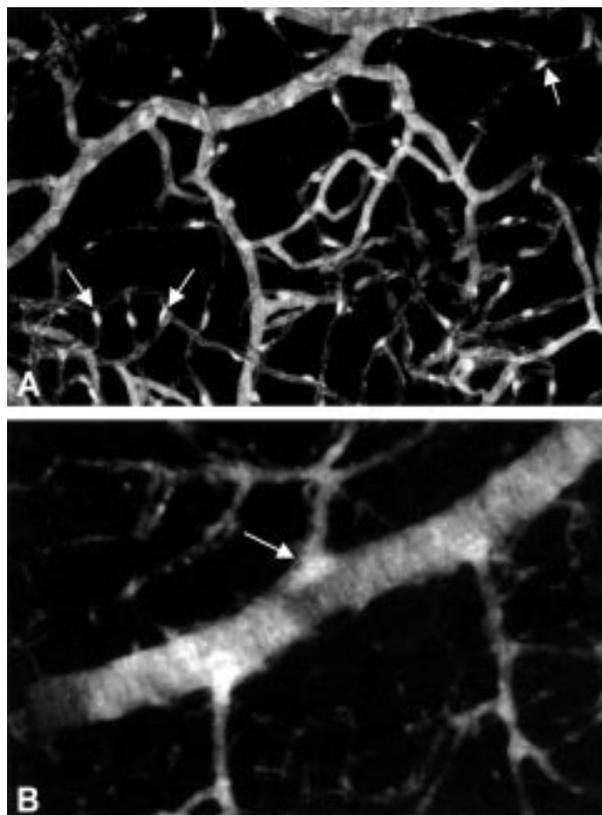
Figure 4　AGE immunoreactivity in diabetic and aged rats. (A) Trypsin digest of retinal vascular tree from an 8 month diabetic rat. AGE immunoreactivity is marked in the arterioles and capillaries. The pericytes of the retinal capillary beds are hyperfluorescent indicating accumulation of AGEs (arrows). (B) AGE immunoreactivity in the retinal vasculature of a 28 month old, non-diabetic rat. The immunofluorescence pattern is different from that observed in the diabetic retina (A) with AGE localisation appearing confined to the vascular basement membranes of arteries, arterioles and, to a lesser extent, the capillaries. Note the bright fluorescence at arteriolar sphincters (arrow).

(hypoxic) conditions[98] with clear implications for barrier dysfunction and disruption of vascular cell growth dynamics. More research is needed to determine AGE pathogenic influences on retinal vascular function, but it is clear that these products, whether as adducts on important serum derived proteins, as reactive intracellular intermediates or as accumulative extracellular matrix crosslinks have the potential to disrupt key signalling pathways with significant impact on cellular function. Future mechanistic in vitro and in vivo studies will help to establish the precise role of advanced glycation in diabetic retinopathy.

RETINA (AGE RELATED DYSFUNCTION)

A spectrum of age related changes in the retinal pigment epithelium (RPE) and underlying Bruch's membrane has been described clinically, ultrastructurally, and histopathologically. The most prominent of these changes, coinciding with the early stages of AMD, are extracellular deposits of drusen, basal laminar deposits (BLDs), and changes in the chemical composition, physical structure, and hydrodynamics of Bruch's membrane.[101 102] Such changes are thought to be important in the development of AMD.[103] Drusen and BLDs form between Bruch's and the RPE[102] and although the histopathological characteristics of the deposits are well documented, their precise chemical composition has only been partly resolved. BLD and drusen have deleterious effects on RPE structure and function and the accumulation of lipofuscin and undigested phagosomes in RPE cells with age has a direct influence on cellular function and outer retinal integrity.[104 105] Lipofuscin

accumulation in RPE cells may reflect accelerated phagocytosis of defective rod outer segments and/or impaired degradation of engulfed photoreceptor remnants due to altered digestibility or failure of lysosomal activity.[106 107] Impaired RPE processing of shed photoreceptor outer segments is associated with drusen formation[108 109] although the precise pathogenesis is poorly understood.

There are growing links between advanced glycation and ageing changes at the outer retina. Recent reports suggest that AGEs accumulate in drusen and in Bruch's membrane with age and occur at a higher level in patients with AMD.[110–113] Further evidence linking AGE accumulation to AMD can be surmised from the composition of drusen which contains lipids, apolipoprotein E, amyloid, and vitronectin[114 115] (proteins which are modified by Maillard chemistry during ageing).[116–118] Advanced glycation is a feature of BM thickening and extracellular matrix dysfunction during diabetes and it is significant that Bruch's membrane is also known to thicken progressively in older patients and become less permeable.[101 119]

RPE cells are radically influenced by exposure to AGEs in vitro where they express abnormal levels of vascular endothelial growth factor (VEGF) and platelet derived growth factor B (PDGF-B).[98 112] This may have a bearing on RPE cell function, maintenance of the choriocapillaris, and integrity of the RPE/photoreceptor complex. The accumulation of lipofuscin and reduction of lysosomal degradative capacity in RPE cells may reflect AGE formation and receptor mediated transport of these adducts to the lysosomal compartment (Fig 5). Significantly, intracellular sequestration of these highly reactive adducts can
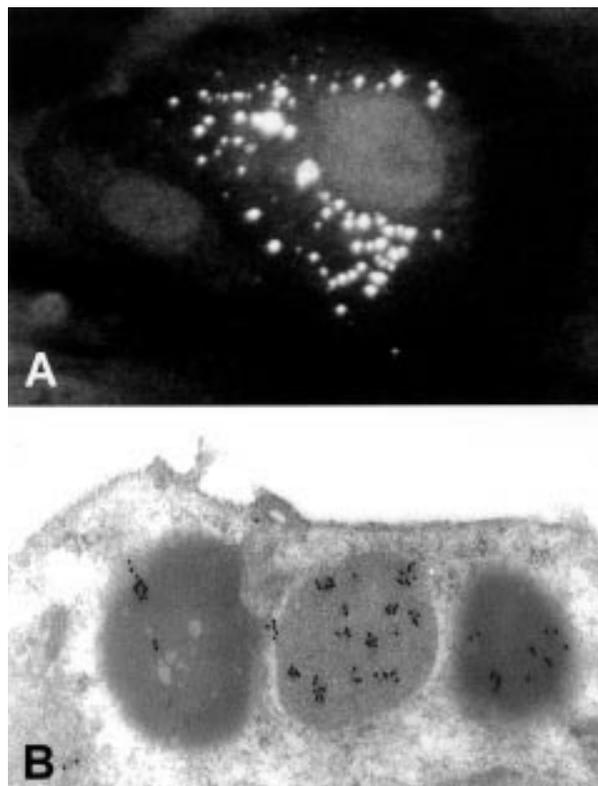

Figure 5　AGEs and AGE receptor accumulation in lysosomes. (A) AGE receptor component (AGE-R1) (see Stitt et al [33]) immunoreactivity in a human RPE cell which was exposed to AGE albumin for 4 days before fixation. Note the hyperfluorescent areas in a perinuclear position—a distribution pattern which is indicative of RPE lysosomal compartments. (B) AGE immunoreactivity in a glomerular epithelial cell from a diabetic dog. Note the high density of gold particles in the lysosomes which indicate AGE accumulation in these organelles.

markedly reduce lysosomal enzymatic activity in other epithelial cell types.[120–122] Incomplete degradation of phagocytosed photoreceptor outer segments is linked to the formation of lipofuscin in RPE cells[123] and it is notable that advanced glycation reactions appear to play an important part in the formation of age related intracellular fluorophores and lipofuscin granules in post-mitotic epithelial cells.[124]

OTHER OCULAR TISSUES

Age related changes to retinal ganglion cells and the optic nerve head is a recognised phenomenon with an aetiological role in the pathogenesis of chronic open angle glaucoma.[125] Since AGE adducts accumulate with age on many long lived macromolecules it is perhaps unsurprising that these products have been detected within the collagenous matrix of the lamina cribrosa where levels correlate with age.[126] The lamina cribrosa has an important role in supporting the optic nerve axonal structure and the AGE mediated crosslinking of this matrix may reduce flexibility and perhaps induce age related axon damage which is characteristic of this degenerative glaucomatous disease.[126] Indeed, inhibition of AGE formation in diabetic rats effectively prevented diabetes induced myelinated optic nerve atrophy.[127]

Anti-AGE therapeutic strategies

Prevention or amelioration of AGE mediated cell toxicity has been a key strategy in the prevention of diabetic complications and some age related pathology. To date there have been a range of approaches which seek to either prevent AGE formation, reduce AGE effects on cells, or even break established AGE crosslinks.

Amadori product formation is the basis of advanced glycation biochemistry because progression to protein crosslinks requires slow chemical rearrangement to create reactive intermediates before the formation of irreversible AGEs. An important pharmacological strategy for the inhibition of this process utilises the small nucleophilic hydrazine compound aminoguanidine, which is a potent inhibitor of AGE mediated crosslinking.[128] This drug can prevent some diabetic vascular complications in experimental animals,[88 129–133] while clinical trials of aminoguanidine were shown to effectively reduce AGE-Hb while leaving HbA_{1c} unaffected.[134] Such optimism has been tempered by the gradual realisation that aminoguanidine also inhibits a range of other important pathways, most notably generation of nitric oxide by eNOS,[135] which may increase non-specific and unwanted side effects of the drug. Nevertheless, aminoguanidine and/or other related AGE inhibitors may eventually find a place in the management of diabetics or in individuals at risk of age related sequelae. Other AGE inhibiting drugs have been recently developed, such as the thiazolidine derivative OPB-9195,[136] pyridoxamine,[137] and 2,3 diaminophenazine (2,3 DAP).[138]

Prevention of AGEs interacting with their receptors or other body proteins is a valid therapeutic approach. The use of neutralising antibodies against glycated albumin has been shown to prevent BM thickening in diabetic (db/db) mice despite the fact that the antibodies did not alter the glycaemic status of the animals.[87] Likewise, the use of the AGE binding properties of lysozyme has succeeded in reducing AGE levels in dialysate from diabetic patients with kidney disease[139] and presents a real possibility for reduction of toxic AGE groups in the body fluids of patients with renal failure. Furthermore, elucidation of AGE receptor signal transduction pathways may also offer intracellular strategies to control receptor mediated sequelae.

Recently, a novel therapeutic strategy has been to attack the AGE crosslinks formed in biological systems. This is an exciting approach since it would "break" pre-accumulated AGEs and subsequently allow clearance via the kidney. Such an AGE crosslink "breaker" prototype has been described to attack dicarbonyl derived crosslinks in vitro.[140] There are now at least two such chemical agents which have the ability to reduce the tissue content of AGEs in experimental diabetes,[141 142] reverse hyperglycaemia related arterial distensibility,[136] and ameliorate age related myocardial stiffness.[143]

Conclusion

It is evident that AGEs may play a significant pathogenic part in diabetic complications and many age related disorders. The pathogenesis of such disorders are multifactorial and it is clear that advanced glycation, while perhaps having a significant role, is not the only processes leading to cell and tissue dysfunction. Nevertheless, key events in diabetes and ageing such as free radical generation and inappropriate activation of signalling molecules (for example, protein kinase C, NFκB gene transcription) may have important links to or are secondary consequences of advanced glycation processes. It must also be stated that in many ageing ocular tissues the accumulation of AGEs may represent a function of the ageing process and their direct aetiological function needs to be directly and unequivocally proved. Whatever their place in the pathogenic hierarchy of ocular disease, AGEs may play an important part in diabetic retinopathy and cataract formation while their putative involvement in glaucoma, diabetic keratopathy, and AMD requires much more evaluation. As research intensifies into Maillard chemistry and the cellular and molecular consequences of advanced glycation, and as pharmacological intervention strategies evolve, we may be close to reducing some of the sight threatening complications affecting diabetic and older individuals.

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