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MINIREVIEW

Advanced Glycation End-Products and the Progress of Diabetic Vascular Complications

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Summary

Epidemiological studies have confirmed that hyperglycemia is the most important factor in the onset and progress of vascular complications, both in Type 1 and 2 diabetes mellitus. The formation of advanced glycation end-products (AGEs) correlates with glycemic control. The AGE hypothesis proposes that accelerated chemical modification of proteins by glucose during hyperglycemia contributes to the pathogenesis of diabetic complications including nephropathy, retinopathy, neuropathy and atherosclerosis. Recent studies have shown that increased formation of serum AGEs exists in diabetic children and adolescents with or without vascular complications. Furthermore, the presence of diabetic complications in children correlates with elevated serum AGEs. The level of serum AGEs could be considered as a marker of later developments of vascular complications in children with Type 1 and 2 diabetes mellitus. The careful metabolic monitoring of young diabetics together with monitoring of serum AGEs can provide useful information about impending AGE-related diabetic complications. It is becoming clear that anti-AGE strategies may play an important role in the treatment of young and older diabetic patients. Several potential drug candidates such as AGE inhibitors have been reported recently.

Key words

Diabetic vascular complications • Hyperglycemia • Advanced glycation end-products • Oxidative stress • Inhibitors of glycation

Diabetes mellitus is increasing at an alarming rate, particularly the non-insulin dependent diabetes mellitus (Type 2 diabetes or NIDDM). In 1997, 124 million people worldwide had diabetes, 97 % of these having Type 2 diabetes. By the year 2010, the total number of people with diabetes is estimated to reach 221 millions (Amos *et al.* 1997). Although there is some variation in the distribution and occurrence of complications between insulin-dependent diabetes (IDDM or Type 1 diabetes) and Type 2 diabetes, both groups are highly prone to increase in number. The development of diabetic complications is a major cause of morbidity and mortality and is an ever-increasing

burden to healthcare authorities in both developed and developing nations.

Large prospective clinical studies have shown a strong relationship between glycemia and diabetic microvascular complications in both Type 1 and Type 2 diabetes. Both hyperglycemia and insulin resistance seem to have important roles in the pathogenesis of macrovascular complications (Anon 1993, 1998).

Diabetes is characterized by high glucose concentrations that lead, *via* several mechanisms (the polyol pathway, hexosamine pathway, AGE pathway and protein kinase C) to increased production of free radical intermediates (Brownlee 2001, Bonnefont-Rousselot 2002, Spitaler and Graier 2002). The resulting glycative, glycoxidative, carbonyl and oxidative stress can play a key role in pathogenesis of diabetes (Bonnefont-Rousselot *et al.* 2000, Jakuš 2000, West 2000, Miyata 2002).

Hyperglycemia, glycation, glycoxidation and the AGE concept

Epidemiological studies have confirmed that hyperglycemia is the most important factor in the onset and progress of diabetic complications. Hyperglycemia is generally accepted to be the major cause of diabetic microvascular complications and may play an important role in the development of macrovascular diseases. It is clear that hyperglycemia is a primary factor that initiates and promotes diabetic complications (Hanssen 1997). However, the exact mechanism of the deleterious effect of hyperglycemia on the small and large blood vessels is not known.

Glycation has both physiological and pathophysiological significance. Under physiological conditions, glycation can be detected in the ageing process, and the reactions are more rapid and more intensive with frequently increased glucose concentrations. Acute and chronic hyperglycemia is known to enhance early, intermediate and advanced glycation.

It is now thought that advanced glycation end-products (AGEs) and advanced lipoxidation end-products (ALEs) contribute to accelerated micro- and macrovasculopathy observed in diabetes. The enhanced formation of AGEs exists in the blood and tissues of diabetics and also in various pathophysiological states, such as atherosclerosis, Alzheimer´s disease, end-stage renal disease (ESRD), rheumatoid arthritis and liver cirrhosis (Šebeková *et al.* 2002) (Table 1).

Table 1. The presence of AGEs in the serum and tissues in various diseases

Disease	Level in serum	Accumulation in tissues	Enhanced formation by glycation	Enhanced formation by oxidative stress
Diabetes mellitus	↑	↑	↑↑	↑
Atherosclerosis	↑	↑	↑↑	↑
ESRD	↑↑↑	↑	↑	↑↑↑
Alzheimer´s disease	normal	↑	↑	↑
Rheumatoid arthritis	↑	↑*	-	↑
Liver cirrhosis	↑	↑	-	↑

* synovial fluid

The AGE concept proposes that chemical modification and crosslinking of tissue proteins, lipids and DNA affect their structure, function and turnover, contributing to a gradual decline in tissue function and to the pathogenesis of diabetic complications.

AGE-modified proteins are formed from the covalent reaction between free amino groups of amino acids, such as lysine, arginine or protein terminal amino acids and oxo group of sugars (glucose, fructose, ribose etc.) to create, first, the Schiff base and then Amadori products of which the best known is HbA_{1c} and fructosamine (fructoselysine) (Fig. 1). The AGE formation from fructoselysine involves the non-oxidative dissociation of fructoselysine to form new reactive intermediates that again modify proteins to form AGEs of various chemical structures (Figs 2 and 3). Alternatively, fructoselysine may decay releasing its carbohydrate moiety either as glucose or as more reactive hexoses,

such as 3-deoxyglucosone, which themselves may modify proteins. It has recently been found that glucose can probably autoxidize to form reactive cabonyl compounds (glyoxal and methylglyoxal) which may react with protein to glycoxidation products (Thornalley 1990, Lyons and Jenkins 1997). Thus, carboxymethyllysine (CML) moieties of proteins may be formed either from an oxidative modification of fructoselysine (Ahmed *et al.* 1986) or from a reaction of glyoxal, the main dicarbonyl compound (Fig. 4). Recently it was shown that peroxynitrite can induce the formation of CML by oxidative cleavage of the Amadori product and also by the generation of reactive dicarbonyl compounds from glucose (Nagai *et al.* 2002).

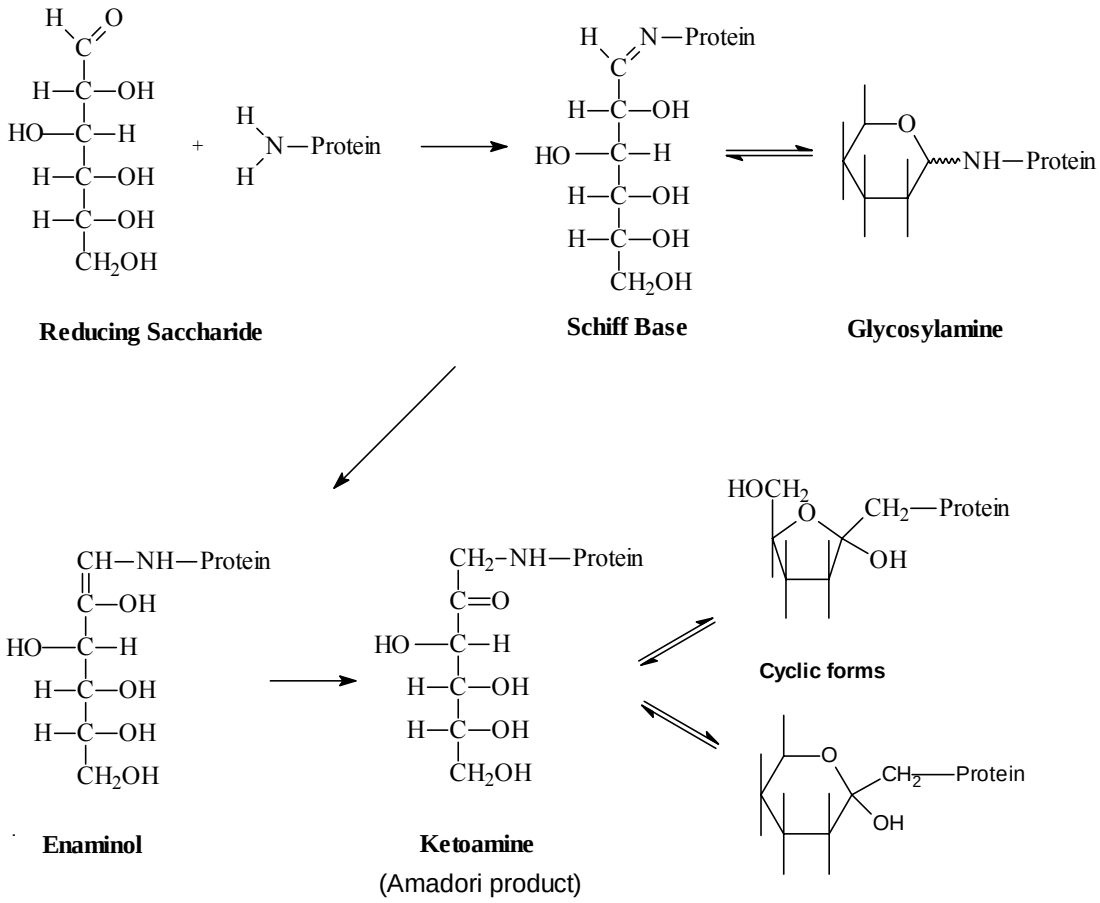


Fig. 1. The formation of Schiff base and Amadori compound.

Thus, AGEs can arise from the decomposition of Amadori products, from fragmentation products of the polyol pathway and as glycoxidative products which all react with protein amino groups.

Arguments for AGEs in microvascular complications

It has been well documented that AGEs progressively accumulate on the tissues and organs which develop chronic complications of diabetes mellitus, such as retinopathy, nephropathy, neuropathy and also macrovascular disease atherosclerosis.

There are three lines of evidence for the role of AGEs in microvascular complications. The first concerns the association between the accumulation of AGE-modified proteins and the severity of microvascular complications in both diabetic animals and man (Sell *et al.* 1992, McCance *et al.* 1993, Beisswenger *et al.* 1995). The second stems from the fact that typical microvascular complications develop following injections of AGE-modified proteins in non-diabetic animals (Vlassara *et al.* 1994). The third line of evidence indicated that development and progression of microvascular complications is inhibited by aminoguanidine (Hammes *et al.* 1995, Soulis *et al.* 1997) and pyridoxamine (Degenhardt *et al.* 2002a,b, Stitt *et al.* 2002).

Early diabetic microangiopathy is characterized by vasodilation, increased blood flow and increased capillary permeability. AGE-modified proteins may lead to all these changes. They can also impair the binding of heparan sulfate to the extracellular matrix, which results in a loss of anionic sites and thus in an increase in endothelial permeability.

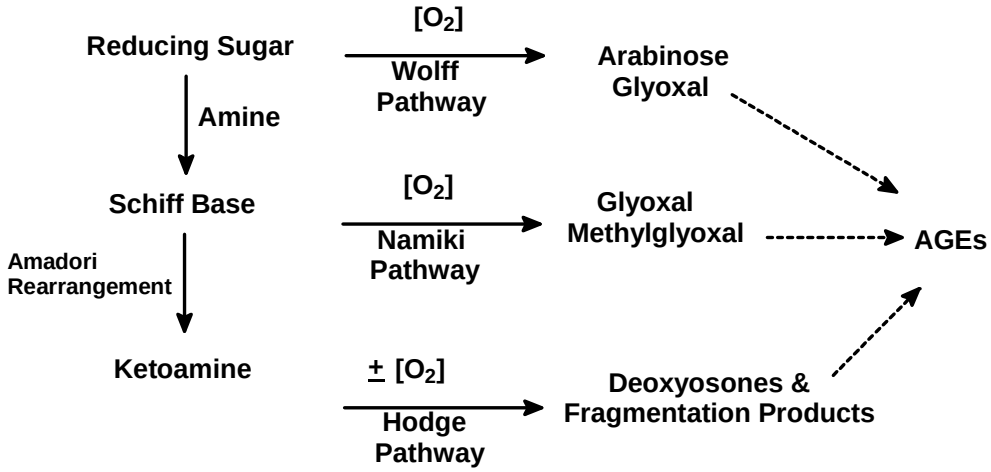


Fig. 2. Pathways of AGEs formation. Wolff pathway – glucose may undergo metal-catalyzed autoxidation to produce reactive carbonyl precursors of AGEs, Namiki pathway – Schiff bases formed on reaction of glucose with protein, undergo reverse aldol reactions and autoxidative cleavage to produce AGE precursors, Hodge pathway – AGE precursors are formed by rearrangement and autoxidation of the Amadori product.

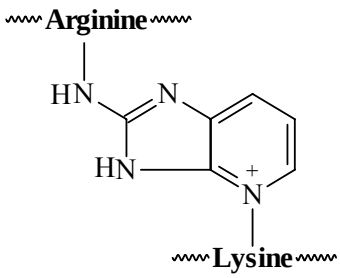
Immunohistochemical analysis has demonstrated different AGEs in both glomerular and tubular cells in experimental and human diabetic nephropathy (Horie *et al.* 1997, Schleicher *et al.* 1999). It has been shown that AGEs are directly linked to an increased retinal vascular endothelial growth factor expression, a factor which is important in the development of proliferative retinopathy (Lu *et al.* 1998).

Significant role of AGEs in atherosclerosis

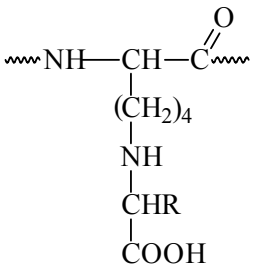
Lipids may also be modified by AGEs (Baynes and Thorpe 2000). AGE-modified substances, especially carboxymethyllysine (CML), have been found in atheromas by immunohistochemical and chemical analysis (Schleicher *et al.* 1999). AGE-modified proteins may be taken up by scavenger receptors. AGE-modified LDL have been identified in the serum. For instance, irreversible and reticulated LDL from the circulation binds to AGE-modified collagen of blood vessel walls. In the majority of blood vessels such reticular binding delays normal outflow of LDL particles which had penetrated the vessel wall and thus enhance cholesterol deposition in the intima. Such reticulation of AGEs increases lipoprotein deposition regardless of the plasma LDL level. This is followed by an accelerated development of atherosclerosis. Turk *et al.* (2002) showed a significant correlation between the content of AGEs measured by ELISA and circulating LDL containing immune complexes LDL-IC in Type 2 diabetics with vascular complications that contribute to the development of atherosclerosis.

The role of AGE receptors

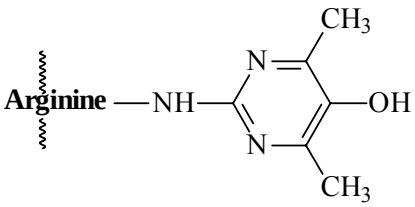
AGE receptors have been identified in macrophages, endothelial cells and several other cell types, and are implicated in protein turnover, tissue remodeling and inflammation (Schmidt *et al.* 2001, Vlassara 2001). The level of AGE proteins reflects the kinetic balance of two opposite processes: the rate of formation of AGEs and the rate of their degradation by means of AGE receptors. AGE protein binding to macrophage cell receptors causes a cascade of events in the homeostasis of blood vessel walls and their milieu by mediation of cytokines and tissue growth factors. AGE receptors participate in the elimination and change of aged, reticular and denatured molecules of extracellular matrix as well as other AGE molecules. However, AGE protein accumulation in diabetes mellitus may exceed the ability of their elimination due to hyperglycemia and the enhanced glycation process. It is possible that gene diversion in AGE receptors can explain variations in the level of AGEs and the differences in susceptibility to diabetic complications.



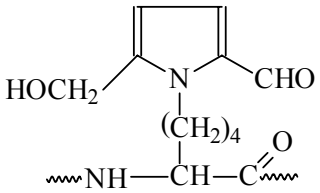
Pentosidine



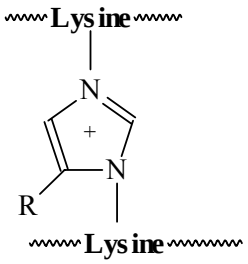
CML: R=H
CEL: R=CH_3



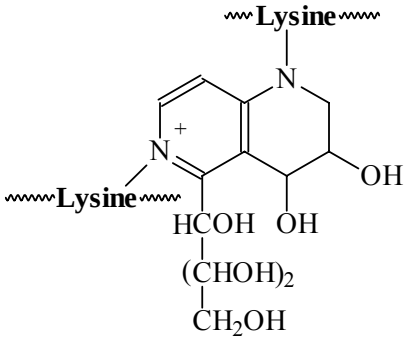
Argpyrimidine



Pyrraline



GOLD: R=H
MOLD: R=CH_3



Crosslines

Fig. 3. Structures of some most important AGEs: pentosidine, argpyrimidine, carboxymethyllysine (CML), carboxyethyllysine (CEL), pyrraline, glyoxal-lysine dimer (GOLD), methylglyoxal-lysine dimer (MOLD) and crosslines.

The role of AGE peptides (glycotoxins)

Circulating AGEs are detoxified by various enzymes. In this process AGE peptides are released as degradation products, partly occurring through proteolysis of matrix components, commonly called glycotoxins. AGE peptides entering the blood circulation are highly reactive. When these glycotoxins are not eliminated by the kidneys, recirculating AGE peptides can generate new AGEs reacting with plasma or tissue components. At this stage glycation accelerates the progress of deterioration.

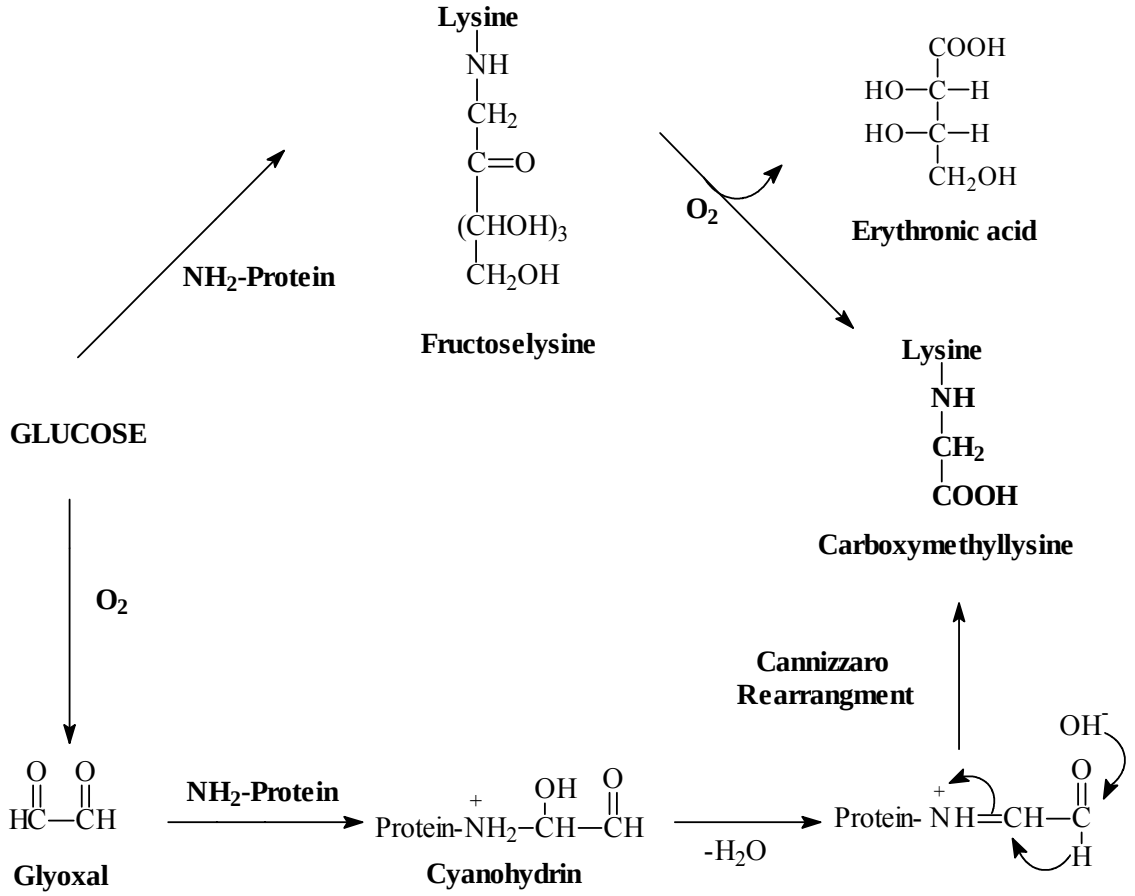


Fig. 4 The formation of carboxymethyllysine (CML) (Onorato *et al.* 2000).

The role of oxidative stress in the creation of diabetic vascular complications

What is the relationship between oxidative stress (OS) and AGEs in the production of micro- and macrovascular complications? OS may be involved in AGE formation, and AGEs may induce oxidative stress. The level of oxidizable substrates such as Amadori adducts, reactive carbonyl and dicarbonyl compounds and polyunsaturated fatty acids is increased in the blood and various tissues in diabetes. Many of the identified AGEs are glycoxidation products. CML modification of proteins is one of the major glycoxidation products formed *in vitro* by the reaction between glucose and proteins (Reddy *et al.* 1995). Many studies have investigated the accumulation of different AGEs (CML, pentosidine, pyrraline) in diabetic animals and man, and have correlated their results with diabetic microvascular complications.

In most studies a correlation has been found between the severity of microvascular complications and the quantitative accumulation of AGEs, especially in the skin (Sell *et al.* 1992, McCance *et al.* 1993, Beisswenger *et al.* 1995). However, a large variability in accumulation has been reported. The relevance of skin measurements to diabetic complications is hitherto uncertain.

Recent studies have suggested that nuclear NF-κB polymorphisms may be involved in the development of diabetic microvascular complications (Hegazy *et al.* 2001). This important transcription factor is involved in the response to OS and inflammation.

It is evident that decreased antioxidative protection and simultaneous overproduction of free radicals occur in diabetic children (Dominguez *et al.* 1998, Jakuš *et al.* 2000, Davison *et al.* 2002, Varvarovská *et al.* 2003). The changes in ascorbate, α-tocopherol and glutathione concentration are consistent with decreased antioxidant protection (Bonnefont-Rousselot *et al.* 2000, West 2000). The resulting enhanced OS as an imbalance between reactive oxygen species production and the antioxidant defense may be present in diabetes, but this

may be both a cause and an effect of tissue damage (Jakuš 2000). A supplementation with antioxidants has been proposed as complementary treatment, and some antidiabetic agents may have antioxidant propeties independently of their role in glucose control (Bonnefont-Rousselot 2001).

Serum measurements of Amadori products and AGEs in human diabetes

Most Amadori albumin and serum AGEs research has been performed in diabetic animals with or without complications. It is therefore important to study especially human diabetes.

Measurement of serum AGEs in human diabetes has been difficult to carry out because there is no consequently recognized standard. Therefore, different groups may not measure in exactly the same way. Serum levels of AGEs (s-AGEs) are measured spectrofluorometrically and by sensitive HPLC, LC/MS or GC/MS methods (Jakuš and Baynes 2001). Enzyme linked immunosorbent assays (ELISA) are now also available for various chemically defined s-AGEs, such as carboxymethylsine and pentosidine (Makita *et al.* 1992, Takeuchi and Makita 2001). The assessment of high-titer polyclonal and monoclonal anti-AGE antibodies has been applied successfully to ELISA and immuno-histochemical studies. The competitive ELISA method is most frequently used for the measurement of AGEs concentrations in body fluids. The reaction principle is as follows: the immunoplate wells are overcoated by AGE-antigen, and the serum containing an unknown quantity of AGE-antigen is incubated together with an anti-AGE antiserum. At the end of the incubation period the wells are treated with labeled secondary antibody enzyme. Then a substrate is added, which gives the absorbance difference to be measured. Competitive immunoreactivity of the samples is read from the calibration curve (Turk *et al.* 2001).

Several investigators have addressed the following questions: a) Do measurements of serum Amadori albumin predict the progression of microvascular complications? b) Do measurements of serum AGEs predict the progression of microvascular complications? c) Can measurements of glycoxidation products in diabetes mellitus elucidate whether they play a role in diabetes?

Amadori-albumin, a major glycated protein, is involved in experimental hyperglycemia-induced microvascular complications (Chen *et al.* 2000, 2001) and is associated with advanced nephropathy in Type 1 diabetic humans. Recently, Schalkwijk *et al.* (2002) showed that Amadori albumin was associated with early nephropathy and with retinopathy status in Type 1 diabetic patients and preceded an increase in albumin excretion rate, but not in the development or progression of retinopathy.

Wolffenbuttel *et al.* (1996) have measured hemoglobin-AGE (Hb-AGE) in Type 2 diabetic patients before and after initial insulin treatment and found that Hb-AGE decreased in parallel according to blood glucose control. Hb-AGE did not correlate with HbA_1c and diabetic retinopathy or nephropathy (Turk *et al.* 1998). It seems that Hb-AGE represents only the metabolic status, equally in subjects with or without diabetic microangiopathy. Makita *et al.* (1992) showed that the presence of diabetic complications in Type 1 diabetic patients correlates with the elevated s-AGEs and low expression of mononuclear cell AGE-receptor-1. Recent immunological studies have shown that the levels of s-AGEs correlate with pre-clinical stages of nephropathy and early retinopathy, and may thus prove to be useful as early biochemical markers of microangiopathy in patients with diabetes mellitus (Beisswenger *et al.* 1995). The increased level of s-AGEs has been shown to predict the progression of morphological changes (basal membrane thickness, mesangial fraction) in the kidney (Berg *et al.* 1997a).

In our study, spectrofluorimetrically measured s-AGEs were found to be increased in poorly metabolically controlled children with Type 1 diabetes mellitus and without vascular complications (Jakuš *et al.* 2000, 2001). In Type 1 diabetes mellitus, only a significant correlation was found between s-AGEs and HbA_1c (Jakuš *et al.* 2001, Kalousová *et al.* 2002). Recent observations suggest that pathological processes leading to late diabetic complications in children begin even before puberty. The level of serum AGEs could be considered as a marker of later developments of diabetic vascular complications. Careful metabolic monitoring of young diabetics together with monitoring of s-AGEs represent the best prevention of AGE-related diabetic complications.

Significant elevation of glucose-derived s-AGEs measured by ELISA was associated with severe diabetic retinopathy in type 2 diabetic patients without renal dysfunction (Koga *et al.* 2002). However, no significant correlations were found here between serum levels of glucose-, glyceraldehyde-, or methylglyoxal-derived

s-AGEs and HbA_1c levels, systolic and diastolic pressure, the age, the duration of diabetes, serum creatinine or blood urea nitrogen levels in Type 2 diabetic patients.

Serum AGEs and serum CML (s-CML) have been found to be elevated in Type 1 (Berg *et al.* 1997a,b) and Type 2 diabetics (Kilhovd *et al.* 1998). However, spare data are available on the relationship between s-AGEs/s-CML and diabetic complications. CML is a dominant epitope in tissue-AGEs and s-AGEs. Possibly, the CML part or non-CML part in the serum might be relevant to diabetic complications. It has recently been shown that serum levels of another AGE product, pentosidine (s-pentosidine), is significantly higher in diabetic children (Type 1) despite normal renal function (Misselwitz *et al.* 2002). Serum AGEs and CML were also significantly elevated in children with chronic renal failure (CRF) and ESRD (Šebeková *et al.* 2001). Miura *et al.* (2003) showed that non-CML advanced glycation end-products are correlated to the severity of microvascular complications in patients with Type 1 diabetes.

AGEs can exert their immunogenicity. Turk *et al.* (2001) demonstrated the presence of AGE-immune complexes (AGE-IC) predominantly in the sera of Type 2 diabetic subjects. Interactions of AGE autoantibodies with AGEs as a continuously produced antigen result in the formation of AGE-IC complexes that may play role in atherogenic processes.

Recently, imidazolone has been identified *in vivo*. Specific immunoreactivity was detected in nodular lesions and expanded mesangial matrix of glomeruli as well as in atheromas (Niwa *et al.* 1997). This product of glycation, but not glycoxidation, may be a very important AGE product for the development of diabetic complications.

Taking into account the involvement of oxidative stress in the pathogenesis of diabetes, antioxidant or anti-AGE treatments are theoretically interesting proposals for adjunct therapy in diabetic patients.

Advanced glycation end-product inhibitors

Advanced glycation end-product inhibitors (AGEIs) appear to show beneficial effects against diabetic complications in tissues. It is becoming clear that these anti-AGE strategies play an important role in the treatment of diabetic patients. Hitherto, two possible approaches are available.

The first approach, concerns the inhibition of rearrangement from early to advanced glycation end-products by means of aminoguanidine, Schiff base resorcylideneaminoguanidine (Jakuš *et al.* 1999), an aminoguanidine pyridoxal Schiff base adduct (Miyoshi *et al.* 2002),pyridoxamine (Khalifah *et al.* 1999, Onorato *et al.* 2000, Degenhardt *et al.* 2002a,b, Stitt *et al.* 2002), thiamine pyrophosphate (Booth *et al.* 1996), OPB-9195, penicillamine (Jakuš *et al.* 1994a,b), ACE inhibitors (Jakuš *et al.* 1999, Miyata *et al.* 2002, Forbes *et al.* 2002) and other synthetic and natural compounds with antiglycation, chelation and antioxidant activity (Hrnčiarová *et al.* 1998, Price *et al.* 2001).

Unfortunately, clinical trials of aminoguanidine in diabetic patients have been suspended due to their adverse efffects. For example, aminoguanidine traps pyridoxal, so that its long-term administration in animals results in vitamin B_6 deficiency and neurotoxicity.

Pyridoxamine (Pyridorin^{TM}) is currently being investigated in phase 3 of clinical trials for the treatment of diabetic nephropathy and is now showing highly promising results (Degenhardt *et al.* 2002b). It is being reported that all doses of Pyridorin^{TM} are being well tolerated, with no serious adverse effects. Patients taking Pyridorin^{TM} exhibited a 32 % decrease from the baseline 24-hour urinary albumin excretion on day 45. Three out of 12 patients taking Pyridorin^{TM} (300 mg/day) became converted from macro- to microalbuminuria by the end of the trial.

The second approach concerns the breakdown of already existing AGEs with substituting thiazolium salts (Cooper *et al.* 2000). Intensive investigations of new compounds, which would break down already existing AGEs, have been recently started and tested *in vivo*.

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