

## Review Article

**Significance of Advanced Glycation End Products in Aging-Related Disease**Ryoji Nagai<sup>1)</sup>, Takefumi Mori<sup>2)</sup>, Yasuhiko Yamamoto<sup>3)</sup>, Yuichi Kaji<sup>4)</sup>, Yoshikazu Yonei<sup>5)</sup>

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**Abstract**

Carbohydrates are indispensable nutrients for life. However, due to the presence of a carbonyl group, reducing sugars such as glucose react non-enzymatically with amino groups on proteins in glycation (or Maillard) reactions. This reaction is divided into early and advanced phase reactions: the former covers the reaction progression up to the Amadori rearrangement, and the latter covers the reaction through the subsequent alterations of oxidation, dehydration, condensation, and so on, eventually generating advanced glycation end products (AGEs). Among post-translational modifications, processes such as phosphorylation or acetylation are known as "post-translational modifications with order," because the target proteins and the time of modification are controlled. However, glycation is a "post-translational modification with disorder" because post-translational modification of proteins by glucose progresses non-enzymatically, irreversibly, and at random, and because the progression depends on the existing time (aging) and the concentration of glucose, rather than the variety of proteins involved. While AGEs were initially considered simply as rust or waste matter in the body, significant attention is now afforded these compounds as a target molecule for newly-developing drugs, for the following reasons: AGEs damage the body by changing the structure and affecting the function proteins; receptors that recognizes AGEs, such as RAGE (receptor for AGE), exist in the body and mediate cellular derangements to induce morbidity; and inhibitors for AGE generation, such as aminoguanidine, pyridoxamine, and benfotiamine, delay the pathogenesis of diabetic nephropathy and retinopathy. Further, racemization proteins containing D-amino acids have been noted, along with AGEs, in drusen, which are characteristic of age-related macular degeneration (AMD). Here, we introduce current topics and future goals of AGEs research, particularly with regard to kidney disease associated with aging and diabetes mellitus, and touch on the involvement of AGEs in the progression of ocular diseases and the function of RAGE.

**KEY WORDS:** AGEs, RAGE, D-amino acids, diabetic nephropathy, age-related macular degeneration (AMD)**Introduction**

While nearly 100 years have passed since French researcher Louis Camille Maillard's discovery of the reaction associated with browning during heating, cooking, and storing food, the history of research on advanced glycation end products (AGEs) in the human body is surprisingly short. Hemoglobin A1c (HbA1c) is measured clinically as an index of blood glucose control and is an Amadori rearrangement product formed from the N-terminal valine residue of a hemoglobin  $\beta$  chain reacted with glucose, as clarified by research in the 1970s. In the decade following, further studies found that the material which showed fluorescence intensity (excitation: 370 nm, emission: 440 nm) was accumulated in collagen in the dura mater with aging<sup>1)</sup>. In addition, this fluorescence intensity was remarkably higher in patients with

diabetes mellitus than in healthy individuals. These findings have drawn attention to the progress of Maillard reactions in the human body.

However, given that a number of fluorescent materials are known to exist in the human body, a quantitative method involving only fluorescence intensity as the index cannot be said to specifically detect AGEs, and instead, measurement using anti-AGE antibodies has been found useful for evaluating multiple-specimen materials. Studies have also confirmed that AGEs accumulate in the kidneys with diabetic nephropathy and chronic renal failure, or in the blood vessels with atherosclerosis, indicating that presence of AGEs is closely related to aging or aging-related diseases.

## Metabolic networks and pathways of AGEs

During glycation, a non-enzyme reaction occurs in the early stages between the aldehyde group of reducing sugars and the amino residue of proteins, and a similar reaction has been found to occur with non-glucose materials containing an aldehyde group<sup>2)</sup>.

These production pathways involving non-glucose aldehydes that are able to modify proteins are divided into two categories: non-enzymatic and enzymatic production pathways. Non-enzymatic pathways include glyoxal, produced by auto-oxidation of glucose, and 3-deoxyglucosone (3DG), generated by hydrolysis from Amadori rearrangement products<sup>3,4)</sup>. Enzymatic pathways include the progression of myeloperoxidase, derived from inflammatory cells such as activated macrophages, to hypochlorite, which then reacts with serine to generate glycolaldehyde<sup>4)</sup>. Among other enzymatic pathways, methylglyoxal (MG) is generated by degradation of triosephosphate in the glycolytic system (Embden-Meyerhof pathway), and 3DG is produced by fructosamine-3-kinase from fructoselysine, an Amadori product (Fig. 1).

Blood MG concentration has been reported to be increased six-fold in patients with type I diabetes mellitus compared with normal individuals (two-fold in the corpus vitreum)<sup>5)</sup>. This finding suggests that production of highly reactive aldehydes, as mentioned above, may be elevated due to continuous hyperglycemic status associated with oxidative stress, inflammation, and disorders of glucose and lipid metabolism, subsequently resulting in rapid formation of AGEs from proteins in a short amount of time. In addition, aldehyde, glucosone, and glyoxal can also be generated by peroxynitrite (ONOO<sup>-</sup>), which is a reaction product of the known vasodilator nitric monoxide (NO) and a superoxide anion radical (O<sub>2</sub><sup>•-</sup>)<sup>6)</sup>. Since NO generation is controlled by NO synthase, this pathway is taken in the broad sense to be an enzymatic pathway for aldehyde generation (Fig. 2).

However, recent research has uncovered a new pathway for post-translational modification. In adipocytes, S-(2-succinyl) cysteine (2SC) is generated by reaction of fumaric acid, a Krebs cycle intermediate, with cysteine, and is formed from various

proteins such as cytoskeleton proteins, cytokines, heat shock protein<sup>7)</sup>, and adiponectin<sup>8)</sup>. These findings clearly indicate that AGE generation can differ greatly according to tissues involved and pathologic condition.

## AGEs and renal disease

Deterioration of renal function due to renal diseases, particularly chronic kidney disease, increases the prevalence of cardiovascular diseases such as cardiac infarction and stroke and is a risk factor for total death<sup>9,10)</sup>. Given that renal function declines gradually with age, the renal function of a 70-year-old is essentially similar to that of a chronic kidney disease patient<sup>11)</sup>. In addition to aging, primary glomerular diseases such as nephritis and lifestyle-related diseases such as hypertension or diabetes mellitus can also accelerate the deterioration of renal function.

Of particular, subjective symptoms of deterioration of renal function typically are evident only after function drops below 20% of normal levels, and renal function is in many cases irreparable by the point when patients consult their physician. While hemodialysis is available as a treatment even if the kidneys cease to function due to renal failure, sparing a patient from immediate death, the five-year survival rate is only 65%<sup>12)</sup>. Protection of renal function is therefore a major goal of anti-aging research.

Patients with chronic kidney disease and renal failure are known to have increased blood levels of carbonyl compounds generated from the glycolysis pathway, such as glyoxal, MG, and 3DG, and AGEs such as pentosidine<sup>13)</sup>. Because levels of these compounds become elevated in the stage of chronic kidney disease characterized by renal dysfunction, a decreased clearance of carbonyl compounds is suspected to be involved in this disease. In addition, blood levels of uric toxins are also increased in these

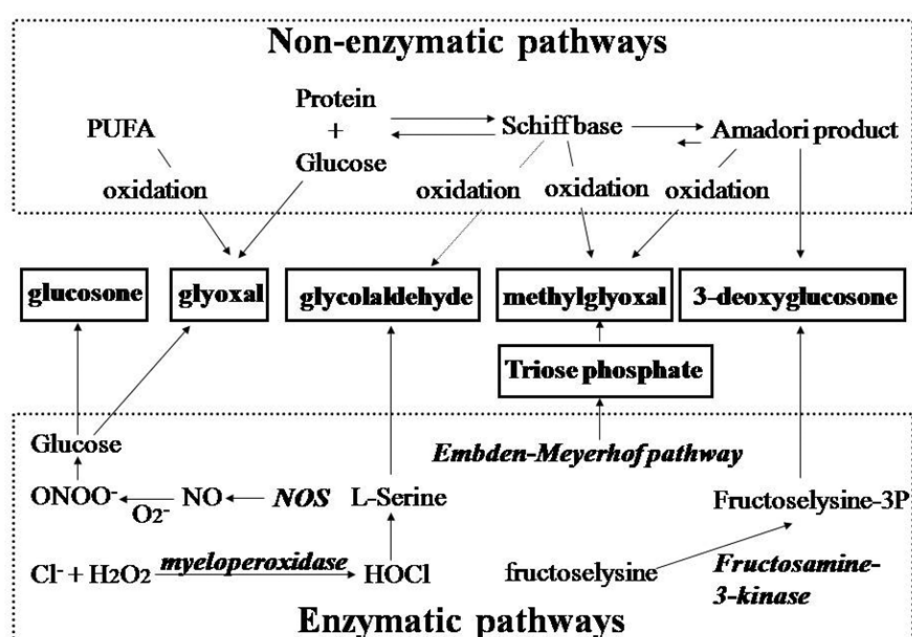


Fig. 1. Production pathway of AGEs generating aldehyde

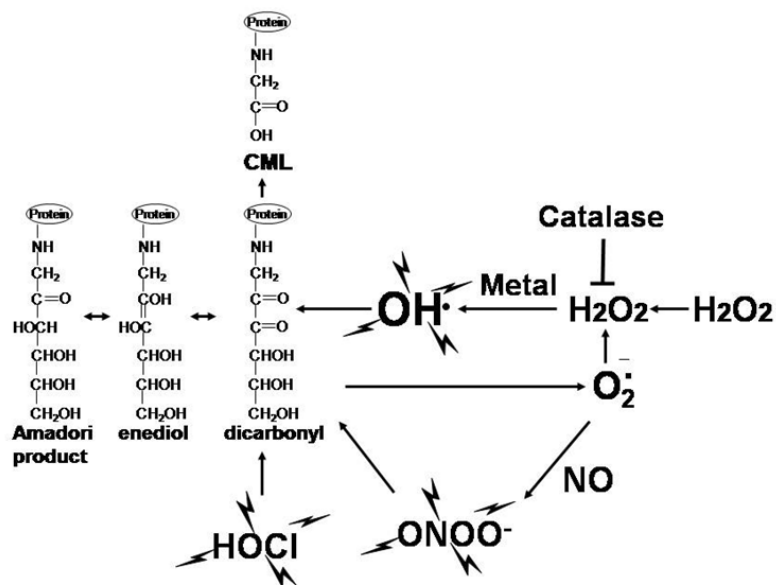


Fig. 2. CML generation accelerated by oxidative reaction

patients due to impaired clearance of toxins, indicating another potential mechanism involving enhanced production of carbonyl compounds stimulated by uremic toxin<sup>14</sup>.

Because levels of carbonyl compounds and AGEs are increased in patients with chronic kidney disease, these proteins are expected to be useful as biomarkers. Our laboratory at Tohoku University (Sendai-city, Miyagi) recently looked into the usefulness of MG and 3DG, measuring their levels in patients with diabetic nephropathy. We found that levels of MG and 3DG were significantly correlated with an increase in urinary albumin excretion over five years, another index of renal damage<sup>15</sup>. MG was also shown to be significantly correlated with increased intima-media thickness (IMT), an index of arteriosclerosis, over five years. We determined MG to be an independent risk factor for increased IMT and hypertension over five years, even after correction for age, blood pressure, BMI, and levels of triglyceride and HbA1c—correlations which were not noted for 3DG. Taken together, these findings indicate that levels of carbonyl compounds increase independently according to various disease conditions.

Carbonyl compounds and AGEs were not only found to be increased in patients with chronic kidney disease, but also involved in the pathogenesis of the disease itself. Cellular stress caused by these carbonyl compounds and AGEs is known as “carbonyl stress”<sup>16</sup>. When MG dissolved in drinking water was administered to rats to achieve a blood MG concentration equivalent to that of patients undergoing hemodialysis, salt-sensitive hypertension was observed. On immunostaining 3-nitrotyrosine as a marker for oxidative stress in the kidney, an obvious increase in immunostaining was observed in kidney samples after administering MG with high salt intake<sup>17</sup>. Since an increase in oxidative stress in the kidney has been found to inhibit urinary sodium excretion and thereby induce hypertension<sup>18</sup>, these results indicate that MG induces salt sensitive hypertension through enhanced renal oxidative stress. Given that MG is known to react with hydrogen peroxide to generate reactive oxygen species<sup>19</sup>, increased MG was expected to be accompanied by a slight increase in hydrogen peroxide induced by high salt intake, resulting in further oxidative stress in the kidney.

Oral administration of MG actually increased insulin resistance, as measured by the glucose clamp technique. The results suggest that both oxidative stress and carbonyl stress may be involved in the mechanism of insulin resistance, since the resistance was improved by administering N-acetylcysteine or anti-carbonyl agents<sup>17</sup>. These previous findings in animal experiments indicate that carbonyl stress is closely associated with the pathogenesis of chronic kidney disease, which is often accompanied by conditions such as salt-sensitive hypertension and high insulin resistance. However, the potential involvement of carbonyl stress in insulin resistance in humans remains unclear, requiring further investigation in future studies.

Results from a number of studies have determined the role of carbonyl stress and AGEs on the renal damage. In a transgenic mouse overexpressing the gene for receptor for advanced glycation end products (RAGE), renal dysfunction was observed at the same level as under diabetic conditions<sup>20</sup>. In animals overexpressing the gene for glyoxalase 1 (GLO1), a metabolizing enzyme of MG, renal dysfunction induced by ischemia and reperfusion was improved<sup>21</sup>. In our laboratory, we modeled chronic kidney disease using Dahl salt-sensitive hypertension rats, in which oxidative stress is elevated in the kidneys, and demonstrated that oral administration of MG with water induced hypertension without addition of salt and increased urinary excretion of albumin<sup>22</sup>. We also observed tubulo-glomerular injury in the kidneys, accompanied by elevated levels of N<sup>ε</sup>-(carboxyethyl)lysine (CEL), a marker of renal AGEs, and 8-hydroxy-guanosine (8-OHdG), a marker of oxidative stress. These changes were inhibited by the administration of an angiotensin II receptor blocker, indicating that the renin-angiotensin system is involved in hypertension and renal injury induced by MG.

Although carbonyl compounds and AGEs are known to be closely involved in the pathogenesis of chronic kidney disease, hypertension, and diabetes mellitus, as described above, data from human studies are still insufficient, and markedly few drugs currently being developed target carbonyl stress for treatment of these diseases. A clinical trial using pyridoxamine to treat diabetic

nephropathy is undergoing, with results expected soon. Pyridoxamine has been shown to inhibit renal dysfunction in non-diabetes kidney disease in animal models<sup>23</sup>, and pyridoxamine synthesized with the GMP standard in our laboratory has been used in clinical trials and confirmed to be safe for single or repeated, continuous administration in humans. In the future, we plan to examine the role of carbonyl compounds and AGEs in chronic kidney disease in humans using this pyridoxamine, and to use the results to develop a new drug targeting carbonyl compounds and AGEs for treating kidney disease.

## AGEs and ocular diseases

A number of ocular diseases are closely related to the formation of AGEs. Accumulation of proteins containing high levels of AGEs has been noted with diabetic complications such as retinopathy and keratopathy and age-related ocular alterations such as cataracts, pinguecula, spheroid degeneration, and age-related macular degeneration (AMD)<sup>24,25</sup>. In addition, D-amino acids produced by racemization<sup>26,27</sup>, which were not believed to exist *in vivo*, have been found coexisting with AGEs. Proteins containing high concentrations of AGEs and D-amino acids are unable to perform their originally intended function due to structural alterations. Ocular complications which occur with diabetes and age-related ocular diseases are commonly believed to be due to changes at the molecular and atomic levels, such as changes in amino acid composition in the eyes. Such complications can therefore be considered associated with deposition of proteins comprised of AGEs or D-amino acids generated by post-translational modification.

AMD is a leading cause of blindness in a number of advanced countries, and reducing the number of patients suffering from this disease will require clarification of its pathogenesis and development of prevention and treatment methods. In this section, we will discuss the pathogenesis of AMD, focusing in particular on AGEs and D-amino acids.

Symptoms of AMD vary in severity from negligible change without visual impairment to the final stage of blindness. In the early stages, abnormal accumulation of yellow-brownish-colored proteins, known as drusen, without angiogenesis can be noted in the macular area. Histological examination has shown that drusen are deposited between the retinal pigment epithelium and basal membrane. Proteome analysis of the protein contained in drusen has identified 129 kinds of proteins, including clusterin, TIMP3, and albumin<sup>28</sup>. Similarly, a proteome analysis of drusen in a *Macaca fascicularis* model of AMD identified proteins such as annexin V, clusterin, complement components, and vitronectin<sup>29</sup>. Drusen have shown to be comprised of accumulated proteins containing high levels of AGEs<sup>30</sup>. AGEs have also been observed in the hypertrophic area of Bruch's membrane in elderly subjects<sup>31</sup>. Drusen may thus be deposited not only on aging but also by hemorrhage and exudation, implying its involvement in the pathogenesis of AMD. Autoantibodies against AGEs are occasionally produced in the body, and accumulation of drusen in the macular region may thereby cause a local inflammatory reaction<sup>32</sup>.

AGEs formed in proteins are believed to exert activity by binding to a specific receptor, RAGE<sup>33-35</sup>. Immunohistochemical analyses have shown that RAGE localizes to a number of different points, including retinal pigment epithelium in the retina<sup>36,37</sup>, and

expression of RAGE increases on application of AGEs to this epithelium<sup>38</sup>. Coupled with the observation that drusen deposits between the retinal pigment epithelium and Bruch's membrane, a basal membrane, this finding indicates that interaction between AGE and AGE receptors occurs continuously in AMD, affecting the adjacent tissue<sup>36,39</sup>. Inflammatory reactions and angiogenesis are known to play a key role in AMD, and these changes may be due to continuous contact between the retinal pigment epithelium expressing RAGE and drusen abundantly containing AGEs.

However, results from a histochemical study using a polyclonal antibody against D-aspartic acid-containing protein<sup>40</sup> demonstrated the presence of proteins containing D-aspartic acid in the drusen and hypertrophic Bruch's membrane in elderly subjects<sup>24</sup>. At present, little is known regarding the precise biological role of D-amino acid-containing proteins. However, these proteins' structures differ greatly from those of L-amino acid-containing proteins due to the change in bond angle with the adjacent amino acid which occurs on chirality conversion<sup>26,27</sup>. Both AGEs and D-amino acids are known to perform key roles in age-related alterations<sup>26,27</sup>. While receptors to D-amino acid-containing proteins have yet to be identified, AGE formation in proteins and D-amino acid-containing proteins often coexist and are both known to be deeply involved in age-related alterations. Further clarification of how D-amino acid-containing proteins are involved in the pathogenesis of AMD is therefore necessary.

## Role of RAGE in aging-related diseases

Two pathways have been proposed for glycation-induced cellular and tissue derangement: direct damage by protein modification or structural alteration of secretion, membrane, and intracellular proteins and the extracellular matrix; and damage mediated by cell-surface receptors which recognize AGEs as ligands. At present, the following cell-surface proteins and receptors which can bind AGEs have been identified: RAGE; macrophage type-I and type-II class A scavenger receptors (MSR-A); class B scavenger receptor family members of CD36, SR-BI, and lectin-like oxidized low density lipoprotein receptor-1 (LOX-1); galectin-3 complex; fasciclin, EGF-like, laminin-type EGF-like, and link domain-containing scavenger receptor-1,2 (FEEL1,2); megalin; and toll-like receptor (TLR) 4. Of these, RAGE is well-known as a functional receptor for AGEs, causing cellular responses via intracellular RAGE signaling. AGE-binding proteins and receptors other than RAGE may also be involved in cellular and tissue damages by influencing a systemic or local accumulation or clearance of AGEs.

RAGE belongs to an immunoglobulin super-family with a single membrane-spanning domain and was originally isolated from bovine lung samples and identified as a cell-surface receptor able to bind AGEs<sup>41</sup>. RAGE has been found expressed in a wide variety of cells and tissues, including lung alveolar epithelial cells, vascular cells, and immune cells. Under physiologically healthy conditions, expression of RAGE is generally low in organs and tissues, except for lungs. However, expression is known to be increased in pathological lesions such as atheromatous plaques where AGEs have accumulated.

A representative intracellular signaling pathway of RAGE involves activation of the transcription factor NFκB through generation of intracellular oxidative stress and the ras/MAP kinase pathway. In vascular endothelial cells, RAGE signaling caused by AGEs is able to induce gene expression of vascular endothelial



growth factor (VEGF) and vascular cell adhesion molecule-1 (VCAM-1), leading to enhancement of vascular permeability and angiogenesis and to localized inflammation, respectively<sup>42)</sup>. Secretion of various cytokines such as tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), interleukin 1 $\beta$  (IL1 $\beta$ ), IL6, and monocyte chemoattractant protein-1 (MCP-1) have been found to be induced by AGE-RAGE signaling in monocytes and macrophages, and RAGE promoter assays have shown that AGE-RAGE signaling itself promotes transcriptional upregulation of the RAGE gene by activating NF $\kappa$ B<sup>43)</sup>. TNF $\alpha$  and estrogen also enhanced transcription of the RAGE gene by activating NF $\kappa$ B and the transcription factor Sp1, respectively<sup>43)</sup>.

Recently, a mammalian homologue of the *Drosophila* gene Diaphanous 1 (mDial) has been identified as a direct binding molecule with an intracellular domain for RAGE as a part of the machinery involved in RAGE intracellular signaling<sup>44)</sup>. As a Formin homology protein, mDial exists across a wide range of species, from yeast to mammals, and is known to be related to cell division, polarity formation, and movement by actin polymerization. However, the molecular pathways and machinery involved in RAGE intracellular signaling are not yet fully understood, and further studies will be required to clarify the molecular network of RAGE signaling.

### ***AGEs associated with RAGE which elicit RAGE signaling***

In addition to N $^{\epsilon}$ -(carboxyl)methyllysine (CML) and AGEs generated from glyceraldehyde or glycolaldehyde, which have been found to bind to RAGE<sup>45)</sup>, other RAGE ligands have also been identified, including advanced oxidation protein products (AOPP) generated by oxidative stress, amyloid  $\beta$  protein (seen in the brain with Alzheimer's disease), transthyretin associated with familial amyloid polyneuropathy, high-mobility group B-1 (HMGB-1)/amphoterin and S100 proteins as inflammatory mediators secreted from immune cells, and leukocyte cell surface Mac1. With its multi-ligand receptor, RAGE is thus implicated in the pathogenesis of a number of diseases and is now recognized as a pattern-recognition receptor (PRR), similar to toll-like receptors.

To investigate the *in vivo* functional role of RAGE in the development of diabetic vascular diseases, RAGE transgenic mice overexpressing RAGE protein in vascular endothelial cells were created, and then crossbred with insulin-dependent diabetic mice. The resultant diabetic transgenic mice showed advanced diabetic nephropathy<sup>20)</sup>. In addition, disruption of the *RAGE* gene was found to ameliorate progression of diabetic nephropathy when compared with wild-type diabetic control mice<sup>46)</sup>. These findings clearly show that RAGE is functionally involved in the pathogenesis and progression of diabetic nephropathy, presenting this molecule as a potential target for prophylaxis and treatment of diabetic vascular complications.

Structural variation of RAGE, *i.e.* isoforms, has been recently reported, complicating the pathogenesis of RAGE-related diseases. RAGE isoforms are produced by alternative splicing of RAGE pre-mRNA and further modified by enzymatic proteolysis: ectodomain shedding of RAGE generates soluble RAGE (sRAGE). Another sRAGE isoform, produced by alternative splicing, which lacks a C-terminal transmembrane and an intracellular domain of full-length membrane-bound RAGE was named endogenous secretory RAGE (esRAGE)<sup>47)</sup>. esRAGE has a ligand-binding site and can capture RAGE ligands such as AGEs, thereby functioning

as a decoy receptor by inhibiting the interaction of the cell surface RAGE with the ligands. esRAGE circulates in human blood and can be detected in various organs and tissues by immunohistochemistry<sup>48)</sup>, and the mouse orthologue of human esRAGE has also been identified<sup>49)</sup>.

To clarify the functional roles of esRAGE in the pathogenesis of various diseases, our laboratory at Kanazawa University (Kanazawa-city, Ishikawa) and collaborators have established a sandwich ELISA system (esRAGE ELISA; B-Bridge, Daiichi Fine Chemical Co., Ltd, Takaoka-city, Toyama) that is able to specifically measure human esRAGE levels<sup>50)</sup>. In cases with low serum levels of esRAGE, the prevalence of metabolic syndrome<sup>51)</sup>, atherosclerosis, and diabetic retinopathy<sup>50)</sup> tends to be high, suggesting that esRAGE may be a novel biomarker and potential protective factor against these diseases. However, care should be taken when analyzing data from patients with nephropathy, since serum esRAGE level is strongly influenced by kidney dysfunction (increased serum creatinine and decreased GFR)<sup>52)</sup>.

In addition, sRAGE is produced by the ectodomain shedding from full-length membrane-bound RAGE (signal transducer) via activation of matrix metalloproteinase (MMP) 9 or a disintegrin and metalloproteinase (ADAM) 10. Reinforcing this ectodomain shedding will decrease the total amount and expression of full-length membrane-bound RAGE, in turn increasing the amount of sRAGE working as a decoy receptor. This process can modify AGEs-RAGE signaling and subsequent cellular and tissue derangement. Further clarification of this mechanism will be required to develop new methods of controlling RAGE ectodomain shedding (Fig. 3).

Suppression of RAGE action may be useful in preventing or at least slowing aging and development of various diseases such as diabetic vascular complications, atherosclerosis, cancer, and inflammation. Potential candidate compounds to achieve this suppression include inhibitors of expression of full-length membrane-bound RAGE, RAGE-specific antagonists, blockers against RAGE intracellular signaling, enhancers of esRAGE production, supplementation of esRAGE or sRAGE, and inducers of RAGE shedding. Angiotensin-converting enzyme inhibitor, thiazolidine, and statin (hydroxymethylglutaryl-CoA reductase inhibitor) have been reported to stimulate esRAGE secretion (53,54). In addition, low-molecular-weight heparin, depolymerized and fractionated from heparin, has been shown to work as a RAGE antagonist by inhibiting AGE-RAGE signaling<sup>46)</sup>. TTP488 is a specific RAGE antagonist presently under clinical trials for treating Alzheimer's disease and diabetic nephropathy in the United States, with encouraging results anticipated.

### ***Current known and in-development AGE generation inhibitors***

Given the well-documented involvement of AGEs in the pathogenesis of aging-related diseases, as described above, a number of AGE generation inhibitors are being developed around the globe to address these conditions. Methods for prevention and treatment of human AGE-related disorders are mainly classified as follows: inhibition of AGE generation, degradation of already-generated AGEs (AGE breakers)<sup>55,56)</sup>, and competitive inhibition of RAGE<sup>57)</sup>. In this section, we will discuss the AGE generation inhibitors for which the research in humans is the most advanced.

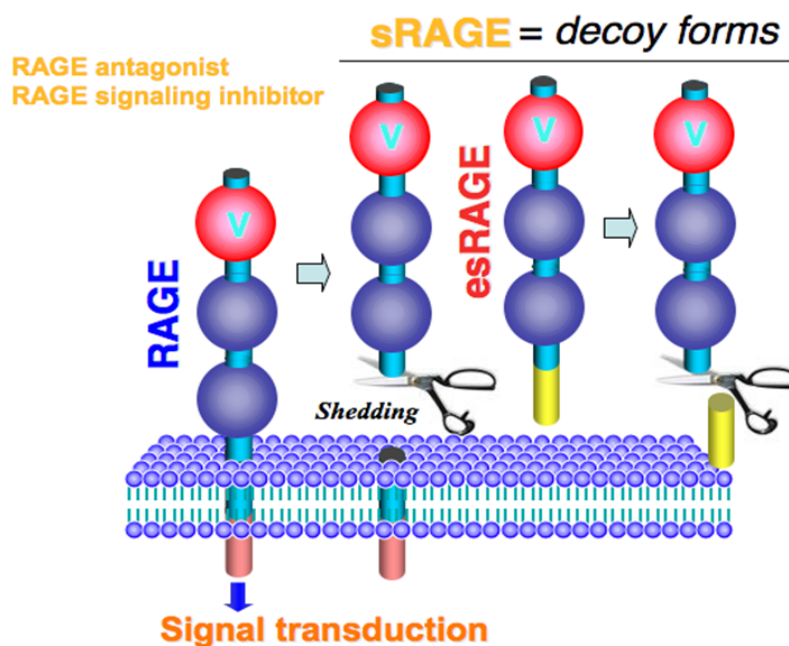


Fig. 3. Strategies for blocking RAGE signaling. esRAGE, endogenous secretory RAGE.

Methods of inhibition of AGE generation include trapping the aldehyde generated by a Maillard reaction during metabolism, and inhibition of oxidation using metal-chelating agents, for example, since cross-linkage formation of proteins by Maillard reaction or formation of AGEs such as CML and pentosidine tends to accelerate under oxidative conditions<sup>58</sup>. Aminoguanidine is the first AGE inhibitor with an amino residue which traps the aldehyde group of reducing sugars<sup>59</sup>. This compound has reduced prevalence of nephropathy and retinopathy in animal models of diabetes mellitus. In addition, a US team in 1999 reported that aminoguanidine significantly reduced prevalence of proteinuria in patients with diabetic nephropathy in a phase III trial, although no significant reduction in serum creatinine was achieved. Similarly, the vitamin B6 derivative pyridoxamine has been found capable of trapping an aldehyde group via its amino group and is also known to inhibit formation of AGEs, as well as lipid peroxide. Similarly to findings with aminoguanidine, pyridoxamine has been shown to significantly inhibit the progress of nephropathy<sup>60</sup> and retinopathy<sup>61</sup>, although the serum glucose concentration was not changed in a rat model of streptozotocin-induced diabetes. Clinical trials for these compounds are presently under way in a number of countries. Administration of thiamin or benfotiamine, a hydrophobic derivative of thiamin, has been shown to induce expression of transketolase in renal mesangium cells and to reduce AGE content in those cells, thereby improving microalbuminuria in diabetic rats<sup>62</sup>. This finding indicates that AGE formation in cells under hyperglycemic conditions may be involved in the pathogenesis of diabetic nephropathy. These previous findings indicate the usefulness of AGEs as target molecules for in-development drugs for treating diabetic complications, with still more effective medicines expected after further analyzing the mechanism of AGE generation in humans at a molecular level in the future.

AGE detection using antibodies is more effective than instrumental analysis, as previously described, facilitating detection of AGEs in the body and identification of inhibitors against AGE generation. Our laboratory at Japan Women's

University has confirmed high correlation in measured values of CML and pentosidine using ELISA and high-performance liquid chromatography (HPLC). We screened *Astragalus radix* samples for the natural herb-derived compound that inhibits generation of CML and pentosidine from the reaction of bovine serum albumin and ribose. Our findings confirmed that astragaloside significantly inhibits both of these AGEs<sup>63</sup>, although the inhibitory effect of the compound has not yet been confirmed *in vivo*. A mixture of herbal extracts from chamomile (*Anthemis nobilis*), hawthorn berry (*Crataegus oxyacantha*), doku-dami (*Houttuynia cordata*), and grape leaf (*Vitis vinifera*) has been found effective in inhibiting formation of AGEs<sup>64,65</sup>.

Given that a large number of AGEs are generated by oxidizing reactions, it is generally believed that antioxidant flavonoids can inhibit AGEs generation. However, high concentrations of flavonoids such as catechins promote production of hydrogen peroxide<sup>66</sup>, and therefore, excessive intake of flavonoids may actually enhance generation of AGEs<sup>67</sup>. Using the anti-AGE antibody library, we recently demonstrated the possibility that CEL is generated from ketone bodies and identified a new pathway of CEL generation, involving reacting proteins with acetol generated from acetone by acetone monooxygenase. We further hypothesized that levels of ketone bodies might be decreased by oral administration of citric acid, which plays a role in the TCA cycle. When citric acid was orally administered to streptozotocin-induced diabetic rats, development of cataracts and deterioration of renal function was significantly inhibited on reduction of ketone body generation, and CEL accumulation decreased in the crystalline lens. This effect was confirmed to be similar to that achieved with insulin injection<sup>68</sup>.

Diethylenetriaminepentaacetic acid (DTPA), a metal-chelating agent, inhibits CEL generation from acetol, indicating that oxidation is essential in this reaction. However, citric acid, known to possess metal-chelating abilities, unexpectedly did not inhibit CEL generation from acetol, possibly due to the chelating ability of citric acid being weaker than that of DTPA. We ultimately

found that, while it did not directly inhibit CEL formation, citric acid significantly inhibited CEL generation in the crystalline lens of rats, an *in vivo* effect which may be due to indirect activity to reduce ketone body generation. It should be noted, however, that citric acid's inhibitory effect against ketone body generation is still uncertain. Blood levels of ketones are elevated in patients with type I diabetes as well as those experiencing morning sickness during pregnancy and patients who engage in excessive exercise or acute dieting. Citric acid, which is abundant in many fruits, may indeed be useful in preventing diabetic complications or other diseases. Easier detection of AGEs will greatly facilitate our search for AGE generation inhibitors.

## References

- Monnier VM, Kohn RR, Cerami A: Accelerated age-related browning of human collagen in diabetes mellitus *Proc Natl Acad Sci U S A* 81; 583-587: 1984
- Mera K, Takeo K, Izumi M, et al: Effect of reactive-aldehydes on the modification and dysfunction of human serum albumin. *J Pharm Sci* 99; 1614-1625: 2010
- Nagai R, Matsumoto K, Ling X, et al: Glycolaldehyde, a reactive intermediate for advanced glycation endproducts, plays an important role in the generation of an active ligand for the macrophage scavenger receptor. *Diabetes* 49; 1714-1723: 2000
- Nagai R, Hayashi CM, Xia L, et al: Identification in human atherosclerotic lesions of GA-pyridine, a novel structure derived from glycolaldehyde-modified proteins. *J Biol Chem* 277; 48905-48912: 2002
- McLellan AC: Glyoxalase system in clinical diabetes mellitus and correlation with diabetic complications. *Clin Sci (London)* 87; 21-29: 1994
- Nagai R, Unno Y, Hayashi MC, et al: Peroxynitrite induces formation of *N*<sup>ε</sup>-(carboxymethyl) lysine by the cleavage of Amadori product and generation of glucosone and glyoxal from glucose: novel pathways for protein modification by peroxynitrite. *Diabetes* 51; 2833-2839: 2002
- Nagai R, Brock JW, Blatnik M, et al: Succination of protein thiols during adipocyte maturation - a biomarker of mitochondrial stress. *J Biol Chem* 282; 34219-34228: 2007
- Frizzell N, Rajesh M, Jepson MJ, et al: Succination of thiol groups in adipose tissue proteins in diabetes: Succination inhibits polymerization and secretion of adiponectin. *J Biol Chem* 284; 25772-25781: 2009
- Go AS, Chertow GM, Fan D, et al: Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. *N Engl J Med* 351; 1296-1305: 2004
- Nakayama M, Sato T, Sato H, et al: Different clinical outcomes for cardiovascular events and mortality in chronic kidney disease according to underlying renal disease: the Gonryo study. *Clin Exp Nephrol* 14; 333-339: 2010
- Weinstein JR, Anderson S: The aging kidney: physiological changes. *Adv Chronic Kidney Dis* 17; 302-307: 2010
- Held PJ, Pauly MV, Diamond L: Survival analysis of patients undergoing dialysis. *JAMA* 257; 645-650: 1987
- Nakayama K, Nakayama M, Iwabuchi M, et al: Plasma alpha-oxoaldehyde levels in diabetic and nondiabetic chronic kidney disease patients. *Am J Nephrol* 28; 871-878: 2008
- Kakuta T, Tanaka R, Satoh Y, et al: Pyridoxamine improves functional, structural, and biochemical alterations of peritoneal membranes in uremic peritoneal dialysis rats. *Kidney Int* 68; 1326-1336: 2005
- Ogawa S, Nakayama K, Nakayama M, et al: Methylglyoxal is a predictor in type 2 diabetic patients of intima-media thickening and elevation of blood pressure. *Hypertension* 56; 471-476: 2010
- Miyata T, Ishikawa N, van Ypersele de Strihou C: Carbonyl stress and diabetic complications. *Clin Chem Lab Med* 41; 1150-1158: 2003
- Guo Q, Mori T, Jiang Y, et al: Methylglyoxal contributes to the development insulin resistance and salt sensitivity in Sprague-Dawley rats. *J Hypertens* 27; 1664-1671: 2009
- Mori T, Cowley AW Jr, Ito S: Molecular mechanisms and therapeutic strategies of chronic renal injury: physiological role of angiotensin II-induced oxidative stress in renal medulla. *J Pharmacol Sci* 100; 2-8: 2006
- Nakayama M, Saito K, Sato E, et al: Radical generation by the non-enzymatic reaction of methylglyoxal and hydrogen peroxide. *Redox Rep* 12; 125-133: 2007
- Yamamoto Y, Kato I, Doi T, et al: Development and prevention of advanced diabetic nephropathy in RAGE-overexpressing mice. *J Clin Invest* 108; 261-268: 2001
- Kumagai T, Nangaku M, Kojima I, et al: Glyoxalase I overexpression ameliorates renal ischemia-reperfusion injury in rats. *Am J Physiol Renal Physiol* 296; F912-921: 2009
- Mori T, Chen X, Hu C, et al: Methylglyoxal induced hypertension with angiotensin II mediated pathway in Dahl salt sensitive rats. *J Hypertens* 26; S524: 2008 (abstract)
- Alderson NL, Chachich ME, Youssef NN, et al: The AGE inhibitor pyridoxamine inhibits lipemia and development of renal and vascular disease in Zucker obese rats. *Kidney Int* 63; 2123-2133: 2003
- Kaji Y, Oshika T, Takazawa Y, et al: Localization of D-beta-aspartic acid-containing proteins in human eyes. *Invest Ophthalmol Vis Sci* 48; 3923-3927: 2007
- Kaji Y: Ocular diseases caused by accumulation of proteins with post-translational modifications. *Nippon Ganka Gakkai Zasshi* 113; 424-442: 2009 (in Japanese)
- Fujii N: D-amino acid in elderly tissues. *Biol Pharm Bull* 28; 1585-1589: 2005
- Fujii N, Saito T: Homochirality and life. *Chem Rec* 4; 267-278: 2004
- Crabb JW, Miyagi M, Gu X, et al: Drusen proteome analysis: an approach to the etiology of age-related macular degeneration. *Proc Natl Acad Sci U S A* 99; 14682-14687: 2002
- Umeda S, Suzuki MT, Okamoto H, et al: Molecular composition of drusen and possible involvement of anti-retinal autoimmunity in two different forms of macular degeneration in cynomolgus monkey (*Macaca fascicularis*). *FASEB J* 19; 1683-1685: 2005
- Ishibashi T, Murata T, Hangai M, et al: Advanced glycation end products in age-related macular degeneration. *Arch Ophthalmol* 116; 1629-1632: 1998
- Handa JT, Verzijl N, Matsunaga H, et al: Increase in the advanced glycation end product pentosidine in Bruch's membrane with age. *Invest Ophthalmol Vis Sci* 40; 775-779: 1999
- Shibayama R, Araki N, Nagai R, et al: Autoantibody against *N*<sup>ε</sup>-(carboxymethyl)lysine: an advanced glycation end product of the Maillard reaction. *Diabetes* 48; 1842-1849: 1999
- Schmidt AM, Hasu M, Popov D, et al: Receptor for advanced glycation end products (AGEs) has a central role in vessel wall interactions and gene activation in response to circulating AGE proteins. *Proc Natl Acad Sci U S A* 91; 8807-8811: 1994

- 34) Schmidt AM, Mora R, Cao R, et al: The endothelial cell binding site for advanced glycation end products consists of a complex: an integral membrane protein and a lactoferrin-like polypeptide. *J Biol Chem* 269; 9882-9888: 1994
- 35) Yamamoto H, Watanabe T, Yamamoto Y, et al: RAGE in diabetic nephropathy. *Curr Mol Med* 7; 752-757: 2007
- 36) Yamada Y, Ishibashi K, Bhutto IA, et al: The expression of advanced glycation endproduct receptors in rpe cells associated with basal deposits in human maculas. *Exp Eye Res* 82; 840-848: 2006
- 37) Hammes HP, Hoerauf H, Alt A, et al: N<sup>ε</sup>-(carboxymethyl)lysine and the AGE receptor RAGE colocalize in age-related macular degeneration. *Invest Ophthalmol Vis Sci* 40; 1855-1859: 1999
- 38) Zhou J, Cai B, Jang YP, et al: Mechanisms for the induction of HNE-MDA- and AGE-adducts, RAGE and VEGF in retinal pigment epithelial cells. *Exp Eye Res* 80; 567-580: 2005
- 39) Barile GR, Schmidt AM: RAGE and its ligands in retinal disease. *Curr Mol Med* 7; 758-765: 2007
- 40) Fujii N, Awakura M, Takemoto L, et al: Characterization of alphaA-crystallin from high molecular weight aggregates in the normal human lens. *Mol Vis* 9; 315-322: 2003
- 41) Neeper M, Schmidt AM, Brett J, et al: Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. *J Biol Chem* 267; 14998-15004: 1992
- 42) amagishi S, Yonekura H, Yamamoto Y, et al: Advanced glycation end products-driven angiogenesis *in vitro*. Induction of the growth and tube formation of human microvascular endothelial cells through autocrine vascular endothelial growth factor. *J Biol Chem* 272; 8723-8730: 1997
- 43) Tanaka N, Yonekura H, Yamagishi S, et al: The receptor for advanced glycation end products is induced by the glycation products themselves and tumor necrosis factor-alpha through nuclear factor-kappa B, and by 17 beta-estradiol through Sp-1 in human vascular endothelial cells. *J Biol Chem* 275; 25781-25900: 2000
- 44) Hudson BI, Kalea AZ, Del Mar Arriero M, et al: Interaction of the RAGE cytoplasmic domain with diaphanous-1 is required for ligand-stimulated cellular migration through activation of Rac1 and Cdc42. *J Biol Chem* 283; 34457-34468: 2008
- 45) Yamamoto Y, Yonekura H, Watanabe T, et al: Short-chain aldehyde-derived ligands for RAGE and their actions on endothelial cells. *Diabetes Res Clin Pract* 77; S30-40: 2007
- 46) Myint K.M., Yamamoto Y., Doi T, et al: RAGE control of diabetic nephropathy in a mouse model: effects of RAGE gene disruption and administration of low-molecular weight heparin. *Diabetes* 55; 2510-2522: 2006
- 47) Yonekura H, Yamamoto Y, Sakurai S, et al: Novel splice variants of the receptor for advanced glycation end-products expressed in human vascular endothelial cells and pericytes, and their putative roles in diabetes-induced vascular injury. *Biochem J* 370; 1097-1109: 2003
- 48) Cheng C, Tsuneyama K, Kominami R, et al: Expression profiling of endogenous secretory receptor for advanced glycation end products in human organs. *Mod Pathol* 18; 1385-1396: 2005
- 49) Harashima A, Yamamoto Y, Cheng C, et al: Identification of mouse orthologue of endogenous secretory receptor for advanced glycation end-products: structure, function and expression. *Biochem J* 396; 109-115: 2006
- 50) Sakurai S, Yamamoto Y, Tamei H, et al: Development of an ELISA for esRAGE and its application to type 1 diabetic patients. *Diabetes Res Clin Pract* 73; 158-165: 2006
- 51) Koyama H, Shoji T, Yokoyama H, et al: Plasma level of endogenous secretory RAGE is associated with components of the metabolic syndrome and atherosclerosis. *Arterioscler Thromb Vasc Biol* 25; 2587-2593: 2005
- 52) Yamamoto Y, Miura J, Sakurai S, et al: Assaying soluble forms of receptor for advanced glycation endproducts. *Arterioscler Thromb Vasc Biol* 27; e33: 2007
- 53) Forbes JM, Thorpe SR, Thallas-Bonke V, et al: Modulation of soluble receptor for advanced glycation end products by angiotensin-converting enzyme-1 inhibition in diabetic nephropathy. *J Am Soc Nephrol* 16; 2363-2372: 2005
- 54) Tam HL, Shiu SW, Wong Y, et al: Effects of atorvastatin on serum soluble receptors for advanced glycation end-products in type 2 diabetes. *Atherosclerosis* 209; 173-177: 2010
- 55) Vasani S, Zhang X, Zhang X, et al: An agent cleaving glucose-derived protein crosslinks *in vitro* and *in vivo*. *Nature* 382; 275-278: 1996
- 56) Yang S, Litchfield JE, Baynes JW: AGE-breakers cleave model compounds, but do not break Maillard crosslinks in skin and tail collagen from diabetic rats. *Arch Biochem Biophys* 412; 42-46: 2003
- 57) Bucciarrelli LG, Wendt T, Qu W, et al: RAGE blockade stabilizes established atherosclerosis in diabetic apolipoprotein E-null mice. *Circulation* 106; 2827-2835: 2002
- 58) Fu MX, Wells-Knecht KJ, Blackledge JA, et al: Glycation, glycooxidation, and cross-linking of collagen by glucose. Kinetics, mechanisms, and inhibition of late stages of the Maillard reaction. *Diabetes* 43; 676-683: 1994
- 59) Brownlee M, Vlassara H, Kooney A, et al: Aminoguanidine prevents diabetes-induced arterial wall protein cross-linking. *Science* 232; 1629-1632: 1986
- 60) Degenhardt TP, Alderson NL, Arrington DD, et al: Pyridoxamine inhibits early renal disease and dyslipidemia in the streptozotocin-diabetic rat. *Kidney Int* 61; 939-950: 2002
- 61) Stitt A, Gardiner TA, Alderson NL, et al: The AGE inhibitor pyridoxamine inhibits development of retinopathy in experimental diabetes. *Diabetes* 51; 2826-2832: 2002
- 62) Babaei-Jadidi R, Karachalias N, Ahmed N, et al: Prevention of incipient diabetic nephropathy by high-dose thiamine and benfotiamine. *Diabetes* 52; 2110-2120: 2003
- 63) Motomura K, Fujiwara Y, Kiyota N, et al: Astragalosides isolated from the root of *Astragalus radix* inhibits the formation of advanced glycation end-products. *J Agric Food Chem* 57; 7666-7672: 2009
- 64) Yonei Y, Yagi M, Hibino S, et al: Herbal extracts inhibit Maillard reaction, and reduce chronic diabetic complications risk in streptozotocin-induced diabetic rats. *Anti-Aging Medicine* 5; 93-98: 2008
- 65) Yonei Y, Miyazaki R, Takahashi Y, et al: Anti-glycation effect of mixed herbal extract in individuals with pre-diabetes mellitus: a double-blind, placebo-controlled, parallel group study. *Anti-Aging Medicine* 7; 26-35: 2010
- 66) Akagawa M, Shigemitsu T, Suyama K: Production of hydrogen peroxide by polyphenols and polyphenol-rich beverages under quasi-physiological conditions. *Biosci Biotechnol Biochem* 67; 2632-2640: 2003
- 67) Fujiwara Y, Kiyota N, Motomura K, et al: Some natural compounds enhance CML formation. *Ann N Y Acad Sci* 1126; 152-154: 2008
- 68) Nagai R, Nagai M, Shimasaki S, et al: Citric acid inhibits development of cataracts, proteinuria and ketosis in streptozotocin (type1) diabetic rats. *Biochem Biophys Res Commun* 393; 118-122: 2010