J Am Soc Nephrol 14: S259–S263, 2003

The AGE-RAGE System and Diabetic Nephropathy

SHIGERU SAKURAI, HIDETO YONEKURA, YASUHIKO YAMAMOTO, TAKUO WATANABE, NOBUSHIGE TANAKA,* HUI LI, A.K.M. AZADUR RAHMAN, KHIN-MAR MYINT, CHUL-HEE KIM, and HIROSHI YAMAMOTO

*Department of Biochemistry and Molecular Vascular Biology, and *Department of Ophthalmology, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan.*

Abstract. As is diabetes itself, diabetic vasculopathy is a multifactor disease. Studies revealed advanced glycation end products (AGE) as the major environmental account for vascular cell derangement characteristic of diabetes and the receptor for AGE (RAGE) as the major genic factor that responds to them. AGE fractions that caused the vascular derangement were proved to be RAGE ligands. When made diabetic, RAGE transgenic mice exhibited the exacerbation of the indices of nephropathy and retinopathy, and this was prevented by the inhibition of AGE formation. Extracellular signals and nuclear factors that induce the transcription of human RAGE gene were also identified, which would be regarded as risk factors of diabetic complications. Through an analysis of vascular polysomal poly(A)$^+$RNA, a novel splice variant coding for a soluble RAGE protein was found and was named endogenous secretory RAGE. Endogenous secretory RAGE was able to capture AGE ligands and to neutralize the AGE action on endothelial cells, suggesting that this variant has a potential to protect blood vessels from diabetes-induced injury. The AGE-RAGE system, therefore, should be a candidate molecular target for overcoming this life- and quality-of-life–threatening disease.

In 1912, Maillard (1) reported the generation of brown-colored substances by a nonenzymatic reaction between reducing sugars and amino acids. It begins with linkage between the carbonyl group and the amino group to form Schiff bases and then Amadori compounds, finally yielding irreversibly cross-linked products termed "mélanoïdine." The series of the chemical reactions was named after the discoverer and has been one of the major themes in food chemistry, because melanoidines constitute an essential component of colors, odors, and tastes of a wide variety of foods. The Maillard reaction, however, does not occur merely on kitchen ranges. In 1981, Monnier and Cerami (2) documented that it can take place within our bodies. Because it proceeds as we age, the final products were then termed "AGE." The AGE formation and accumulation are most accelerated under diabetes.

The major sources of the carbonyl group in the glycation reaction *in vivo* include glucose and carbonyl compounds, such as glyceraldehyde, glyoxal, glycolaldehyde, methylglyoxal, and 3-deoxyglucosone, which are derived from glucose, Schiff bases, and Amadori compounds (3) (Figure 1). Long half-lived proteins, such as serum albumin, lens crystallin, and collagen in the extracellular matrix, are akin to be glycated.

Correspondence to Dr. Hiroshi Yamamoto, Department of Biochemistry and Molecular Vascular Biology, Division of Cardiovascular Medicine, Kanazawa University Graduate School of Medical Science, 13-1 Takara-machi, Kanazawa 920-8640, Japan. Phone: 81-76-265-2180; Fax: 81-76-234-4226; E-mail: yamamoto@med.kanazawa-u.ac.jp

1046-6673/1408-0259
Journal of the American Society of Nephrology

DOI: 10.1097/01.ASN.0000077414.59717.74

AGE Actions on Vascular Cells

Microvessels, which are first deranged in diabetic retinopathy and nephropathy, are composed of endothelial cells (EC) and pericytes. By co-culture experiments, Yamagishi *et al.* (4,5) showed that pericytes not only regulate the growth of neighboring EC but also preserve EC-specific functions, including the production of prostacyclin, an antithrombogenic prostanoid. This indicates that when the pericyte–EC interaction is impaired, angiogenesis and thrombogenesis should result. Such a state does occur in the early phase of retinopathy and is known as "pericyte loss." It was AGE that we noticed as a cause of pericyte loss. We prepared AGE-BSA by incubating BSA with glucose and administered the resultant material to bovine retinal pericytes in culture. AGE were found to retard the growth of pericytes and also to exert an acute toxicity to this cell type (6). Yonekura *et al.* (7) also prepared AGE-BSA with glyceraldehydes, glyoxal, glycolaldehyde, methylglyoxal, and 3-deoxyglucosone and examined their effects on pericytes. Among those AGE fractions, glyceraldehyde- and glycolaldehyde-derived AGE strongly retarded the pericyte growth.

AGE also act on EC. Contrasting with the case of pericytes, AGE-BSA supershifted upward the growth curve of human microvascular EC (8,9). EC synthesis of DNA was also stimulated by the exposure to AGE-BSA but not to nonglycated BSA. These results indicate that AGE are potentially angiogenic. A thrombogenic activity of AGE was also noted. AGE-BSA inhibited EC ability to synthesized prostacyclin on one hand (8,9) and stimulated the production of plasminogen activator inhibitor-1 on the other (10). AGE, therefore, can cause angiogenesis and thrombogenesis by dual mechanisms: first by

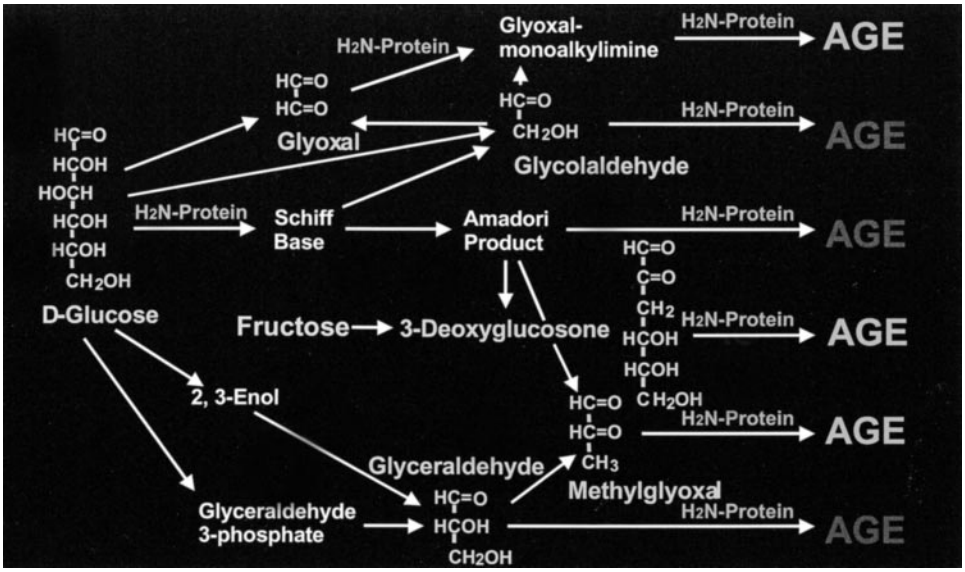

Figure 1. Nonenzymatic glycation (3). Red, RAGE-binding, biologically active AGE fractions.

impairing EC-pericyte interactions and second by the direct action on EC.

The angiogenic activity of AGE is mediated by autocrine vascular endothelial growth factor (VEGF) (11,12), as evidenced by the facts that AGE induced the expression of VEGF gene in EC and that AGE-induced EC proliferation and tube formation were abolished by the anti-VEGF neutralizing antibody (9).

Receptor for AGE

Receptor for AGE (RAGE) is a multi-ligand cell surface receptor initially isolated from bovine lung by the group of Stern (13). Endogenous ligands, such as amphoterin, calgranulin, amyloid β proteins, and transthyretin, have also been identified. Yamamoto *et al.* (manuscript submitted) conducted a surface plasmon resonance assay using purified human RAGE proteins and glucose- and aldehyde-derived AGE. As a result, in addition to glucose-derived AGE, glyceraldehydes- and glycolaldehyde-derived AGE fractions were found to bind to RAGE. These ligands were those that were observed to elicit pericyte and EC derangement, suggesting that certain AGE structures effect vascular cells through interactions with RAGE (Figure 2). This idea seems to be supported by the findings that the AGE-induced decrease in pericyte proliferation, increase in EC proliferation, inhibition of EC synthesis of prostacyclin, and stimulation of EC production of plasminogen activator inhibitor-1 all were abolished by RAGE antisense (6,8,10). Tsuji *el al*. (14) showed that AGE induction of collagen synthesis is blocked by RAGE ribozyme.

RAGE Transgenic Mice

From these lessons, a hypothesis has been drawn that the AGE-RAGE system may participate in the development of diabetic vascular complications. To evaluate this hypothesis *in vivo*, Yamamoto *et al.* (15,16) created transgenic mice that overexpress human RAGE proteins in vascular cells and crossbred them with another transgenic mouse line that develops insulin-dependent diabetes early after birth. The resultant double transgenic mice showed statistically significant increases in kidney weight, albuminuria, glomerulosclerosis index, and serum creatinine compared with the diabetic control (Figure 3), whereas blood glucose, hemoglobin A1c, and serum AGE levels were essentially invariant between the two groups. The increases in serum creatinine and sclerosis index were effectively prevented with (±)-2-isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide, an inhibitor of AGE formation (15,16). Indices diagnostic of diabetic retinopathy were also most prominent in double transgenic mice (Figure 3). Thus, this transgenic approach has supported the concept that the AGE-RAGE system plays an active role in the development of diabetic complications and has developed a useful animal model for testing remedies.

Regulation of Human RAGE Gene

In light of the findings with RAGE-overexpressing transgenic mice, it is reasonable to assume that upregulation of the endogenous RAGE gene would aggravate diabetic vascular derangement. Accordingly, it seemed important to know the mechanism of RAGE gene regulation, so we screened factors that can influence the EC level of RAGE mRNA. Tanaka *et al.* (17) identified three inducers of RAGE gene transcription: AGE ligands themselves, TNF-α and 17β-estradiol. The former two shared the same *cis*-element for induction, which was located around nucleotide −671 in the human RAGE 5'-flanking sequence, whereas estradiol-responsible elements resided at −189 and at −172. Electromobility shift assays revealed that nuclear factor-κB is the *trans*-factor that binds to the AGE- and TNF-α–responsible element and that Sp-1/estrogen receptor-α complex is the factor acting on the estradiol-

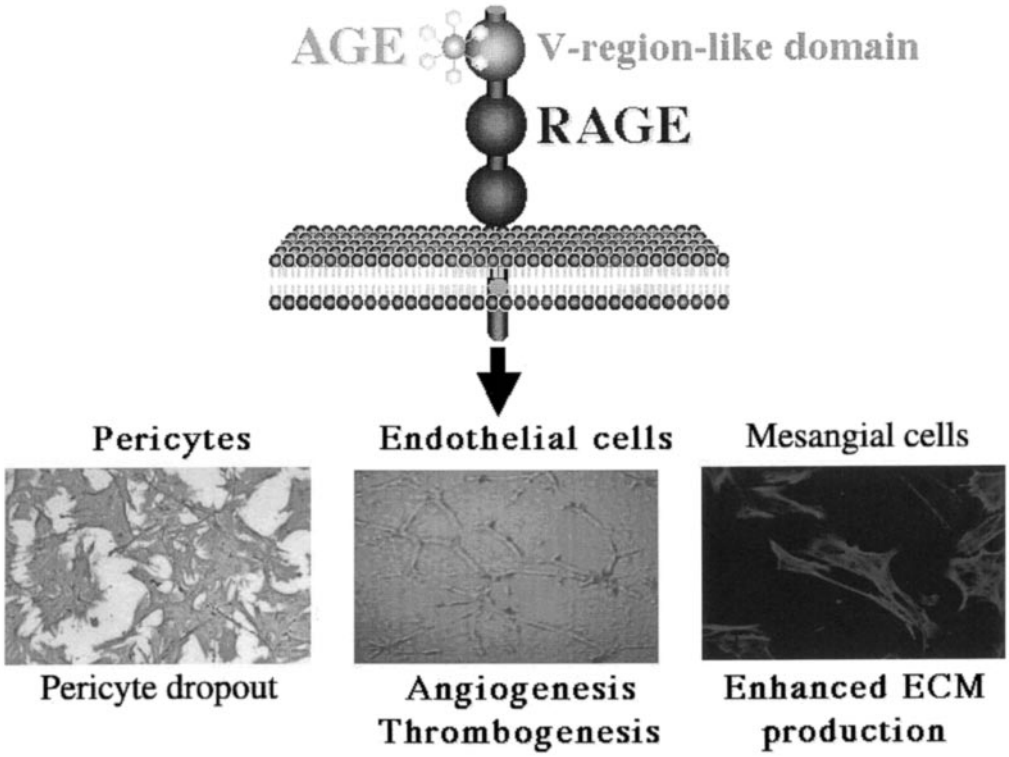

Figure 2. Vascular cell changes caused by AGE-RAGE interactions.

responsive elements estradiol (17) (Figure 3). TNF-α has been known to be responsible for insulin resistance (18). The action on RAGE gene unveiled another side of this cytokine; that is, an increased TNF-α level in diabetic patients may worsen diabetic complications through RAGE induction. The finding that AGE themselves can activate the RAGE gene seems to be consistent with the observation that the AGE-rich vasculature exhibits enhanced RAGE immunoreactivity (19). Such a positive feedback loop may exacerbate diabetic vasculopathy. The RAGE gene activation by estradiol may provide a biochemical basis for the well-known fact that pregnancy worsens diabetic complications (20).

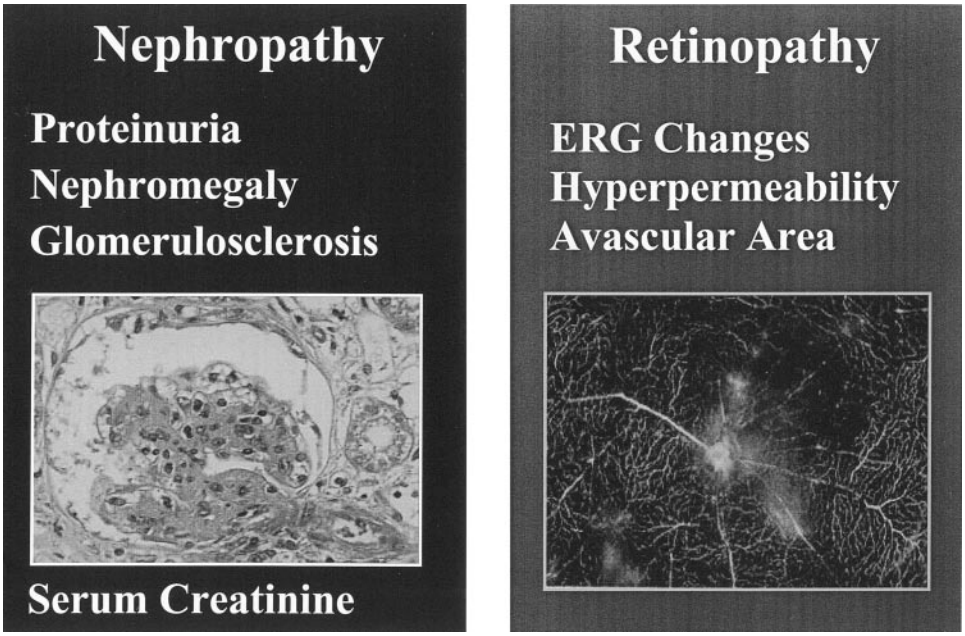

Figure 3. Indices aggravated in RAGE-overexpressing diabetic double-transgenic mice.

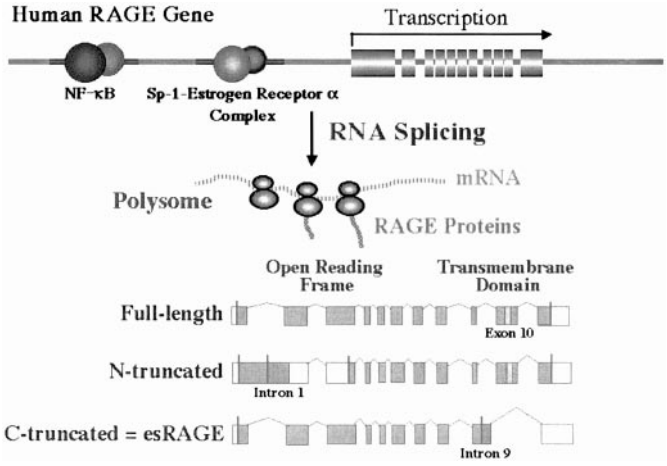

Figure 4. Regulation of human RAGE gene expression and the splice variants.

Endogenous Secretory RAGE

Yonekura *et al.* (21) analyzed poly(A)$^+$RNA isolated from polysomes of human EC and pericytes and isolated previously undescribed splice variants of RAGE mRNA. Three major variants were identified: the known full-length membrane-bound form, a novel N-terminally truncated membrane-bound form, and a novel C-terminally truncated soluble form (Figure 4). The ratio of expression of these variants differed from one cell type to another; C-truncated > full-length = N-truncated in EC; full-length > N-truncated > C-truncated in pericytes. Each cDNA directed synthesis of the protein product of the expected size in COS cells. Both the full-length and the N-truncated forms mainly resided on the plasma membrane, whereas the C-truncated form was liberated into the media. Furthermore, the full-length and C-truncated forms bound to an AGE-immobilized column, whereas the N-truncated form was recovered in the pass-through fractions, thus confirming that the ligand-binding site is located in the amino-terminal V-region–like domain of RAGE proteins. We named the C-truncated form endogenous secretory RAGE (esRAGE). esRAGE would be cytoprotective, because it is able to capture AGE outside cells (Figure 5). In effect, this variant was found to neutralize effectively the AGE action on EC and does exist in human circulation (21). An ELISA system for esRAGE has been developed, and, with it, diabetic subjects with or without complications are now being screened.

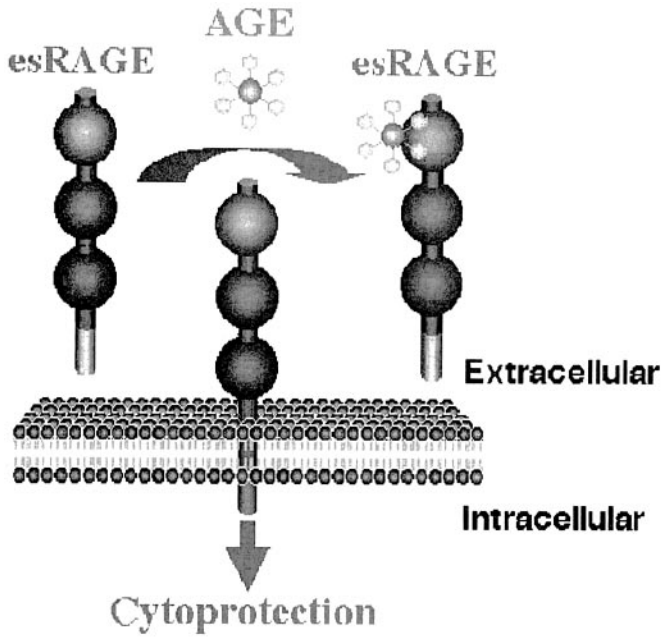

Figure 5. esRAGE.

Acknowledgments

This work was supported by "the Research for the Future Program" of the Japan Society for the Promotion of Science and grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

The authors thank Professor Hi Bahl Lee, Soon Chun Hyang University, the President of the 5th Hyonam Kidney Laboratory International Symposium for providing an opportunity to submit this paper; Professor Toshio Doi, Tokushima University; Professor Hiroshi Okamoto; Dr. Shin Takasawa and Dr. Ichiro Kato, Tohoku University; Dr. Masayoshi Takeuchi, Hokuriku University; Professor Zenji Makita and Dr. Sho-ichi Yamagishi, Kurume University, for collaboration; and Shin-ichi Matsudaira, Reiko Kitamura, and Yoshie Yamamoto for assistance.

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