MECHANISMS OF VASCULAR INJURY IN DIABETES: LESSONS FROM VASCULAR CELLS IN CULTURE AND TRANSGENIC ANIMALS

Hiroshi Yamamoto

Department of Biochemistry, Kanazawa University School of Medicine, Kanazawa, Japan

Microvessels are composed of two cell types, endothelial cells (EC) and pericytes. The decrease in the latter (pericyte loss) and the increase in the former (angiogenesis) are the hallmarks of diabetic retinopathy. Coculture experiments revealed that the growth and function of EC are under a control of neighboring pericytes, thus explaining why endothelial derangement follows pericyte loss. During prolonged hyperglycemic exposure, nonenzymatically glycated protein derivatives termed advanced glycation endoproducts (AGE) are formed at an accelerated rate and accumulate in palsma and various tissues. When bovine retinal pericytes were cultured with AGE-bovine serum albumin, the number of viable pericytes was decreased, which was abolished by a DNA complement of mRNA coding for a cell-surface receptor for AGE (RAGE). AGE can act also on EC, again in a RAGE-dependent manner. They stimulated the proliferation and tube formation of microvascular EC, and inhibited prostacyclin synthesis but induced plasminogen activator inhibitor-1. Thus, AGE predispose to both angiogenesis and thrombogenesis. The angiogenic activity of AGE was found to be mediated by autocrine vascular endothelial growth factor. To address the role of RAGE in the development of diabetic pathology, we created transgenic mice that overexpress RAGE in vascular cells and made them diabetic by crossbreeding with another transgenic line that develops insulin-dependent diabetes early after birth. The resultant double transgenic mice exhibited accelerated nephropathy, as evidenced by significantly higher scores in albuminuria, glomerulosclerosis and serum creatinine compared with single transgenic and nontransgenic littermates. The nephropathy was ameliorated by the administration of an inhibitor of AGE formation. Indices diagnostic of diabetic retinopathy were also prominent in the double transgenic mouse model. Further, the regulators of RAGE gene expression have been identified. Transcription of human RAGE gene was induced by tumor necrosis factor-α and AGE ligands themselves through nuclear factor-κB activation, and by 17β-estradiol through Sp-1. The results thus suggest that the AGE-RAGE system plays an active role in the development of diabetic vasculopathy and is a promising target in the prophylaxis and therapy of this disease. **Biomed Rev 2000; 11:19-27.**

INTRODUCTION

It is vascular complications that directly account for short life expectancy and poor quality of life (QOL) in patients with diabetes mellitus. To understand how blood vessels are impaired in the diabetic state is therefore important to make diabetes a not-terrible disease. As is diabetes *per se*, diabetic complications are thought to be caused by multiple environmental and genetic factors. A concept that interactions between senescent macromolecules termed advanced glycation

endoproducts (AGE) occurring in our internal milieu and a cellular receptor for them are the major account for diabetes-induced vascular derangement has emerged, suggesting the possibility that the AGE and their receptor system will be a promising target for overcoming diabetic vascular complications (1-7). Reviewed here are lessons at cellular, subcellular and molecular levels and from transgenic animals, the results of which have led us to propose this concept.

LESSON FROM COCULTURE

Microvessels, the place at which diabetic microangiopathies exemplified by retinopathy and nephropathy take place, are composed of two cell types, endothelial cells (EC) and pericytes. EC not only serve as the barrier against cellular and acellular blood components but also produce various vasoactive substances, which contribute to the regulation of vascular function. Pericytes are cells that encircle the endothelium. Because they have such an anatomical topology, and because they contain intracellular contractile fibers, pericytes have been regarded as the microvascular equivalent to smooth muscle cells. However, the biological significance of pericytes is not fully understood. To address this issue, EC-pericyte coculture systems were developed (8,9). When human umbilical vein EC were cultivated on the feeder layer of bovine retinal pericytes, the growth curve of EC shifted downward, and this required a physical contact between the two cell types (8). Pericytes were also found to influence EC ability to synthesize prostacyclin, an antithrombogenic prostanoid. This also required EC-pericyte contact. Further, pericytes conferred protection against lipid peroxide-induced injury of EC (8), and human fibroblasts could not substitute for pericytes. Accordingly, pericytes are considered to endow the neighboring EC with instructive signals. Candidate molecules for this are represented by transforming growth factor-beta (TGF-β) (10) and heparan sulfate proteoglycans (11,12).
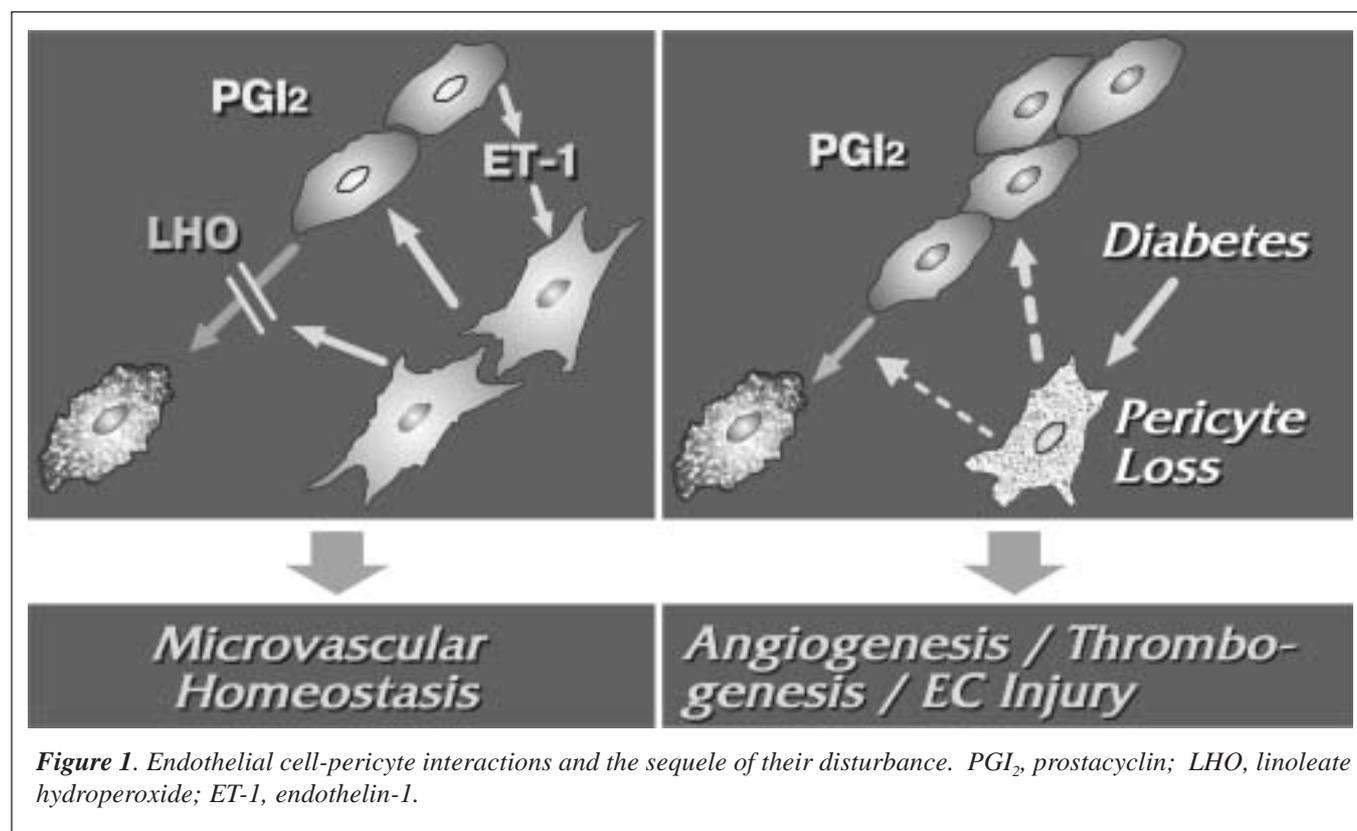
EC-pericyte coculture experiments were also conducted in an inverted orientation; *viz,* pericytes were cultivated on EC feeder. Contrasting with the case of the pericyte effect on EC, EC exerted a stimulatory effect on pericyte growth. This was achieved even when pericytes were separated from the EC layer, suggesting that diffusible molecules are responsible for the stimulation. The molecule was identified to be endothelin-1 (9). As shown in Figure 1, these observations indicate that the growth and functions of EC are under a control of neighboring pericytes, and that the interaction between EC and pericytes contributes to the maintenance of microvascular homeostasis. When the number of pericytes decreases, EC would undergo angiogenesis and thrombogenesis. Such a circumstance does occur *in vivo* under the diabetic state. Pericyte loss or dropout is that, which has been known as the

earliest histopathologic change in diabetic retinopathy. Knowledge from the pericyte-EC coculture experimentation suggests that deficiency in endothelin-1 action may be a possible mechanism. However, plasma endothelin-1 levels in diabetic patients are reported to be rather higher than in normal subjects (13), while pericytogenesis proceeds normally in endothelin-1 deficient mice (14 and unpublished observation by Yamagishi S, Katsuta S, Kurihara Y, Kurihara H, Yamamoto H). Then, other candidates involved in diabetes-induced pericyte derangement were sought.

LESSON FROM PERICYTES

What we have noticed of as a possible cause for pericyte loss is AGE. Reducing sugars like glucose can react non-enzymatically with the amino groups of proteins to form Schiff bases and then Amadori compounds. These early glycation products further undergo reactions, including dehydration, condensation and crosslinking, yielding irreversible fluorescent protein derivatives termed AGE. This reaction was first described in 1912 in the field of food chemistry by Maillard (15). Subsequently, it has been known that AGE also occur within our bodies during ageing and at an extremely accelerated rate in diabetes mellitus. The irreversible nature of diabetic complications and the well known fact that the progression of this disease best correlates to the extent of glycation as reflected by hemoglobin A_{1c}, the Amadori rearrangement product of hemoglobin, prompted us to see whether AGE participate in pericyte loss. For this, Yamagishi *et al* (2) incubated bovine serum albumin (BSA) with high concentration of glucose. The resultant material exhibited spectrophotometric features characteristic of AGE, migrated more slowly than nonglycated BSA on reducing SDS-polyacrylamide gel electrophoresis, and was thus regarded as AGE-BSA. This preperation was then administered to the culture of bovine retinal pericytes. Their growth was found to be retarded by AGE-BSA in a dose-dependent manner (4). Though modest, AGE-BSA also exerted an acute toxicity to pericytes. Accordingly, AGE seemed to take an active part in the decrease in pericytes during prolonged diabetic exposure.

The presence of cellular receptors that can bind AGE has been described. Among them is a cell surface receptor for AGE (RAGE), which was initially isolated from bovine lung by the group of Dr Stern (16). It belongs to the immunoglobulin superfamily, having three immunoglobulin-like domains in the N-terminal extracellular segment, one transmembrane region and a short C-terminal intracytoplasmic stretch. To test whether this receptor molecule is required for the AGE action on pericytes, we employed antisense strategy. A septadecamer DNA complement of bovine mRNA[RAGE] and its sense control were synthesized by phosphorothioate chemistry (17), and administered to pericyte culture with or

Figure 1. *Endothelial cell-pericyte interactions and the sequele of their disturbance. PGI₂, prostacyclin; LHO, linoleate hydroperoxide; ET-1, endothelin-1.*

without AGE-BSA. As a result, RAGE antisense, but not sense control, was found to abolish the AGE-induced decrease in viable pericytes (4). This indicated that the AGE effect on pericytes is mediated by receptor-ligand interactions.

LESSON FROM ENDOTHELIAL CELLS

AGE also act on EC. Contrasting with the case of pericyte, AGE-BSA supershifted upward the growth curve of human microvascular EC (2). EC synthesis of DNA was also stimulated by the exposure to AGE-BSA, but not by non-glycated 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 (1,2), and stimulated the production of plasminogen activator inhibitor-1 on the other (3). These AGE actions on EC were also RAGE-dependent, as evidenced by their cessation with RAGE antisense. The most effective dosage of AGE-BSA was 50 μg/ml in each of the three indices. This concentration is comparable with the level of circulating AGE documented in human diabetic patients (18).

Vascular growth in the adult may occur via vasculogenesis (angioblast mobilization), angiogenesis (capillary growth) or arteriogenesis (collateral growth) (reviewed in 19). Angiogenesis is the process by which new vascular networks are formed from preexisting capillaries, and is physiologically essential for development, growth, reproduction, and wound healing. Various pathologic states also depend on angiogenesis; these include cancer growth and metastasis, proliferative diabetic retinopathy, rheumatoid arthritis, psoriasis vulgaris, and atherosclerotic lesions. Hypoxia has been known as the principal factor for angiogenesis. However, how hypoxia induces it is not yet fully understood. To address this issue, we developed a hypoxic culture system, in which microvascular cells are cultured in a controlled atmospheric culture chamber containing defined concentrations of oxygen (20,21).

Both microvascular EC and pericytes were found to grow more rapidly under hypoxic conditions than under normoxic conditions. The O_2 tension that caused the maximal growth promotion was 5% in EC (22) and 2.5% in pericytes (21). Expression of the gene coding for vascular endothelial growth factor (VEGF) was found to increase as the atmospheric O_2 tension decreased, and the hypoxia induction of EC and pericyte growth was completely neutralized by VEGF antisense (21) and anti-VEGF antibodies (22). This indicates that VEGF synthesized by microvascular cells themselves participates in the hypoxia-driven angiogenesis, and that VEGF can also act as a pericyte mitogen under hypoxic conditions (23). Recent studies have provided information that both EC and pericytes

express not only VEGF but also other members of the VEGF gene family, including placental growth factor (PLGF), VEGF-B, VEGF-C, VEGF-D and their receptors VEGFR2, VEGFR3 and neuropilin-1 (19,24). Among them, PLGF was found to play an important role in the hypoxia-induced stimulation of EC growth; this factor is expressed in EC as abundantly as two orders of magnitude higher than VEGF, and antisense DNAs against mRNA^{PLGF} and mRNA^{VEGF} additively halted the EC growth (24).

The lesson from hypoxic culture of vascular cells evoked a question whether the angiogenic activity of AGE is also VEGF-mediated. First, we analyzed VEGF gene expression in EC exposed to varying concentrations of AGE-BSA. Levels of mRNA^{VEGF165} and mRNA^{VEGF121}, the secretory forms of VEGF, were most increased at 50 µg/ml, the concentration at which AGE exerted the strongest biologic actions on EC. On the other hand, the levels of mRNA for VEGF receptors were essentially invariant. Second, functional roles of VEGF were assessed with VEGF neutralizing antibodies. The antibody was found to abolish not only AGE-induced EC proliferation but also AGE-induced tube formation of EC. Therefore, it was concluded that it is autocrine VEGF that mediates the angiogenic activity of AGE (2), as it does in hypoxia-driven EC growth and tube formation (21).

LESSON FROM MESANGIAL CELLS

The AGE-RAGE system is also related to mesangial expansion, the histopathologic hallmark of diabetic glomerulosclerosis caused by enhanced production of extracellular matrix proteins. The group of Doi (5) developed a hammerhead ribozyme against mRNA^{RAGE} and introduced it into mouse mesangial cells in culture. The level of collagen type IV mRNA doubled when exposed to AGE, but this induction was abolished in cells that expressed the RAGE ribozyme.

A HYPOTHESIS FOR DIABETES-INDUCED MICROVASCULAR DERANGEMENT

From those lessons, we propose a mechanism for diabetes-induced microvascular derangement in retinopathy and nephropathy (Fig. 2). During prolonged hyperglycemia, AGE are formed and accumulate in circulating blood and in various tissues. AGE act on pericytes through interactions with RAGE, leading to pericyte loss. This results in deterioration of EC-pericyte interactions, which in turn causes angiogenesis and thrombogenesis. AGE directly act on EC again through RAGE, which further leads to angiogenesis and thrombogenesis, the former being mediated by autocrine VEGF. When microthrombus is formed within microvessels, hypoxic regions would be
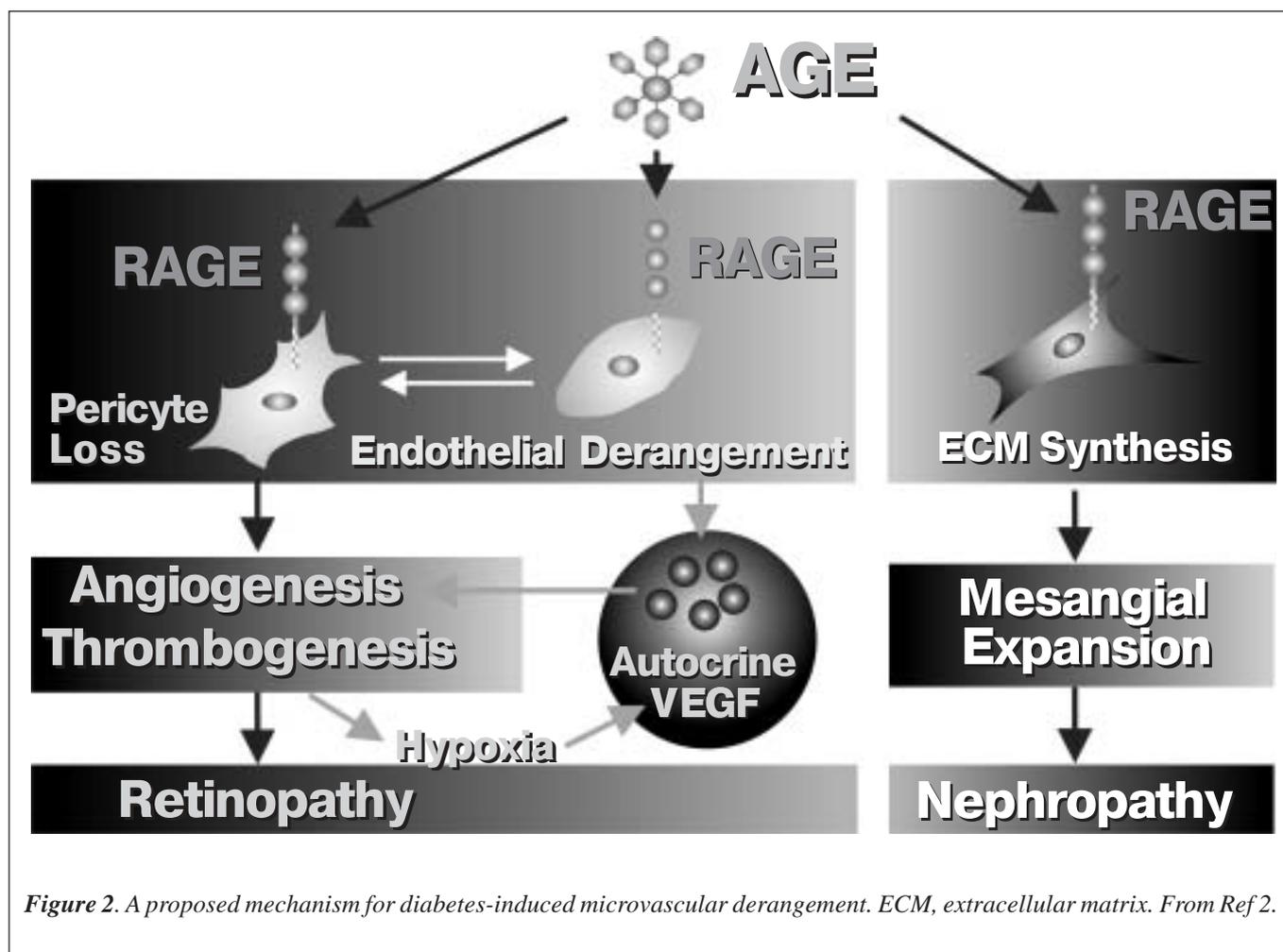
generated downstream, which can superdrive VEGF production, thus forming a vicious cycle. These changes would contribute to the development and progression of retinopathy. The AGE-RAGE system also participates in mesangial expansion, leading to the development of nephropathy.

LESSON FROM TRANSGENIC MICE

To evaluate the hypothesis *in vivo*, Yamamoto *et al* (25) created transgenic mice that overexpress human RAGE proteins in vascular cells by introducing fertilized ova a transgene carrying human RAGE genomic DNA under the control of the murine VEGF receptor promoter, which acts specifically in EC (Fig. 3). Integration into the mouse genome, active transcription and translation of the RAGE transgene were confirmed by Southern blot analysis, reverse transcription-polymerase chain reaction, and immunoblotting. Renal glomeruli of the transgenic mice were positively stained for human RAGE in an EC pattern.

There are several means to induce diabetes in experimental animals (26). Chemical or surgical maneuvers for diabetes induction, however, cause some diversity among individual animals in terms of the extent of severity and the onset of diabetes. Accordingly, we employed a genetic approach by which both a diabetic state and an advanced glycation would be most stably induced. *Viz*, RAGE transgenic mice were crossbred with another transgenic mouse line that consistently develops insulin-dependent diabetes as early as 1 week after birth due to the inducible nitric oxide synthase (iNOS)-mediated selective destruction of insulin-producing pancreatic β cells (27), yielding four groups of littermates which carried both, either or neither of the transgenes (Fig. 3). They were designated double Tg, RAGE Tg, iNOS Tg and nontransgenic control. Blood analysis revealed sustained hyperglycemia, high hemoglobin A_{1c} levels and the progressive accumulation of AGE in the former two but not in the latter two groups.

The gross phenotypic abnormality that we initially noticed in double Tg was enlarged kidneys. The kidney weight : body weight ratios in double Tg were about 30% higher above the values in the other three groups. Accordingly, we first focused the study on nephropathy, a representative life-threatening diabetic complication which approximately 30% of patients with insulin-dependent diabetes mellitus suffer from (28). This renal complication of diabetes is characterized by increased albuminuria and glomerular hypertrophy as well as nephromegaly in the early phase. In its late phase, glomerulosclerosis and increased serum creatinine follow. Double Tg showed a significant acceleration of all the early- and late-phase indices of diabetic nephropathy. The diabetic RAGE-overexpressing mice finally developed typical nodular lesions and hyaline arteriosclerosis. The increases in serum creati-

Figure 2*. A proposed mechanism for diabetes-induced microvascular derangement. ECM, extracellular matrix. From Ref 2.*

nine and in the sclerosis index were effectively prevented with (±)-2-isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide (25), a thiazolidine derivative that can inhibit AGE formation by blocking carbonyl groups on glycation intermediates (29). Indices diagnostic of diabetic retinopathy were also most prominent in double Tg. This included the delay in the peaks of oscillatory potentials on electroretinogram, increased permeability of retinal vessels and an increase in hypovascular or avascular areas in the retina.

Therefore, this transgenic technology-aided approach has provided firm *in vivo* support to the concept that AGE-RAGE system plays an active role in the development of diabetic complications, and that the AGE-RAGE system is an effective target for intervention in this disease. Further, there was no single animal model that develops renal changes similar to those seen in humans (30). The double transgenic mice will serve as a most useful animal model for studying the pathogenesis of diabetic complications and for testing remedies.

LESSON FROM THE NUCLEUS

In light of the findings with RAGE-overexpressing tansgenic mice, it is reasonable to assume that upregulation of the endogenous RAGE gene would aggravate diabetic vascular derangement. To know the regulatory mechanism of RAGE gene expression is, therefore, important. Hence, we pursued factors that can influence EC expression of the RAGE gene. Tanaka *et al* (31) found that AGE ligands themselves, tumor necrosis factor-alpha (TNF-α) and 17β-estradiol (E2) can activate RAGE gene transcription. AGE and TNF-α shared the same *cis*-element for induction, which was located around nucleotide -671 in the human RAGE 5'-flanking sequence, while E2-responsible elements resided at -189 and at -172 (32). Electromobility shift assays revealed that nuclear factor-κB (NF-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 E2-responsive elements
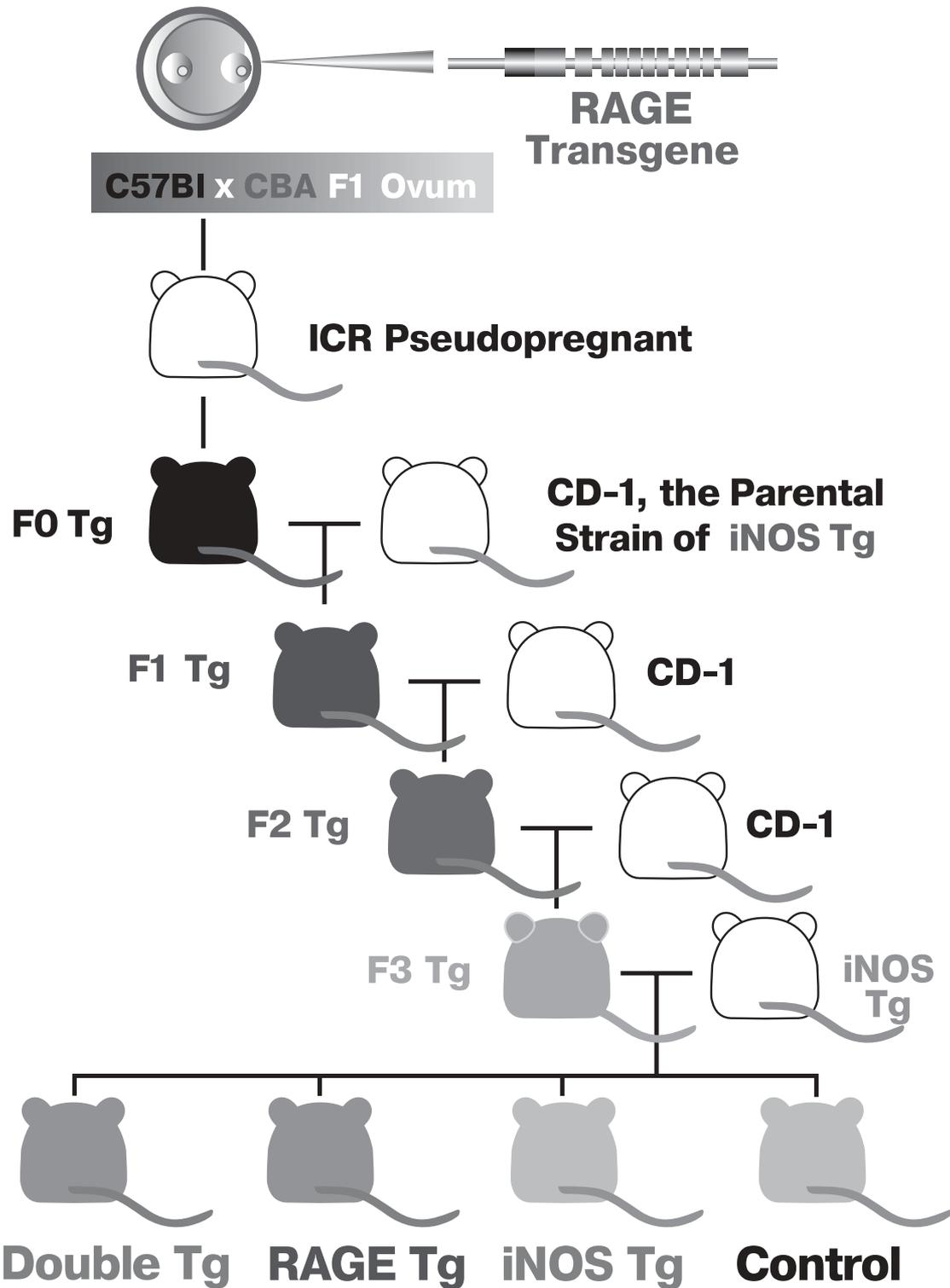
Figure 3. *Generation of diabetic RAGE-overexpressing mice. RAGE Tg was backcrossed to the nontransgenic parental strain CD-1 of iNOS Tg for more than 3 generations to unify the genetic background. From Ref 25.*

(31) (Fig. 4). The finding indicated that AGE themselves can activate the RAGE gene expression, and that increased RAGE may further transduce AGE signals within microvascular cells. Being consistent with this observation, it has been reported the AGE-rich vasculature exhibits enhanced RAGE immunoreactivity in the sites of diabetic microvascular injury (32). Such a positive feedback loop in the diabetic state may exacerbate diabetic vasculopathy. TNF-α is known to be overexpressed in adipose tissue under obese and diabetic states and to cause insulin resistance, the central and early component of non-insulin-dependent diabetes mellitus (33,34). The action on RAGE gene unveiled another role of this cytokine in diabetes; that is, an increased TNF-α level in diabetic patients may worsen diabetic complications via RAGE induction. The Sp1/estrogen receptor α-mediated RAGE gene activation by E2 would seem to provide a biochemical basis for the well known fact that pregnancy worsens diabetic retinopathy (35).

CONCLUSION

Some actions of nurture and nature are obviously at variance with the purposes of evolution, and rather assist with the development of diseases. Endogenous RAGE ligands have recently been identified, and studies with them suggest that this receptor molecule is also related to neuronal network forma-

tion in the cerebrum (36) and to proinflammatory reactions (37). Diabetic complications appear to exploit the molecular devices primarily evolved for development and survival. In all the likelihoods, AGE would hardly be encountered by ancestral *homo sapiens*, who was placed in starved conditions under usual circumstances and was relatively short-lived, but occur as we become nourished and get longevity, eventually being recognized by the cell surface molecule probably due to their steric mimicry of natural ligands. Nevertheless, the lessons presented in this review have taught us that we should develop effective means to interrupt such an abusage within for overcoming this life- and QOL-threatening disease.
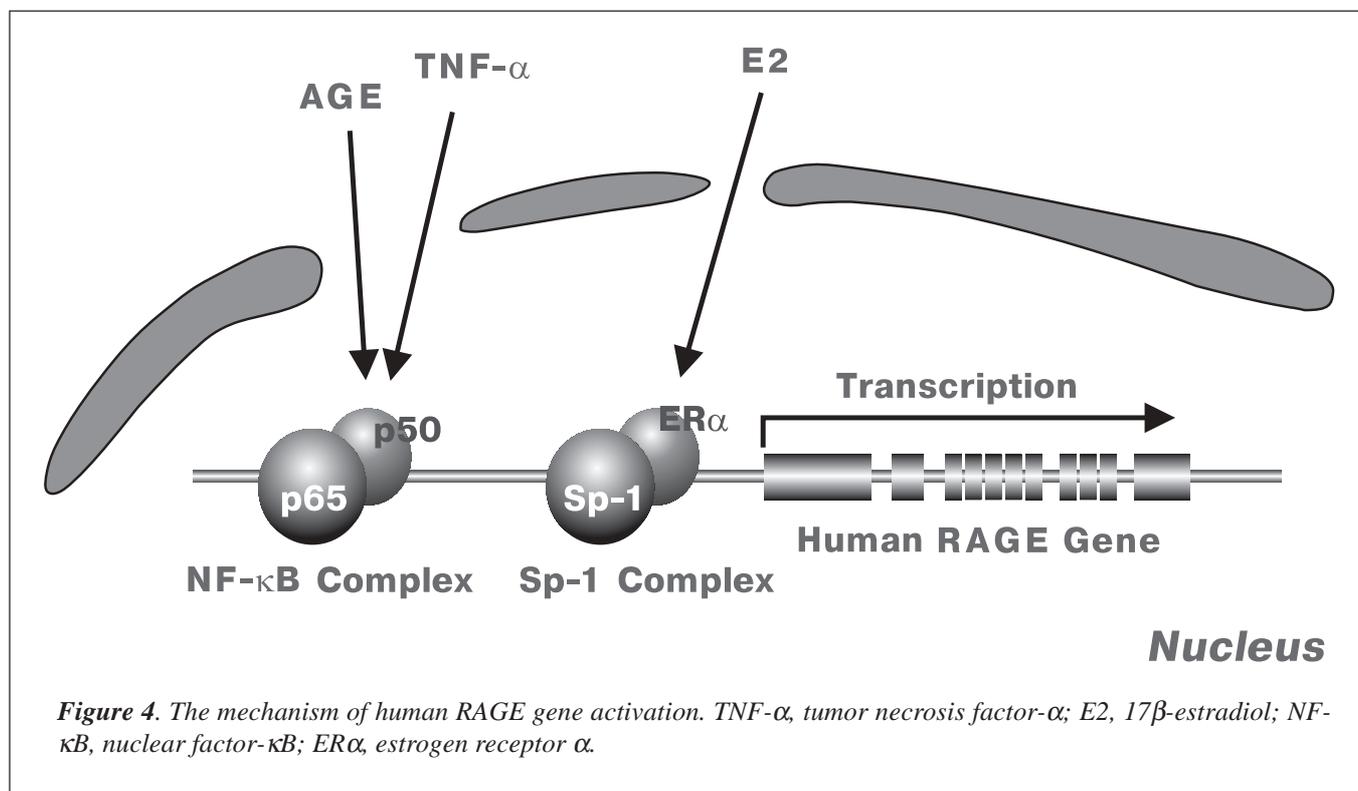
Figure 4. The mechanism of human RAGE gene activation. TNF-α, tumor necrosis factor-α; E2, 17β-estradiol; NF-κB, nuclear factor-κB; ERα, estrogen receptor α.

REFERENCES

1. Yamagishi S, Yamamoto Y, Harada S, Hsu C-C, Yamamoto H. Advanced glycosylation end products stimulate the growth but inhibit the prostacyclin-producing ability of endothelial cells through interactions with their receptors. *FEBS Lett* 1996; 384: 103-106.

2. Yamagishi S, Yonekura H, Yamamoto Y, Katsuno K, Sato F, Mita I, *et al*. Advanced glycation endproducts-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* 1997; 272: 8723-8730.

3. Yamagishi S, Fujimori H, Yonekura H, Yamamoto Y, Yamamoto H. Advanced glycation endproducts inhibit prostacyclin production and induce plasminogen activator inhibitor-1 in human microvascular endothelial cells. *Diabetologia* 1998; 41: 1435-1441 .

4. Yamagishi S, Hsu C-C, Taniguchi M, Harada S, Yamamoto Y, Ohsawa K, *et al*. Receptor-mediated toxicity to pericytes of advanced glycosylation end products: A possible mechanism of pericyte loss in diabetic microangiopathy. *Biochem Biophys Res Commun* 1995; 213: 681-687.

5. Tsuji H, Iehara N, Masegi T, Imura M, Ohkawa J, Arai H, *et al*. Ribozyme targetting of receptor for advanced glycation end products in mesangial cells. *Biochem Biophys Res Commun* 1998; 245: 583-588.

6. Vlassara H, Striker LJ, Teichberg S, Fuh H, Li YM, Steffes M. Advanced glycation end products induce glomerular sclerosis and albuminuria in normal rats. *Proc Natl Acad Sci USA* 1994; 91:11704-11708.

7. Park L, Raman KG, Lee KJ, Lu Y, Ferran LJ Jr, Chow WS, *et al*. Suppression of accelerated diabetic atherosclerosis by the soluble receptor for advanced glycation endproducts. *Nat Med* 1998; 4:1025-1031.

8. Yamagishi S, Kobayashi K, Yamamoto H. Vascular pericytes not only regulate growth, but also preserve prostacyclin-producing ability and protect against lipid peroxide-induced injury of co-cultured endothelial cells. *Biochem Biophys Res Commun* 1993; 190: 418-425.

9. Yamagishi S, Hsu C-C, Kobayashi K, Yamamoto H. Endothelin 1 mediates endothelial cell-dependent proliferation of vascular pericytes. *Biochem Biophys Res Commun* 1993; 191: 840-846.

10. Sato Y, Rifkin DB. Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor-β1 like molecule by plasmin during co-culture. *J Cell Biol* 1989; 109: 309-315.

11. Orlidge A, D'Amore PA. Cell specific effects of glycosaminoglycans on the attachment and proliferation of vascular wall components. *Microvasc Res* 1986; 31: 41-53.

12. Stramm LE, Li W, Aguirre GD, Rockey JH. Glycosaminoglycan synthesis and secretion by bovine retinal capillary pericytes in culture. *Exp Eye Res* 1987; 44: 17-28.

13. Takahashi K, Ghatei MA, Lam HC, O'Halloran DJ, Bloom SR. Elevated plasma endothelin in patients with diabetes mellitus. *Diabetologia* 1990; 33: 306-310.

14. Kurihara Y, Kurihara H, Suzuki H, Kodama T, Maemura K, Nagai R, *et al*. Elevated blood pressure and craniofacial abnormalities in mice deficient in endothelin-1. *Nature* 1994; 368: 703-10.

15. Maillard ML-C. Action des acides amine's surles sucres; formation desmélanoïdines par voie méthodique. *COMPTES PENDUS* 1912; 599-601.

16. Neeper M, Schmidt AM, Brett J, Yan SD, Wang F, Pan YC, *et al*. Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. *J Biol Chem* 1992; 267: 14998-5004.

17. Yonekura H, Migita H, Sakurai S, Wang H, Harada S, Abedin MJ, *et al*. Antisense display - a method for functional gene screening: evaluation in a cell-free system and isolation of angiogenesis-related genes. *Nucleic Acids Res* 1999; 27: 2591-2600.

18. Makita Z, Vlassara H, Cerami A, Bucala R. Immunochemical detection of advanced glycosylation end products *in vivo*. *J Biol Chem* 1992; 267: 5133-5138.

19. Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. *Nat Med* 2000; 6: 389-395.

20. Yamamoto H. "Vascular VEGF" in hypoxia-induced proliferation of vascular cells. *Microcirc Annu* 1996; 11: 11-12.

21. Nomura M, Yamagishi S, Harada S, Hayashi Y, Yamashima T, Yamashita J, Yamamoto H. Possible participation of autocrine and paracrine vascular endothelial growth factors in hypoxia-induced proliferation of endothelial cells and pericytes. *J Biol Chem* 1995; 270: 28316-28324.

22. Yamagishi S, Kawakami T, Fujimori H, Yonekura H, Tanaka N, Yamamoto Y, *et al*. Insulin stimulates the growth and tube formation of human microvascular endothelial cells through autocrine vascular endothelial growth factor. *Microvasc Res* 1999; 57: 329-339.

23. Yamagishi S, Yonekura H, Yamamoto Y, Fujimori H, Sakurai S, Tanaka N, Yamamoto H. Vascular endothe-

lial growth factor acts as pericyte mitogen under hypoxic conditions. *Lab Invest* 1999; 79: 501-509.

24. Yonekura H, Sakurai S, Liu X, Migita H, Wang H. Yamagishi S, *et al*. Placenta growth factor and vascular endothelial growth factor B and C expressions in microvascular endothelial cells and pericytes: implication in autocrine and paracrine regulation of angiogenesis. *J Biol Chem* 1999; 274: 35172-35178 .

25. Yamamoto Y, Yamagishi S, Yonekura H, Doi T, Tsuji H, Kato I, *et al*. Roles of the AGE-RAGE system in vascular injury in diabetes. *Ann NY Acad Sci* 2000; 902: 163-172.

26. Yamamoto H, Uchigata Y, Okamoto H. Streptozotocin and alloxan induce DNA strand breaks and poly(ADP-ribose) synthetase in pancreatic islets. *Nature* 1981; 294: 284-286.

27. Takamura T, Kato I, Kimura N, Nakazawa T, Yonekura H, Takasawa S, *et al*. Transgenic mice overexpressing type 2 nitric-oxide synthase in pancreatic beta cells develop insulin-dependent diabetes without insulitis. *J Biol Chem* 1998; 273: 2493-2496.

28. Krolewski M, Eggers PW, Warram JH. Magnitude of end-stage renal disease in IDDM: A 35 year follow-up study. *Kidney Int* 1996; 50: 2041-2046.

29. Nakamura S, Makita Z, Ishikawa S, Yasumura K, Fujii W, Yanagisawa K, *et al*. Progression of nephropathy in spontaneous diabetic rats is prevented by OPB-9195, a novel inhibitor of advanced glycation. *Diabetes* 1997; 46: 895-899.

30. Velasquez MT, Kimmel PL, Michaelis OE IV. Animal models of spontaneous diabetic kidney disease. *FASEB J* 1990; 4: 2850-2859.

31. Tanaka N, Yonekura H, Yamagishi S, Fujimori H, Yamamoto Y, Yamamoto H. The receptor for advanced glycation endproducts is induced by the glycation products themselves and TNF-α through NF-κB, and by 17β-estradiol through Sp-1 in human vascular endothelial cells. *J Biol Chem* 2000; 275: 25781-25790.

32. Soulis T, Thallas V, Youssef S, Gilbert RE, McWilliam BG, Murray-McIntosh RP, *et al*. Advanced glycation end products and their receptors co-localise in rat organs susceptible to diabetic microvascular injury. *Diabetologia* 1997; 40: 619-628.

33. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* 1993; 259: 87-91.

34. Chaldakov G, Fiore M, Hristova M, Stankulov I, Triaca V, Ghenev P, Aloe L. Adipose tissue-secreted molecules (adipokines): neuroimmune implications. *Clin Appl Immunol Invest* 2000; 1: 11-16.

35. Moloney JB, Drury MI. The effect of pregnancy on the natural course of diabetic retinopathy. *Am J Ophthalmol* 1982; 93: 745-56.

36. Hori O, Brett J, Slattery T, Cao R, Zhang J, Chen JX, *et al*. The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and co-expression of rage and amphoterin in the developing nervous system. *J Biol Chem* 1995; 270: 25752-61.

37. Hofmann MA, Drury S, Fu C, Qu W, Taguchi A, Lu Y, *et al*. RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. *Cell* 1999; 97: 889-901.