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**The role of AGE-RAGE system
in the development of diabetic nephropathy *in vivo***

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Abstract: Vascular complications are what eventually threaten the lives of diabetic patients. Here we show direct in vivo evidence that the interaction between advanced glycation endproducts (AGE), the formation of which is accelerated during prolonged hyperglycemic exposure, and a cell surface receptor for AGE (RAGE) is the major cause of such complications. We created transgenic mice that overexpress human RAGE in vascular cells and crossbred them with another transgenic line which develops insulin-dependent diabetes early after birth. The resultant double transgenic mice exhibited accelerated kidney changes compared with single transgenic littermates, and the nephropathy was ameliorated by an inhibitor of AGE formation. The AGE-RAGE system will thus be a promising target for overcoming diabetic complications.

Key words: diabetes, glycation, transgenic mouse, diabetic nephropathy, RAGE

Abbreviations: advanced glycation endproducts, AGE; receptor for AGE, RAGE; diabetes mellitus, DM; endothelial cell, EC; RAGE transgenic mice, RAGETg; inducible nitric oxide synthase, iNOS; iNOS transgenic mice, iNOSTg; N⁻-carboxymethyl-lysine, CML; reverse transcription-polymerase chain reaction, RT-PCR; hemoglobin A1c, HbA1c; periodic acid-Schiff, PAS; (±)-2-isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide, OPB-9195

Background: Exposure of proteins to reducing sugars like glucose results in nonenzymatic glycation which forms reversible Schiff bases and Amadori compounds [1]. A series of further complex molecular rearrangements then yield irreversible AGE [1]. In diabetes mellitus (DM), prolonged hyperglycemia superdrives this reaction and AGE accumulate in the circulating blood and in various tissues [1]. A hypothesis that interactions between

AGE and receptor for AGE (RAGE) [2, 3] are the major causes of diabetic vascular derangement has emerged from experiments with vascular endothelial cells (EC) [4, 5], pericytes [6] and renal mesangial cells [7] in culture and from AGE- or soluble RAGE-infusion studies in animals [8, 9]. To evaluate directly this hypothesis in vivo, we created RAGE transgenic mice (RAGETg) and analyzed the renal changes in these mice following the induction of diabetes.

Material and Methods:

1. Construction of transgenic mice and induction of diabetes. RAGETg was produced by introducing a transgene that carried human RAGE genomic DNA under the control of the murine *flk-1* promoter, which acts specifically in EC [10]. RAGETg was crossbred with another transgenic mouse carrying human cDNA for inducible nitric oxide synthase (iNOS) under the control of the insulin promoter (iNOSTg) [11].
2. Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated by the guanidinium thiocyanate method and reverse-transcribed as described [12].
3. Isolation of EC and monocytes, and western blot analysis. EC from mouse renal cortex and peripheral blood monocytes were isolated as described [12]. Proteins were extracted from the cells and underwent western blot analyses as described [12].
4. Determination of AGE. Serum N -carboxymethyl-lysine (CML) and non-CML AGE were differentially measured by a competitive ELISA as described [13]. Immunofluorescence analysis of the kidney sections was carried out with anti-CML and anti-non-CML AGE antibodies [13] as described [14].
5. Albuminuria. Urinary albumin and creatinine levels were determined as described [15], and used to calculate the albumin : creatinine ratio.
6. Renal histology. The severity of the renal sclerosis was scored on an arbitrary scale from

0 to 4 [14]. The mean glomerular volume was determined as described [14, 15]. Periodic acid-Schiff (PAS)-positive area in the mesangium was measured to quantify mesangial expansion [16].

Results: We could successfully create 5 lines of RAGETg. Two lines of them, 102 and 103, carrying high copy numbers of the transgene were used for subsequent experiments. RT-PCR analysis with human RAGE-specific primers revealed active transcription of the transgene in each line, and immunoblotting confirmed the overexpression of RAGE proteins in whole kidney, renal EC and peripheral monocytes. Renal glomeruli of RAGETg were positively stained for human RAGE in an EC pattern. Next, we employed a genetic approach by which a diabetic state and advanced glycation as well would be most stably induced. Viz, RAGETg was crossbred with another transgenic line-iNOSTg that consistently develops insulin-dependent diabetes as early as 1 week after birth, yielding four groups of littermates designated DoubleTg, iNOSTg, RAGETg, and non-transgenic control. Blood analysis revealed sustained hyperglycemia, high HbA1c levels and the progressive accumulation of non-CML AGE in the diabetic groups, but no significant differences between DoubleTg and iNOSTg. Serum CML was marginally increased in the diabetic groups.

Diabetic renal complication is characterized by increased albuminuria, glomerular hypertrophy and mesangial expansion as well as nephromegaly in the early phase [17]. In its late phase, glomerulosclerosis and increased serum creatinine follow [17]. The urinary albumin : creatinine ratio became significantly higher in DoubleTg than in the other groups at 4 months. The microscopic lesions noted in the diabetic groups consisted of glomerular cell proliferation, glomerular hypertrophy and mesangial expansion. DoubleTg showed accelerated increases in glomerular cell proliferation, glomerular volume, and mesangial

area and fraction in comparison with iNOSTg. Diffuse glomerulosclerosis progressed as the mice aged in both groups. However, there was a conspicuous difference between DoubleTg and iNOSTg in the severity of sclerosis, as evidenced by increased accumulation of PAS-positive materials in the mesangial area of the former group of animals. Quantitative examinations of at least 50 glomeruli per mouse revealed a significantly higher sclerosis index in DoubleTg than in iNOSTg at 4 months of age. Immunostaining showed that significant amounts of not only non-CML AGE but also CML accumulated in the mesangial area in the diabetic mice, but not in non-diabetic mice. At 6 months of age, the serum creatinine level of DoubleTg increased to 1.24 ± 0.07 mg/dl, being the highest among the groups. Further, typical nodular lesions and hyaline arteriosclerosis were noted at 8 months of age in DoubleTg.

We then conducted an intervention study with OPB-9195, a thiazolidine derivative that can inhibit AGE formation by blocking carbonyl groups on glycation intermediates. When the mice had received OPB-9195 p.o. for 5 months, the serum non-CML AGE and CML levels were reduced in diabetic groups, with the blood glucose and HbA_{1c} being essentially unaffected. The OPB-9195 treatment significantly suppressed the increase in both serum creatinine and the sclerosis index of DoubleTg at 6 months of age.

Conclusion: Overexpression of human RAGE gene in vascular cells has been demonstrated to cause a significant acceleration of all the early- and late-phase indices of diabetic nephropathy (Table 1), and the development of this complication was reversed by the inhibition of AGE formation. Because circulating levels of AGE and their deposition in renal glomeruli were essentially invariant between the diabetic animals carrying or not carrying the RAGE transgene, the level of RAGE expression was considered to have rate-limited the progression of diabetic nephropathy. The present study indicates that RAGE

engagement by AGE plays an active role in the development of diabetic nephropathy, 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 [18]. The double transgenic mice will serve as a most useful animal model for studying the pathogenesis of diabetic kidney complications and for testing remedies.

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Table 1. Chronological course of renal derangement in the present mouse model compared with type 1 diabetes patients

Stage	Chronology			Main structural changes or lesions	Glomerular filtration ratio	Albumin excretion	Blood pressure
	Human	DoubleTg	iNOSTg				
1. Early renal hypertrophy-hyperfunction	- 2 yrs -	- 2 mo	- 4 mo	Increased kidney size and glomerular size	Increased	maybe increased, but reversible	Normal
2. Renal lesions without clinical signs	Almost all in first 5 yrs	2 mo	4 - 6 mo	Mesangial expansion	Increased	Normal by definition	Normal
3. Incipient diabetic nephropathy	Typically after 6 - 15 yrs	4 mo	> 6 mo	Further mesangial expansion	Still supranormal, predicted to decline	Increased	incipient increase
4. Proteinuria, clinical overt diabetic nephropathy	After 15-25 yrs	6 mo		Clear and pronounced abnormalities	Decline Increased serum creatinine	Progressive clinical proteinuria	High
5. End-stage renal failure	Final outcome, after 25-30 yrs or more	> 6 mo		Glomerular closure and advanced glomerulopathy	Reduced Renal failure	Often some decline due to nephron closure	High

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