【総説】

第五回 高安賞最優秀賞受賞論文

論文 「RAGE deletion and inhibition improve diabetic nephropathy」

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Abstract: Diabetic nephropathy is a major microvascular complication in long-standing diabetic patients who eventually undergo renal dialysis or transplantation. To prevent the development of this disease and advanced kidney injury, effective remedies directed toward the key molecular target are required. We examined whether inhibition of the receptor for advanced glycation endproducts (RAGE) could attenuate changes in the diabetic kidney, and show here that inactivation of the RAGE gene in a mouse model of diabetic nephropathy results in significant suppression of diabetic kidney changes, compared with wild-type diabetic mice. Furthermore, we found that low-molecular weight heparin (LMWH) can bind RAGE at a *KD* value of approximately 17 nM and act as an antagonist to RAGE. LMWH treatment of mice significantly prevented diabetic nephropathy in a dose-dependent manner, and also significantly reversed the indices of advanced diabetic nephropathy. This study should provide insight into the pathological role of RAGE in both the early and advanced phases of diabetic nephropathy, and suggests that RAGE antagonists will be a useful remedy in the treatment of this disease.

Key Words: Diabetic nephropathy, receptor for advanced glycation endproducts (RAGE), advanced glycation endproducts (AGE), low-molecular weight heparin (LMWH).

INTRODUCTION

In recent years, the developed countries have witnessed a dramatic increase in the prevalence of diabetes mellitus (DM), predominantly relating this to lifestyle changes. Because of its chronic nature, several complications appear in patients with DM: the microvascular complications, including retinopathy, neuropathy and nephropathy, the macrovascular complication such as ischemic heart disease, occlusive peripheral vascular disease and cerebrovascular accidents. Among them, nephropathy is one of the most severe complications of diabetes, causing increased mortality due to end-stage renal disease[1], affecting ~ 40% of diabetic patients[2].

After the onset of diabetic nephropathy, albuminuria, glomerular hypertrophy and nephromegaly appear in early phase, followed by mesangial expansion, glomerulosclerosis, and increased serum creatinine in advanced stage. Mesangial expansion and development of glomerulosclerosis destroy the renal filtration unit and eventually lead to renal failure[3].

ADVANCED GLYCATION ENDPRODUCTS (AGE), RECEPTOR FOR AGE (RAGE) AND DIABETIC NEPHROPATHY

Exposure of proteins to reducing sugars like glucose causes non-enzymatic glycation to yield reversible Schiff bases and Amadori compounds. A series of further complex molecular rearrangements finally give rise to irreversible brown-colored compounds termed AGE. In diabetes, because of prolonged hyperglycemia, AGE formation and accumulation proceed at an accelerated rate, and AGE have been implicated in the development of diabetic vascular complications. Various AGE binding proteins and receptors are reported. Among them, receptor for AGE (RAGE) is the receptor that introduces signal transduction. Engagement of RAGE by AGE results in activation of transcription factor NF-kB and its nuclear translocation, thereby up-regulation of various cytokines and adhesion molecules, which would eventually lead to the development of diabetic vascular complications[4]. Our previous *in vivo* study with diabetic transgenic mice that overexpress human RAGE gene in vascular cells, showed statistically significant increases in indices of diabetic

nephropathy such as nephromegaly, albuminuria, glomerulosclerosis and serum creatinine when compared to the control diabetic transgenic mice[5]. Accordingly, we hypothesized that AGE-RAGE system plays an active role in the development of diabetic nephropathy, and that the transgenic animals should be a useful animal model for diabetic nephropathy.

DELETION OF RAGE GENE IMPROVES DIABETIC NEPHROPATHY

To test our hypothesis, we next created a mouse line that lacks endogenous RAGE gene. RAGE null mouse was developed by homologous recombination for gene targeting, and by subsequent Cre-*lox p* recombination. Some of the newborn mice were found to carry the deleted allele that lacks both RAGE exons 1 and 2, and the neo cassette. Mutant RAGE(+/-) mice were obtained, and crossbreeding between RAGE(+/-) mice resulted in the production of RAGE(-/-) homozygous mice. The RAGE(-/-) mice grew apparently normal without gross phenotypic or microscopic abnormalities. RT-PCR and western blot analyses revealed the absence of RAGE mRNA and protein production in the RAGE null mice.

To induce diabetes, RAGE(-/-) mice were crossbred with the other transgenic mice (iNOSTg) which consistently develop hypoinsulinaemic diabetes as early as 1 week after birth due to nitric oxide-mediated selective destruction of pancreatic β cells. iNOSTg mice represented a stable diabetic state (Fig. 1A) with enhanced AGE formation and accumulation. According to our previous findings, the diabetic transgenic mice (iNOSTg) itself showed glomerular hypertrophy until 4 months of age and progressive mesangial expansion and glomerulosclerosis afterward. Male heterozygous iNOSTg/RAGE(+/-) mice were then mated with female RAGE(+/-) and the resultant six groups of male littermates underwent subsequent analyses. Three groups carrying the iNOS transgene developed diabetes and were designated as DM^+RAGE(+/+), DM^+RAGE(+/-) and DM^+RAGE(-/-); the other three devoid of the iNOS transgene never developed diabetes and were designated as DM^-RAGE(+/+), DM^-RAGE(+/-) and DM^-RAGE(-/-). Nephromegaly expressed as KW/BW ratio and albuminuria were significantly reduced in DM^+RAGE(+/-) and DM^+RAGE(-/-), when compared with DM^+RAGE(+/+) mice. When periodic acid-schiff-positive (PAS-positive) area in the mesangium was determined to quantify mesangial expansion, the score was significantly reduced in glomeruli of RAGE(-/-) mice. Sclerosis index was also significantly attenuated in the diabetic RAGE(+/-) and (-/-) mice when compared with diabetic RAGE(+/+) mice (Fig. 1B). We noticed that the values obtained with the DM^+RAGE(+/-) mice were consistently intermediate between those with DM^+RAGE (+/+) and DM^+RAGE(-/-) mice, indicating that the extent

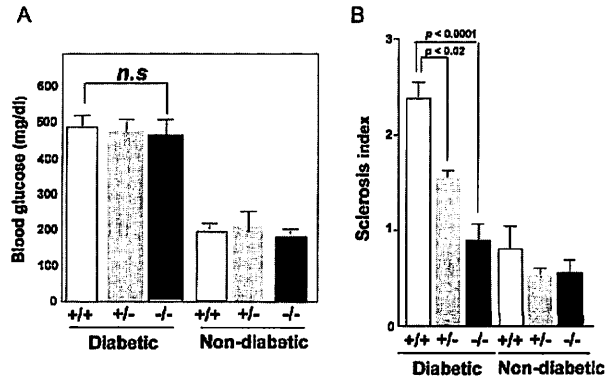

Fig. 1. Amelioration of diabetic nephropathy in RAGE-deficient mice[6]. (A) Diabetic state. (B) Renal glomerulosclerosis.

of attenuation of diabetic nephropathy is proportional to RAGE gene dosage. RAGE ligands - AGE and S100 protein and tissue growth factors, which are known to link to diabetic nephropathy, such as transforming growth factor β (TGF β) connective tissue growth factor (CTGF) and vascular endothelial growth factor (VEGF) more densely accumulated in the glomeruli of diabetic RAGE (+/+) mice when compared to diabetic RAGE(-/-) mice. The evidence suggests that deletion of RAGE gene attenuates the expression of molecular mediators of glomerulosclerosis probably by reducing the ligand actions, thereby improving diabetic kidney changes (6). RAGE would seem, therefore, to be a promising target for overcoming diabetic nephropathy, and if a compound was available to antagonize the AGE action on RAGE, an effective remedy against this disease could be developed.

LOW-MOLECULAR WEIGHT HEPARIN (LMWH) ACT AS AN ANTAGONIST OF RAGE AND IMPROVES DIABETIC NEPHROPATHY

RAGE would thus be a candidate molecule worth targeting for overcoming diabetic nephropathy. However, no RAGE antagonists have so far been available. Since RAGE is a heparin-binding protein, we speculated that heparin might influence AGE-RAGE interactions. We initially tested the unfractionated high molecular weight heparin. It was found to inhibit AGE association with RAGE. Its net action on RAGE, however, was agonistic, when assessed with human endothelial cells in culture. We next examined whether fragmented and fractionated heparin - low-molecular weight heparin (LMWH) can inhibit the AGE-RAGE interaction and antagonize RAGE signaling. LMWH largely lost N-sulfate residues during the fragmentation by nitrous acid and its biological actions were speculated to be different from unfractionated high-molecular weight heparin. First, we employed surface plasmon resonance assay (SPR) to check LMWH binding to the purified extracellular domain of RAGE. The assay

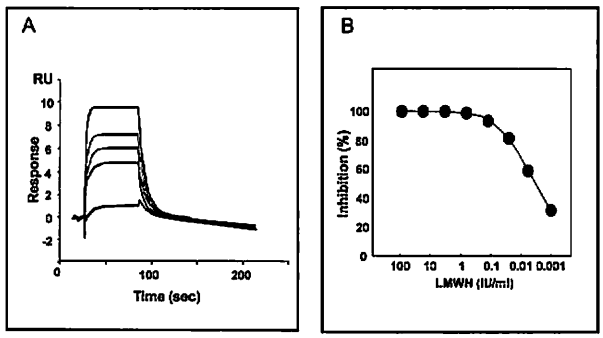

Fig. 2. Physical association between LMWH and purified recombinant RAGE proteins (A) and LMWH competition of AGE-RAGE binding (B)[6].

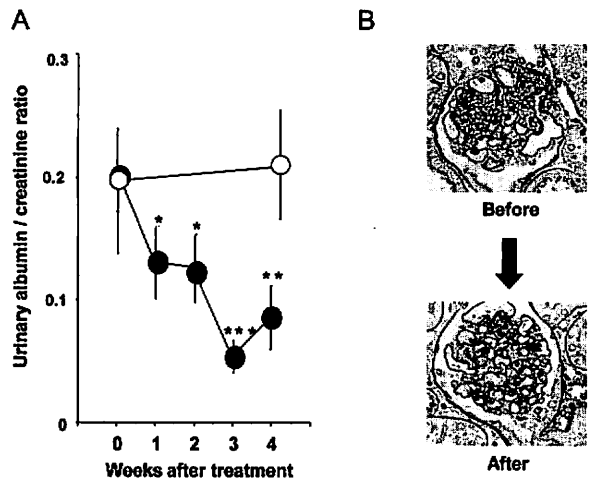

Fig. 3. Reversal by LMWH of albuminuria (A) and glomerulosclerosis (B)[6]. ○, vehicle (PBS); ●, LMWH. *, $p<0.05$; **, $p<0.015$; ***, $p<0.0025$.

revealed that LMWH is able to bind RAGE with an affinity approximately 6 times higher than AGE (i.e., ~ 17 nM versus ~ 100 nM) (Fig. 2A). Secondly, a dose-dependent competitive inhibition by LMWH was demonstrated in a plate assay of AGE binding to RAGE (Fig. 2B). Thirdly, to check its antagonistic activity in RAGE signaling, we performed an NF-κB promoter-luciferase reporter assay with RAGE-overexpressing cells. AGE-induced NF-κB promoter-driven enzyme activity was significantly reduced by the addition of LMWH. Finally, 0.1-1.0 IU/ml of LMWH have significantly inhibited AGE-induced VEGF mRNA upregulation in RAGE-overexpressing endothelial cells (ECV304) and AGE-induced VCAM-1 expression in human umbilical vein endothelial cells (HUVEC). These findings clearly indicate that LMWH exerts an antagonistic action on RAGE[6].

We then conducted an interventional study with LMWH. Mice were divided into five groups; one non-diabetic control group and four iNOSTg diabetic groups that received LMWH via an osmotic pump, daily sc injection of 40 or 80 IU of LMWH and vehicle alone. Each group was treated and observed from one month (subclinical state of diabetic nephropathy) to four months (overt and advanced nephropathy state) of age. Although LMWH treatment did not lower the blood glucose and HbA1c levels of each group, albuminuria expressed as urinary albumin/creatinine ratio, deposition of PAS-positive materials in glomeruli, and glomerulosclerosis, were found to be prevented in LMWH-treated diabetic groups when compared with the control diabetic group. Finally, we performed a therapeutic intervention with LMWH in diabetic mice from 4 (advanced diabetic nephropathy state confirmed by open renal biopsy) to 5 months of age. Treatment with LMWH 80 IU daily sc injection for one month revealed significant reduction in urinary albumin/creatinine ratio, mesangial expansion and glomerulosclerosis (Fig. 3)[6].

CONCLUSION

Diabetic nephropathy is one of the chronic complications of diabetes mellitus that directly account for short life expectancy and poor quality of life. AGE have been regarded as the main molecular target in diabetic complications, and inhibition of AGE-RAGE interaction and of subsequent signal transduction would also seem to be a promising way for overcoming this diseases. LMWH is clinically used in the treatment and prophylaxis of deep vein thrombosis, pulmonary embolism and myocardial infarction. Several reports revealed non-anticoagulant therapeutic functions of LMWH, but the underlying molecular mechanism has not yet been elucidated. Our present findings of its antagonistic action on RAGE as well as the findings in RAGE-deficient animals may contribute to improvement in the management of diabetic nephropathy and other RAGE-related diseases.

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