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Suppression of transforming growth factor beta and vascular endothelial growth factor in diabetic nephropathy in rats by a novel advanced glycation end product inhibitor, OPB-9195

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Abstract

Aims/hypothesis. Advanced glycation end products (AGEs) participate in the pathogenesis of diabetic nephropathy. We reported earlier that OPB-9195, a synthetic thiazolidine derivative and novel inhibitor of advanced glycation, prevented progression of diabetic glomerulosclerosis by lowering serum concentrations of advanced glycation end products and reducing their deposition in the glomeruli. Here, we examined their contribution and that of growth factors, such as transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF), to the progression of diabetic nephropathy. We also investigated the expression of type IV collagen in the kidneys of Otsuka-Long-Evans-Tokushima-Fatty (OLETF) rats, a Type II (non-insulin-dependent) diabetes mellitus model, after treatment with OPB-9195.
Methods. Using northern blots and immunohistochemical techniques, we determined the renal expression of TGF-β and type IV collagen mRNAs and proteins in OLETF rats. We also examined OPB-9195's effects on renal expression of VEGF mRNA and protein.
Results. Concomitant increases in TGF-β and type IV collagen expression were observed at each point in time in OLETF rats not given OPB-9195. In contrast, OPB-9195 treatment greatly suppressed the renal expression of TGF-β, VEGF and type IV collagen mRNAs and proteins to that seen in non-diabetic rats.
Conclusion/interpretation. Since OPB-9195, an AGE-inhibitor, prevented the progression of diabetic nephropathy by blocking type IV collagen production and suppressing overproduction of two growth factors, TGF-β and VEGF, in diabetic rats, this compound warrants further investigation. [Diabetologia (1999) 42: 579–588]

Keywords Type II diabetes mellitus, extracellular matrix, glomerulosclerosis, type IV collagen, Otsuka-Long-Evans-Tokushima-Fatty rat.

The dominant histological feature of diabetic nephropathy is expansion of the extracellular matrix (ECM) in the mesangial areas of the glomeruli and thickening of the basement membrane, with resulting glomerulosclerosis [1, 2]. As the disease progresses, increased mesangial expansion and increased thickening of the basement membrane lead to obliteration of the glomerular capillary lumen, proteinuria and loss of glomerular filtration [3, 4].

Nonenzymatic glycation has been implicated in the pathogenesis of long-term complications of diabetes mellitus, including diabetic nephropathy [5–8]. In particular, advanced forms of Maillard reaction products, named advanced glycation end products (AGEs) [6, 7, 9–11], have been postulated to participate in glomerular injury. They have been shown to accumulate in the kidneys of diabetic rats in parallel

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Abbreviations: AGEs, Advanced glycation end products; AG, aminoguanidine; VEGF, vascular endothelial growth factor; OLETF, Otsuka-Long-Evans-Tokushima-Fatty; LETO, Long-Evans-Tokushima-Fatty; ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

with the development of albuminuria and mesangial expansion [12]. More direct evidence of the contribution of AGEs to diabetic nephropathy is that treating normal rats with exogenous AGEs induces glomerular hypertrophy, mesangial sclerosis, gene expression of matrix proteins and production of growth factors, such as transforming growth factor-beta (TGF-β) and platelet-derived growth factor (PDGF) [10, 13–15]. Advanced glycation end products may cause tissue injury both directly through phenomena such as trapping and cross-linking [8] and indirectly, by binding to specific receptors (AGEs receptors) on the surface of various cells [16–19]. Effects of AGEs are mediated by various growth factors and cytokines which modulate, in a paracrine/autocrine fashion, the altered cell proliferation, enhanced ECM accumulation and haemodynamic, permeability and haemorrheological changes characterizing diabetic microangiopathy [20]. Intensive investigation into the pathogenesis of ECM accumulation in diabetes has consistently implicated the prosclerotic cytokine, TGF-β, as a key mediator [21, 22].

Aminoguanidine (AG) [23], a hydrazine-like compound which blocks AGE formation by interacting with Amadori-derived products [24], has been shown to retard the development of diabetic microvascular and macrovascular complications in experimental animals [10, 12, 13, 15, 25, 26]. In relation to diabetic nephropathy, AG attenuates the rise in albuminuria and prevents mesangial expansion in diabetic rats [27, 28]. Aminoguanidine has a renoprotective effect and the finding that this effect is related to the duration of therapy emphasizes its potential importance in clinical events [26].

The AGE-inhibitor, OPB-9195, was discovered in a Japanese pharmaceutical company, Fuji Memorial Research Institute, which belongs to Otsuka Pharmaceutical. The compound is a synthetic thiazolidine derivative. They initially aimed at developing an insulin sensitizer but then shifted their focus to AGE inhibitors. They had screened many compounds for candidates as insulin sensitizers and accidentally discovered that OPB-9195 was a strong AGE inhibitor. Using Otsuka-Long-Evans-Tokushima-Fatty (OLETF) rats, we found that OPB-9195 inhibits the formation of AGEs but has no effect in lowering blood glucose, dramatically prevents the development of diabetic nephropathy (as noted microscopically) and significantly prevents sclerotic changes [29]. In addition, albumin excretion by OPB-9195-treated OLETF rats was decreased 60 % compared with untreated OLETF rats [29].

Transforming growth factor-β has been implicated in vascular remodelling that accompanies hypertension [30] and in the pathogenesis of diabetic nephropathy [31]. Hyperglycaemia and AGEs increase TGF-β production [22] and TGF-β stimulates the synthesis of individual matrix components such as collagens, fibronectin, laminin and proteoglycans [32, 33] and simultaneously blocks matrix degradation by decreasing the synthesis of proteases and increasing concentrations of protease inhibitors [34, 35]. It also increases the expression of some integrins and changes their relative proportions on the surface of cells in a manner which might facilitate adhesion to the extracellular matrix [36].

Vascular endothelial growth factor (VEGF), a 45 kD protein, was originally discovered to be a tumour-secreted protein which rendered venules and small veins hyperpermeable to circulating macromolecules and has a potency some 50 000 times that of histamine [37]. It is expressed at low concentrations in a wide variety of normal adult human and animal tissues and at higher concentrations in a few select sites, namely podocytes of the renal glomeruli and cardiac myocytes [38, 39]. Human mesangial cells and peripheral blood mononuclear cells produce VEGF in vitro [40]. Since VEGF promotes the growth of vascular endothelial cells and enhances permeability, VEGF produced by podocytes, mesangial cells or monocytes possibly induces the proliferation of glomerular endothelial cells or enhances the permeability of glomerular capillaries. The precise roles of VEGF in the kidney are, however, still not clear.

With the objective of elucidating the precise roles of VEGF in the kidney, we used the OLETF rat model to study diabetic nephropathy because the time course and morphological changes in the kidney of this model closely resemble the nephropathy seen in human Type II (non-insulin-dependent) diabetes mellitus. We examined the contributions of AGEs and growth factors, such as TGF-β and VEGF, to the progression of diabetic nephropathy, and also investigated the expression of type IV collagen in the kidney after treatment with OPB-9195, a novel AGE inhibitor.

Materials and methods

Experimental animals. Male OLETF rats, a model of Type II diabetes mellitus established in 1990 at Tokushima Research Institute, were obtained from that same institute (Otsuka Pharmaceutical) [41]. Male Long-Evans-Tokushima-Fatty (LETO) rats served as normal controls. We prepared three groups of four animals each: control group, diabetes group and diabetes with OPB-9195 (Tokushima Research Institute, Otsuka Pharmaceutical) treatment group [29]. In the OPB-9195 treatment group, OPB-9195 was given daily to the OLETF rats from 24 weeks of age by mixing in the chow at a concentration of 1 mg/g. These rats were killed at 9, 20 (before treatment) and 68 weeks of age. Each animal was weighed, anaesthetized with sodium pentobarbital (50 µg per g of body weight, i. p.) and decapitated and blood was collected for determination of the plasma glucose. For this determination we used the glucose oxidase technique and we also determined the plasma creatinine to assess renal function. One kidney was processed for immunohistochemical studies, as described below. The other kidney was immediately placed in liquid nitrogen and stored at –80 °C for subsequent RNA extraction.

Northern analysis. Each kidney was homogenized, and the total RNA was isolated by the acid guanidium thiocyanate-phenol-chloroform extraction method [42]. For northern blots, 20 µg of total RNA was electrophoresed on a 1.5 % agarose gel containing 2.2 mol/l formaldehyde and the blot was transferred to a nylon membrane (Biodyne Nylon Membranes; PALL, N. Y., USA) by capillary blotting and then crosslinked by exposure to ultraviolet light. The cDNA probes for mouse TGF-β1, mouse α1 (IV) collagen, mouse VEGF and mouse housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were synthesized by the polymerase chain reaction (PCR) using specific oligonucleotide primers and murine kidney cDNA as a template. The primer pair for TGF-β1 (sense, 5′-AAGAACTGCTGTGTGCGG-3′, and antisense, 5′-GCACTTGCAGGAGCGCACAA-3′) yielded a 296-bp PCR product [43]. The primer pair for α1 (IV) collagen (sense, 5′-GTGCGGTTTGTGAAGCACCG-3′, and antisense, 5′-GTTCTTCTCATGCACACTT-3′) yielded a 363-bp PCR product [43]. The primer pair for GAPDH (sense, 5′-AATGCATCCTGCACCACCAA-3′, and antisense, 5′-GTAGCCA TATTCATTGTCATA-3′) yielded a 515-bp PCR product [43]. The primer pair for VEGF (sense, 5′-CTGCTCTCTT GGGTGCACTGG-3′, and antisense, 5′-CACCGCCTTGGC TTGTCACAT-3′ yielded a 563-bp PCR product of VEGF164 and a 431-bp PCR product of VEGF121 [44]. Prehybridization was done for 3 h at 42 °C in hybridization buffer containing 5 × Denhardt's solution [1 × Denhardt's solution = 0.02 % Ficoll-400, 0.02 % polyvinylpyrrolidone, 0.02 % bovine serum albumin (BSA, fraction V)], 5 × standard saline citrate (SSC) (1 × SCC is 150 mmol/l NaCl and 15 mmol/l sodium citrate, pH 7.0), 1 % SDS, 1 % denatured salmon sperm DNA, and 1 % yeast tRNA. Probes were labelled with [α-^{32}P] dCTP (111 TBq/mmol; Amersham, Tokyo, Japan) using a random primed synthesis (DNA labelling kit; Boehringer Mannheim, Tokyo, Japan). For each hybridization, we added the ^{32}P-labelled probe to a final concentration of 1×10^7 counts · min^{-1} · ml^{-1} of fresh prehybridization solution followed by incubation at 42 °C for 16–24 h. Filters were then washed in solutions of decreasing ionic strength and increasing temperature. The final stringency was 0.1 × SSC with 0.3 % SDS for 20 min at 65 °C. The intensity of hybridization was quantified using a phosphorescent image analyser (Fuji BAS5000; Fuji Photo Film, Tokyo, Japan). All results were corrected for differences in RNA loading and transfer by rehybridization with a probe for mouse GAPDH cDNA. The results were expressed as the ratio of image intensity of TGF-β1, α1(IV) collagen and VEGF to GAPDH relative to the control kidneys of LETO rats, which were arbitrarily assigned a value of 1.

Immunohistochemistry. For immunohistochemical staining, isolated kidney tissues were embedded in OCT-compound (Tissue-Tek, Miles, USA) and rapidly frozen at –80 °C. Cryostat sections (4 mm) were fixed in cold acetone for 10 min. Endogenous peroxidase activity was quenched with 0.6 % hydrogen peroxidase in methanol for 5 min. The sections were then blocked with 0.1 % normal rat IgG to prevent nonspecific antibody staining by a secondary antibody. The primary antibodies applied were monoclonal rabbit anti-mouse type IV collagen antibody (LSL, Tokyo, Japan), monoclonal mouse anti-TGF-β1, -β2, -β3 antibody (Genzyme, Cambridge, Mass., USA), and monoclonal mouse anti-human VEGF antibody (IBL, Tokyo, Japan). The sections were then incubated with primary antibodies for 24 h at 4 °C, washed in phosphate-buffered saline (PBS) and incubated with biotinylated anti-mouse rabbit IgG and peroxidase-conjugated streptavidine (DAKO Japan, Sapporo, Japan). Peroxidase conjugates were subsequently localized using diaminobenzidine tetrahydrochloride (DAB) as a chromogen.

Immunohistochemical staining was semiquantitative by using a scale of 0–4 (0 = no staining, 4 = maximum staining) as described elsewhere [45] and the average score of 20 randomly selected glomeruli was calculated. Each slide was scored by an observer blinded to the experimental design. The same set of experiments was repeated at least three times.

Statistical analysis. For all statistical tests, we used raw data, and the means ± SE is given. Data were analysed using the Stat View II program (Abacus Concepts, Berkeley, Calif., USA) on a Macintosh personal computer (Apple Computer, Tokyo, Japan). Comparisons between groups were analysed using the Mann-Whitney U test or Kruskal-Wallis test. A p value of less than 0.05 was considered statistically significant.

Results

Clinical characteristics of study rats. At 50 weeks of age, there were no differences in body weight among the three groups, indicating that OPB-9195 treatment had no influence on weight gain (Table 1). Plasma glucose concentrations were higher in the OLETF rats than in the LETO rats from 20 weeks of age but were similar in OLETF rats at 50 weeks of age regardless of whether OPB-9195 was given or not. There were no significant changes in renal function

Table 1. Clinical characteristics of LETO, OLETF and OPB-9195 treated OLETF rats

Group	*n*	body weight g	Plasma glucose mmol/l	Creatinine µmol/l	Urine albumin mg/mgCr
A 9W					
LETO	4	256 ± 13	7.32 ± 0.94	59.23 ± 1.77	0.52 ± 0.16
OLETF	4	241 ± 17	6.61 ± 1.39	48.62 ± 6.19	1.17 ± 0.18[a]
B 20W					
LETO	4	444 ± 2	10.66 ± 0.94	N. D.	0.54 ± 0.14
OLETF	4	565 ± 12[a]	12.60 ± 1.05	N. D.	18.12 + 2.95[a]
C 50W					
LETO	4	582 ± 14	11.38 ± 1.72	63.65 ± 4.42	12.84 ± 6.83
OLETF	4	570 ± 38	24.26 ± 2.89[a]	77.79 ± 7.07	86.81 ± 15.42[a]
OPB-9195	4	604 ± 24	25.42 ± 8.60[a]	62.76 ± 20.33	47.76 ± 7.87[b]

Data are mean ± SE. N. D.; not done
[a] $p < 0.05$ vs LETO; [b] $p < 0.05$ vs. OLETF

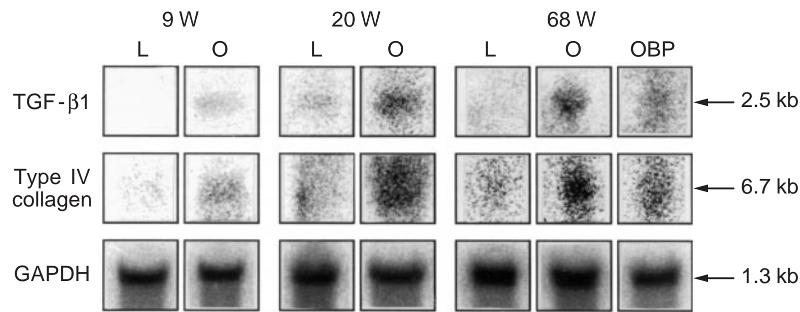


Fig. 1. Gene expression for TGF-β1 and collagen type IV at 9, 20 and 68 weeks in kidneys from diabetic animals (OLETF rats), normal controls (LETO rats) and OPB-9195-treated diabetic animals (OPB-9195 treated OLETF rats). L: LETO (control rats); O: OLETF (diabetic rats); and OPB: OPB-9195-treated OLETF rats. W, weeks

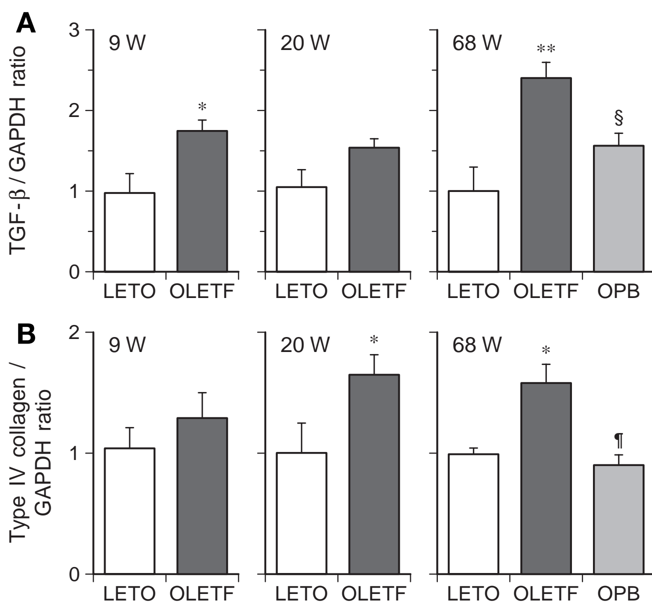


Fig. 2. A, B Quantitation of renal TGF-β1 (**A**) and collagen type IV (**B**) mRNAs. Data are shown as the means ± SEM of the ratio of photo stimulated luminescence (PSL) of specific mRNA to that of GAPDH mRNA, relative to the control animals (designated an arbitrary value of 1) at 9, 20 and 68 weeks. *$p < 0.05$, **$p < 0.01$ vs. LETO; § $p < 0.05$, ¶ $p < 0.01$ vs OLETF. OPB: OPB-9195-treated OLETF rats. W, weeks

among the three groups, although at 50 weeks the plasma creatinine of OLETF rats not treated with OPB-9195 was slightly higher than that of control rats and OLETF rats treated with OPB-9195. Urinary albumin excretion in the OLETF rats was higher than in the LETO rats at each point in time (Table 1). The difference in urinary albumin excretion between the OLETF and LETO rats gradually increased with the duration of diabetes. Albumin excretion by the OLETF rats treated with OPB-9195 was decreased by 45% compared with the untreated OLETF rats at 50 weeks of age ($p < 0.05$) (Table 1).

Expression of TGF-β and type IV collagen mRNAs. Kidney TGF-β1 mRNA was increased in the diabetic rats at 9, 20 and 68 weeks compared with the non-diabetic controls (Fig. 1). Similarly, type IV collagen mRNA expression was increased in the diabetic rats at 20 weeks and 68 weeks compared with the findings in the non-diabetic controls (Fig. 1). The TGF-β1:GAPDH ratio in the OLETF rats at 9, 20 and 68 weeks increased 1.8-fold, 1.5-fold and 2.4-fold compared with the control rats, respectively (Fig. 2A). The type IV collagen:GAPDH ratio in the OLETF rats at 9, 20 and 68 weeks was also increased 1.3-fold, 1.7-fold and 1.6-fold compared with the control rats, respectively (Fig. 2B, lower panel). Thus, the increase in TGF-β1 mRNA paralleled the type IV collagen mRNA expression as well as the progression of glomerulosclerosis. Treatment with OPB-9195 significantly suppressed the overexpression of TGF-β1 and type IV collagen, even at 68 weeks. In particular, at 68 weeks, OPB-9195 reduced the expression of type IV collagen to that of the control (Fig. 2B).

Expression of TGF-β protein. Although TGF-β was weakly stained in normal glomeruli at each point in time, its expression in diabetic glomeruli gradually increased with the duration of diabetes (Figs. 3 and 4). Increased staining of TGF-β in diabetic glomeruli was detected even at 9 weeks of age and maximum staining was seen at 68 weeks (Figs. 3 and 4). Positive staining for TGF-β was mainly within the glomeruli, especially the mesangial area, while being weak in the tubules. This increase in positive staining for TGF-β correlated with the progression of diabetic glomerulosclerosis (Figs. 3 and 4). In contrast, OPB-9195 treatment dramatically reduced the expression of TGF-β protein in the glomeruli, generally to that seen in the control non-diabetic LETO rats (Fig. 4).

Expression of type IV collagen protein. Immunohistochemical studies showed abundant type IV collagen protein expression in the extracellular matrix of the glomeruli and the basement membrane of the tubules

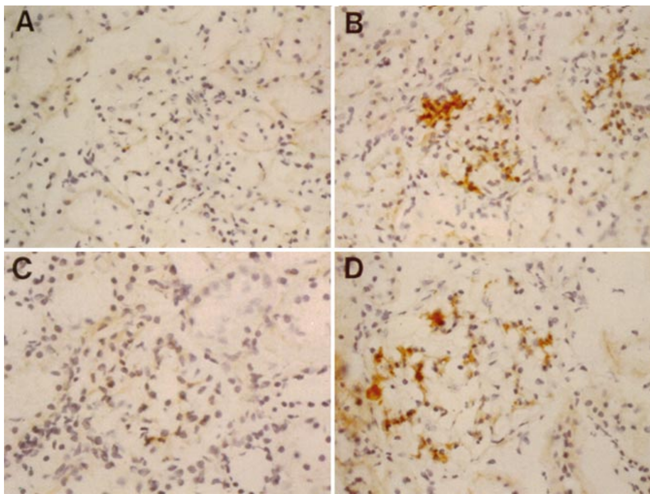


Fig. 3. A–D Immunohistochemical findings for TGF-β protein at 9 weeks in control animals (**A**) and diabetic animals (**B**) and at 20 weeks in control animals (**C**) and diabetic animals (**D**). Magnification × 100

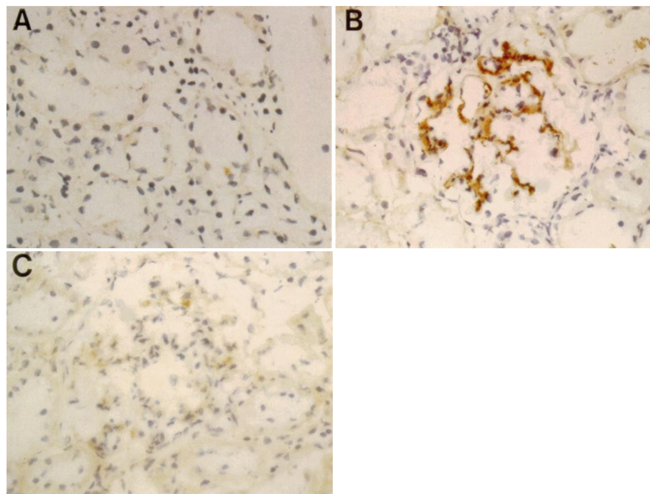


Fig. 4. A–C Immunohistochemical findings of TGF-β protein at 68 weeks in control animals (**A**), diabetic animals (**B**) and OPB-9195-treated diabetic animals (**C**). Magnification × 100

from the diabetic OLETF animals. This indicates a close relation between type IV collagen protein as well as increased type IV collagen mRNA expression and the progression of glomerulosclerosis (Figs. 5 and 6). Type IV collagen began to increase at 20 weeks and continued to increase gradually up to 68 weeks (Figs. 5 and 6). In contrast, type IV collagen protein in the control non-diabetic rats (LETO) remained relatively low throughout the period of observation, although there was a slight increase with aging. In the OLETF rats treated with OPB-9195, type IV collagen protein was dramatically reduced nearly to that seen in the control non-diabetic LETO rats (Fig. 6).

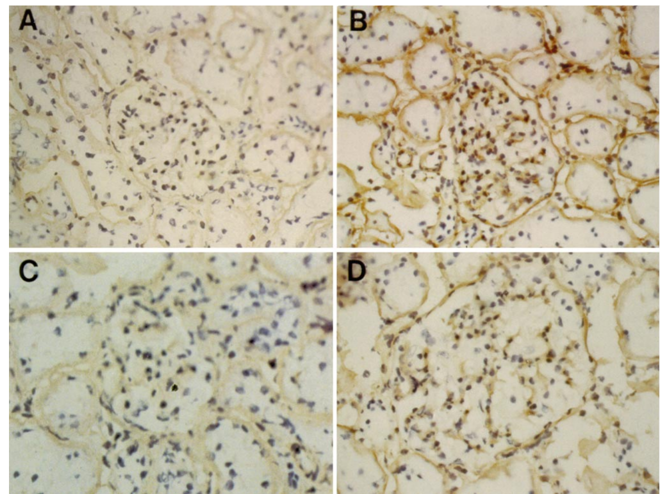


Fig. 5. A–D Immunohistochemical findings for type IV collagen protein at 9 weeks in control animals (**A**) and diabetic animals (**B**) and at 20 weeks in control animals (**C**) and diabetic animals (**D**). Magnification × 100

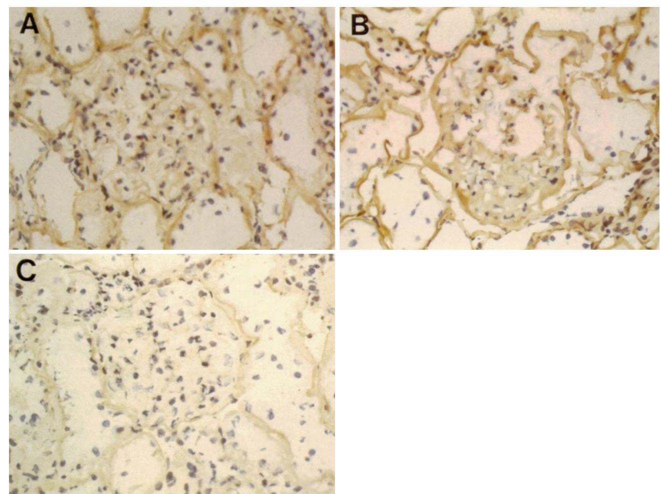


Fig. 6. A–C Immunohistochemical findings for type IV collagen protein at 68 weeks in control animals (**A**), diabetic animals (**B**) and OPB-9195-treated diabetic animals (**C**). Magnification × 100

Expression of VEGF mRNA and VEGF protein. Kidney VEGF mRNA was increased in the OLETF rats at 9, 20 and 68 weeks compared with the non-diabetic controls (LETO) (Fig. 7). The VEGF:GAPDH ratio in the diabetic rats at 9, 20 and 68 weeks was increased 2.1-fold, 1.4-fold and 1.6-fold, respectively, compared with the control rats (Fig. 8). At 68 weeks OPB-9195 prevented the overexpression of VEGF (Fig. 5). Treatment with OPB-9195 greatly decreased VEGF mRNA expression. In contrast to the expression of TGF-β and type IV collagen mRNA, VEGF mRNA expression increased, however, only in the early period of diabetic nephropathy (9 and 20 weeks) and did not increase with the duration of

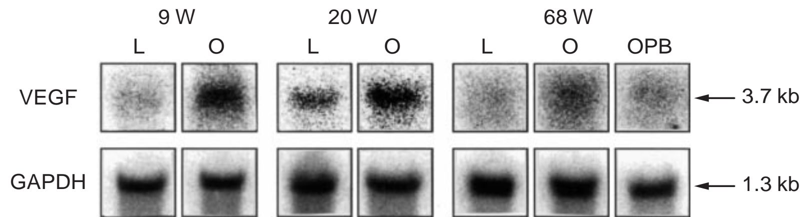


Fig. 7. Gene expression for VEGF at 9, 20 and 68 weeks in kidneys of diabetic animals (OLETF rats), normal controls (LETO rats) and OPB-9195-treated diabetic animals (OPB-9195-treated OLETF rats). L: LETO (control rats); O: OLETF (diabetic rats); and OPB: OPB-9195-treated OLETF rats. W, weeks

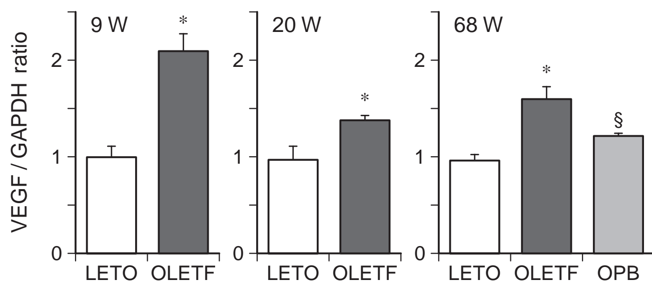


Fig. 8. Quantitation of renal VEGF mRNA. Data are shown as the means ± SEM of the ratio of photo stimulated luminescence of specific mRNA to that of GAPDH mRNA, relative to the control animals (designated an arbitrary value of 1) at 9, 20 and 68 weeks. *$p < 0.05$ vs LETO; § $p < 0.05$ vs OLETF. W, weeks

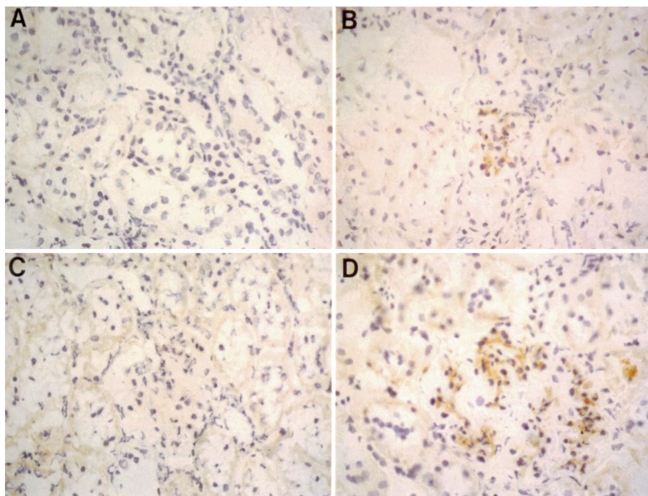


Fig. 9. A–D Immunohistochemical findings for VEGF protein at 9 weeks in control animals (**A**) and diabetic animals (**B**) and at 20 weeks in control animals (**C**) and diabetic animals (**D**). Magnification × 100

diabetes. Increased staining for VEGF in diabetic glomeruli was detected at 9, 20 and 68 weeks. (Figs. 9 and 10). The location of positive staining for VEGF was mainly within the glomeruli. In the diabetic rats treated with OPB-9195, VEGF protein was reduced nearly to that seen in the non-diabetic control rats (Fig. 10).

Semiquantitation of glomerular TGF-β, type IV collagen and VEGF protein. Figure 11A plots the glomerular immunohistochemical staining score of TGF-β. By semiquantitative scoring, an increased TGF-β score was observed in the glomeruli of the diabetic rats compared with the control rats at 9 weeks (control: 0.4 ± 0.2; diabetic: 2.0 ± 0.1; $p < 0.001$), 20 weeks (control: 0.5 ± 0.2; diabetic: 2.8 ± 0.2; $p < 0.001$) and 68 weeks (control: 0.7 ± 0.2; diabetic: 3.8 ± 0.1; $p < 0.001$). Treatment with OPB-9195 reduced the TGF-β staining in diabetic glomeruli to that observed in the control rats (0.8 ± 0.1; $p < 0.001$ vs diabetic). The results of quantitative analysis of type IV collagen protein expression at each point in time are shown in Fig. 11B. By semiquantitative scoring, an increased type IV collagen score was observed in the ECM of the diabetic rats compared with the control rats at 9 weeks (control: 0.9 ± 0.2; diabetic: 1.4 ± 0.2; $p = 0.06$), 20 weeks (control: 1.3 ± 0.2; diabetic: 2.6 ± 0.2; $p < 0.001$) and 68 weeks (control: 2.0 ± 0.2; diabetic: 3.8 ± 0.2; $p < 0.001$). Treatment with OPB-9195 reduced the type IV collagen protein in diabetic ECM to that observed in the control rats (1.6 ± 0.2; $p < 0.001$ vs diabetic). By semiquantitative scoring, an increased VEGF score was observed mainly in the glomeruli of the diabetic animals compared with the control rats at 9 weeks (control: 0.3 ± 0.2; diabetic: 2.0 ± 0.1; $p < 0.001$), 20 weeks (control: 0.5 ± 0.2; diabetic: 2.4 ± 0.2; $p < 0.001$) and 68 weeks (control: 0.5 ± 0.2; diabetic: 3.8 ± 0.1; $p < 0.001$). Treatment with OPB-9195 reduced the VEGF protein in diabetic ECM to that observed in the control rats (1.6 ± 0.2; $p < 0.001$ vs diabetic) (Fig. 11 C).

Discussion

We reported that a novel AGE inhibitor, OPB-9195, greatly inhibited diabetic nephropathy in OLETF rats, a model of Type II diabetes mellitus, both functionally and pathologically. Compared with AG, OPB-9195 had a tenfold effect in inhibiting AGEs'

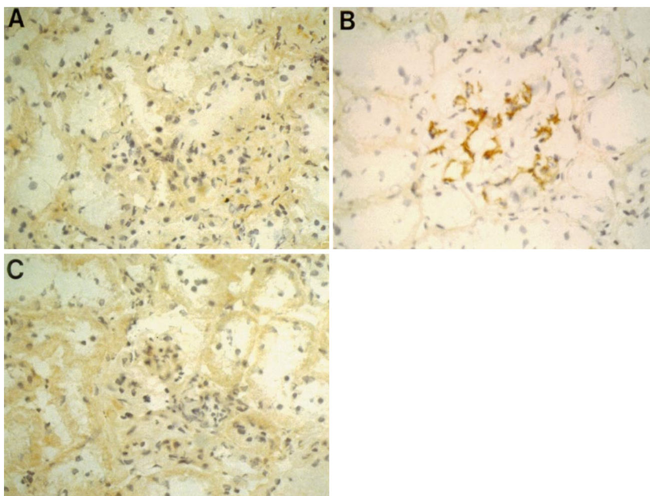


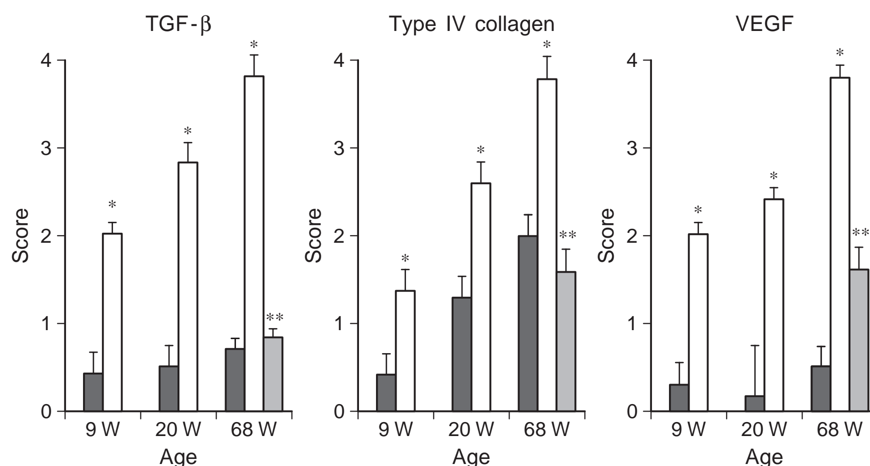
Fig. 10. A–C Immunohistochemical findings for VEGF protein at 68 weeks in control animals (**A**), diabetic animals (**B**) and OPB-9195-treated diabetic animals (**C**). Magnification × 100

formation and diabetic nephropathy. Even a relatively small dose of OPB-9195 ameliorated diabetic nephropathy in OLETF rats by inhibiting the accumulation of AGEs in the kidneys. We found OPB-9195 also decreased albumin excretion during the late phase of diabetic nephropathy. In a histological study, OPB-9195 considerably inhibited mesangial expansion, basement thickness, glomerulosclerosis and AGE accumulation in the glomeruli of OLETF rats [29].

Many studies in experimental animals and humans have implicated overexpression of TGF-β in the pathogenesis of diabetic glomerulosclerosis [46, 47]. There could be various mechanisms responsible for the raised TGF-β mRNA expression observed in diabetic kidneys in our study. High serum glucose induced TGF-β expression by several cell lines in vitro but the precise mechanisms leading to altered gene regulation are not well understood. Mechanisms which have been proposed and may give rise to TGF-β overexpression were shown in a study of glucose-mediated activation of protein kinase C, cell stretch associated with hyperperfusion, the production of Amadori glycated proteins and AGEs [5, 48]. There were several ways in which TGF-β influenced ECM accumulation including increased ECM synthesis, decreased ECM degradation and modulation of the interaction between ECM and neighbouring cells [21]. Neutralizing anti-TGF-β antibodies in vivo suppressed diabetes-related kidney growth [49]. In vivo studies have shown treatment with AGE-modified mouse serum albumin, generated in vitro, induced gene expression of type IV collagen and TGF-β1 in the glomeruli [15]. Intensive investigation into the pathogenesis of ECM accumulation in diabetes has consistently implicated the prosclerotic cytokine, TGF-β, as a key mediator [21, 22].

Combined with these observations regarding TGF-β and AGEs, we hypothesized that the AGE inhibitor, OPB-9195, is perhaps able to ameliorate diabetic nephropathy by blocking TGF-β production. Our current in vivo experiments show that OPB-9195 inhibited the progression of diabetic nephropathy by suppressing expression of TGF-β and VEGF. We also noted that progression of diabetic nephropathy is associated with increased TGF-β, type IV collagen and VEGF mRNA and that overexpression, in conjunction with ECM expansion and glomerulosclerosis, was attenuated when rats were given OPB-9195. These findings suggest that increased AGE formation in diabetes mellitus induces TGF-β and VEGF overexpression and diabetic glomerulosclerosis progresses.

Fig. 11. A–C Semiquantitation of glomerular TGF-β (**A**), collagen type IV (**B**) and VEGF (**C**) protein levels by immunoperoxidase staining at 9, 20 and 68 weeks. Staining was quantitated using a scale of 0–4 (0 = no staining, 4 = maximum staining). Details are described in Methods. The average score was calculated for 20 randomly selected glomeruli. Data are expressed as means ± SEM. *$p < 0.001$ vs LETO, **$p < 0.001$ vs OLETF. ■ LETO, □ OLETF, □ OPB-9195



Our data show that OPB-9195 greatly reduced the TGF-β mRNA expression and protein expression. The finding that OPB-9195 treatment ameliorated glomerulosclerosis, ECM accumulation and TGF-β1 overexpression in diabetic rats without affecting the blood glucose concentration provides evidence that AGE formation in vivo may contribute to the pathogenesis of diabetic nephropathy. Possibly AGEs mediate at least some of their pathogenic effects by induction of TGF-β transcription.

Concomitant increases in TGF-β and type IV collagen mRNAs and proteins were observed at each point in time in the OLETF rats. Treatment with OPB-9195 inhibited the increased expression of TGF-β and type IV collagen mRNAs and reduced the concentrations of the proteins to match those seen in nondiabetic rats (Figs. 1–3). These data suggest that OPB-9195 prevented the progression of diabetic nephropathy by blocking type IV collagen production through the control of TGF-β overproduction in diabetic rats. With regard to macrovascular complications, AGEs may have a role in increased TGF-β1 expression and the vascular hypertrophy observed in experimental diabetes [50].

On the other hand, VEGF is known to have a role in diabetic retinopathy. It is involved in the intraocular neovascularization caused by retinal ischaemia, including diabetic retinopathy [51]. The concentration of VEGF in the intraocular fluid in patients with diabetes has been reported to be raised in proliferative diabetic retinopathy. Retinal endothelial cells have large numbers of high-affinity VEGF receptors [52], and hypoxia increases the content of VEGF mRNA in retinal pericytes, retinal endothelial cells and retinal epithelial cells. In kidney diseases, up-regulation of VEGF and its receptor suggests that VEGF participates in stimulating endothelial cell proliferation after glomerular injury [53]. After the induction of experimental diabetes, the glomerular capillary tuft undergoes hypertrophy accompanied by increased blood flow [54]. These early changes are possibly pathogenetically linked to the subsequent expansion of mesangial ECM and increased permeability to macromolecules [55]. Moreover it has been shown that an increased number of capillaries in the diabetic glomerulus make the eye and the glomerulus more alike [56]. The finding that selective, high-level VEGF expression by epithelial cells located adjacent to highly fenestrated endothelia, including glomerular podocytes, continues in mature animals [57] has been interpreted to indicate that VEGF is important for endothelial cell fenestra formation. In this study, VEGF expression was raised about two to three times in the kidney of the OLETF rats, especially in the early stage of nephropathy, compared with the findings in the non-diabetic control rats and this increase was seen at each point in time. Therefore, VEGF may participate in the progression of the early changes of nephropathy, for example, in hyperfiltration, increased glomerular permeability, urine albumin excretion and, moreover, in the formation of new capillaries. It has been reported that TGF-β strongly stimulates VEGF expression and release by fibroblastic and epithelial cells [58]. Nevertheless, whereas TGF-β stimulates VEGF synthesis and release by non-endothelial cells, TGF-β treatment greatly down-regulates VEGF receptor mRNA and inhibits VEGF receptor protein expression at the endothelial cell surface [59]. It is possible that TGF-β participates in regulating the expression of VEGF in the diabetic kidney. By blocking AGE formation, OPB-9195 considerably inhibited VEGF overexpression in the kidney of OLETF rats. These data suggest that AGE formation may contribute to the pathogenesis of early diabetic nephropathy by increasing the expression of VEGF.

The novel AGE inhibitor, OPB-9195, inhibited diabetic nephropathy by inhibiting TGF-β and VEGF production. We speculate that by blocking AGE formation, OPB-9195 inhibits the overproduction of TGF-β, thereby suppressing gene expression of type IV collagen, ECM accumulation and glomerulosclerosis. Inhibition of overproduction of VEGF by OPB-9195 may improve hyperfiltration, albumin permeability and albumin excretion into the urine.

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