

Inhibition of TGF- β 1 expression by antisense oligonucleotides suppressed extracellular matrix accumulation in experimental glomerulonephritis

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Inhibition of TGF- β 1 expression by antisense oligonucleotides suppressed extracellular matrix accumulation in experimental glomerulonephritis. Overproduction of transforming growth factor- β 1 (TGF- β 1) has been implicated in the pathogenesis of fibrotic diseases. TGF- β 1 plays a crucial role in the accumulation of extracellular matrix (ECM) in human and experimental glomerular diseases. However, it remains unclear whether inhibition of TGF- β 1 overproduction would suppress TGF- β 1-induced ECM accumulation. To inhibit the overproduction of TGF- β 1 in experimental glomerulonephritis induced by anti-Thy 1.1 antibody, we introduced antisense oligodeoxynucleotides (ODN) for TGF- β 1 into the nephritic kidney by the HVJ-liposome-mediated gene transfer method. Sense, scrambled or reverse ODN were also introduced as controls. Transfected ODN accumulated mainly in the nuclei of mesangial cells in the glomeruli of transfected kidneys. In the antisense ODN-transfected rats, a marked decrease in expression of TGF- β 1 mRNA was confirmed by Northern analysis. Consequently, the expression of TGF- β 1 protein in the glomerulus was markedly reduced in the antisense ODN-transfected kidney with a comparable effect in preventing glomerular ECM expansion in experimental glomerulonephritis. In contrast, sense, scrambled and reverse ODNs failed to suppress TGF- β 1 expression and ECM accumulation. Thus, these results suggested that inhibition of TGF- β 1 overproduction could suppress progression to glomerulosclerosis.

Activation of glomerular cells, in particular mesangial cells, plays a key role in the initiation and the progression of glomerular diseases [1, 2]. In the initiation of the glomerular disease, mesangial cells are activated by various stimuli: immune complex and activated complement components in immunological injury, high concentrations of glucose and its metabolites in diabetic nephropathy, physical forces in hyperfiltration and glomerular hypertension [3–5]. Mesangial cells react to these stimuli and undergo phenotypic changes characterized by cell proliferation and alteration in morphology and function [6, 7]. The activated mesangial cells augment secretion of various inflammatory mediators including cytokines and growth factors as well as extracellular matrix (ECM) components [3, 4, 8]. This reaction is basically a step in the

normal tissue healing process against injury [1, 2]. However, the hyperstimulated response of mesangial cells to insult can trigger excessive proliferation of glomerular resident cells and infiltrated cells, and overproduction of ECM components, leading to glomerulosclerosis [1, 2].

Transforming growth factor- β 1 (TGF- β 1), a multifunctional dimeric peptide, regulates biological process such as cell proliferation, differentiation and immunological reaction [9]. Acceleration of tissue healing is another important function of TGF- β 1, which accumulates ECM by increasing the synthesis of ECM components and suppressing the degradation of ECM through decreasing production of metalloproteinases and increasing levels of tissue inhibitors of metalloproteinases (TIMPs) [1, 2]. The role of TGF- β 1 in the progression of glomerulonephritis has been demonstrated by several lines of evidence. The expression of TGF- β 1 protein and its mRNA are up-regulated in glomeruli in experimental and clinical glomerular diseases [10, 11]. In the rat anti-Thy 1.1 model of mesangial proliferative glomerulonephritis, expression of TGF- β 1 is up-regulated and associated with sclerotic lesions of glomeruli [12]. Overexpression of TGF- β 1 in normal rat glomeruli induces glomerulosclerosis characterized by ECM expansion with mild mesangial cell proliferation [13]. Administration of antiserum against TGF- β 1 suppressed ECM accumulation in the anti-Thy 1.1 model [14].

Up-regulation of the TGF- β 1 seems to be linked with sclerotic lesions of glomeruli. Thus, the manipulation of TGF- β 1 expression seems to be a promising strategy for clinical treatment of glomerulosclerosis. For this purpose, antisense oligodeoxynucleotides (ODN) may be a feasible tool because they can be used to suppress specific gene expression. We employed a hemagglutinating virus of Japan (HVJ)-liposome-mediated gene transfer method for manipulating the expression of TGF- β 1 in glomeruli *in situ*. Here, we report that inhibition of TGF- β 1 *in vivo* using antisense ODN suppressed glomerular ECM expansion in the anti-Thy 1.1 glomerulonephritis model.

Methods

Synthetic oligodeoxynucleotides

The sequences of phosphorothioate ODN for rat TGF- β 1 used in this study were: antisense, 5'-CGAGGGCGGCATGGG-3';

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sense, 5'-CCCATGCCGCCCTCG-3'; reverse, 5'-GGGTACG-GCGGGAGC-3'; scrambled, 5'-GCATGGGCGAGGGC-3'. Antisense TGF- β 1 ODN were complementary to rat TGF- β 1 mRNA at the translation initiation region. The specific action of antisense ODN for TGF- β 1 was confirmed in NRK cells. Inhibition of TGF- β 1 action was observed specifically in rats treated with the antisense ODN for TGF- β 1, but not in the sense, reverse or scrambled ODN. The specificity of the effect of antisense TGF- β 1 ODN was observed previously by Itoh et al, who tested the inhibitory action of the same ODN on cultured smooth muscle cells [15]. The ODN were obtained from Toray Industries, Inc., Japan. The ODN were deprotected on the column, dried, resuspended in balanced salt solution (BSS; 140 mM NaCl, 5.4 mM KCl, 10 mM Tris-HCl, pH 7.6), and quantified by spectrophotometry.

Preparation of HVJ-liposome

ODN were transfected to the rat kidney by HVJ-liposome gene transfer method, which has been employed for the transient transfection *in vivo*. The HVJ-liposomes were prepared as described previously [13, 16, 17]. Briefly, phosphatidylserine, phosphatidylcholine, and cholesterol were mixed in a weight ratio of 1:4.8:2. Dried lipid was hydrated in 200 μ l of BSS containing antisense, sense, reverse or scrambled ODN, vortexed vigorously and sonicated to form liposomes. The liposomes were incubated with purified HVJ inactivated by UV irradiation (110 erg/mm²/seconds) for three minutes just before use. The liposome suspensions (containing 10 mg of lipids) were mixed with HVJ (30,000 hemagglutinating units) in a total volume of 4 ml BSS. The mixture was incubated at 4°C for 10 minutes and gently shaken for 60 minutes at 37°C. Free HVJ was removed from the HVJ-liposomes by discontinuous density gradient centrifugation with sucrose. The HVJ-liposome suspensions contained approximately 10 μ g of ODN in 0.75 ml BSS and were maintained at 4°C.

In vivo transfection of FITC-labeled ODN

To estimate the transfection efficiency, fluorescein isothiocyanate (FITC)-labeled phosphorothioate antisense ODN for rat TGF- β 1 was introduced into the left kidney of normal 6-week-old male Sprague-Dawley (SD) rats (Japan SLC, Inc., Hamamatsu, Japan) by the HVJ-liposome-mediated gene transfer method under anesthesia achieved by intraperitoneal injection of pentobarbital (50 mg/kg). FITC-labeled ODN were obtained from Toray Industries, Inc., HVJ-liposome solution including FITC-labeled ODN was injected into the left kidney via the left renal artery and incubated for 10 minutes, and then the kidney was reperused. Kidneys on both sides were removed 20 minutes after transfection. Four- μ m-thick cryostat sections of unfixed snap-frozen specimens were examined by fluorescence microscopy. Specific luminescence of FITC was easily distinguished from the background autofluorescence by treatment with eriochrome black T solution [18]. In addition, the sections were stained with OX-7, a monoclonal antibody to the specific marker of mesangial cells Thy 1.1 (gift from Dr. Kenichi Isobe and Dr. Seiichi Matsuo, Nagoya University, Japan), to examine the glomerular localization of the transfected ODN. Rhodamine-conjugated rabbit anti-mouse IgG (Chemicon international Inc., CA, USA) was used as a second antibody.

Glomerular RNA preparation and Northern blot analysis

To examine the effect of ODN on the level of TGF- β 1 mRNA, Northern analysis was performed with total RNA extracted from isolated glomeruli of nephritic rats induced by the anti-Thy 1.1 antibody OX-7. Two days after injection of OX-7, antisense, sense, reverse and scrambled ODN were transfected into three rats, each. Two days after transfection (4 days after injection of OX-7), total RNA was prepared from glomeruli that were isolated by the graded sieving technique as described previously [19]. For Northern blot analysis, 20 μ g aliquots of total RNA were separated on a 1% agarose formaldehyde gels and transferred onto a nylon membranes (Amersham, Hybond[®]-N). To obtain a probe specific for rat TGF- β 1 [20], rat kidney RNA was reverse transcribed and subjected to polymerase chain reaction (PCR) amplification with specific 5' and 3' primers for rat TGF- β 1 cDNA. The primers sequences were as follows: CGCATCGAAGCCATC-CGTGGC (sense primer, corresponding to nucleotides 545 to 565) and TCAGCTGCACTTGCAGGAGCG (antisense primer, corresponding to nucleotides 1565 to 1585). The PCR fragment was subcloned and its sequence was confirmed by an automated sequencer (Applied Biosystems Inc., Foster City, CA, USA). Partial clones of rat TGF- β 1 cDNA and rat GAPDH cDNA [21] were labeled by the random priming method using [α -³²P] dATP (3,000 Ci/mmol, Amersham). Hybridization was carried out at 42°C overnight in 50% formamide, 10 \times Denhardt's solution, 1% SDS, 5 \times SSC, 50 mM Na phosphate and 200 μ g/ml salmon sperm DNA. The blots were washed three times at 50°C in 0.5 \times SSC and 0.1% SDS, and the signals were quantified by laser densitometry (ScanningImager, Molecular Dynamics, Sunnyvale, CA, USA).

Effects of antisense TGF- β 1 ODN on histological changes

On day 0, to induce experimental glomerulonephritis, the OX-7 antibody (1.0 mg/kg) was administered intravenously to 6-week-old male SD rats [22]. In this anti-Thy 1.1 model of mesangioproliferative glomerulonephritis, the disease progression was strongly associated with elevated TGF- β 1 activity in the glomeruli [14, 23]. On day 2, rats were anesthetized by intraperitoneal injection of pentobarbital, and the left renal artery was surgically exposed. A catheter was inserted into the left renal artery and blood flow to the kidney was occluded by clipping the abdominal aorta. As described above, HVJ-liposome complex containing antisense, sense, reverse or scrambled ODN was introduced selectively into the left kidney of the nephritic rats and incubated for 10 minutes. After transfection, the kidney was reperused. To confirm the successful transfection with the ODN, FITC-labeled ODN was mixed at a ratio of 30% with unlabeled ODN. Biopsy specimens were obtained from the transfected left kidney 20 minutes after transfection. In cases in which successful transfection was ascertained by FITC luminescence, histological examination was subsequently performed. Antisense, sense, scrambled and reverse ODN were successfully introduced into almost all glomeruli of 8, 8, 3 and 3 rats, respectively.

The animals were sacrificed under pentobarbital anesthesia nine days after OX-7 injection. Kidneys on both sides were perfused with 4% paraformaldehyde solution and then removed. Tissues for light microscopy were dehydrated through a graded ethanol series and embedded in paraffin. Histologic sections (2 μ m) of kidneys from both sides were stained with periodic

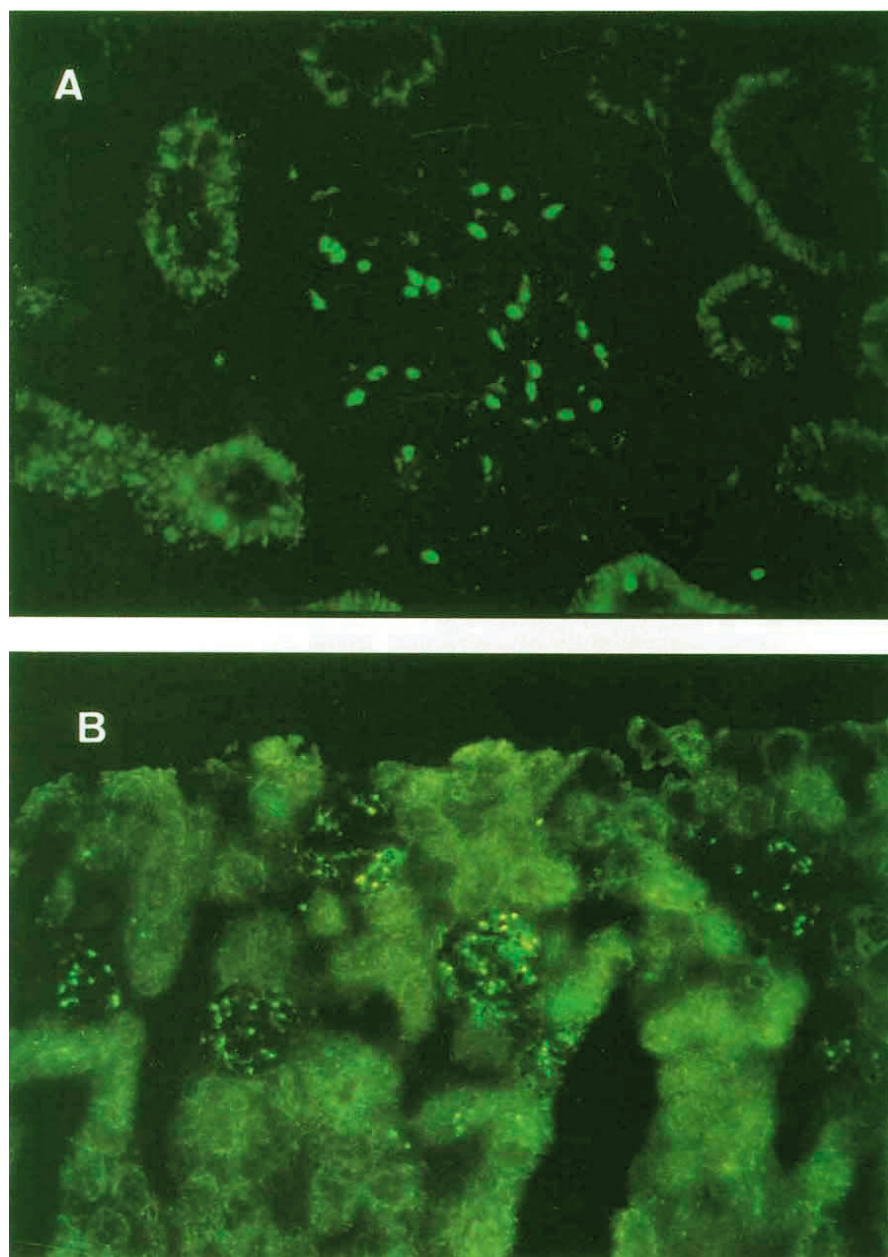


Fig. 1. Representative fluorescence photomicrographs of glomeruli 20 minutes after transfection with FITC-labeled phosphorothioate ODN using the HVJ-liposome method. (A) FITC-labeled ODN were accumulated in the nuclei of glomerular cells in the transfected left kidney ($\times 400$). (B) FITC-labeled ODN were observed in all glomeruli ($\times 100$).

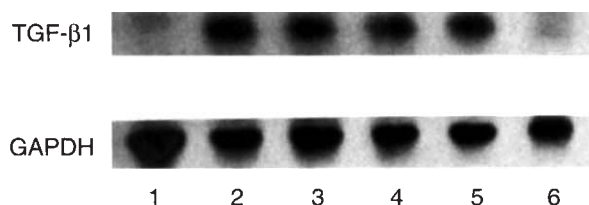


Fig. 3. Northern analysis of the effects of antisense ODN on glomerular TGF- $\beta 1$ mRNA in nephritic rats. Glomeruli were obtained from age-matched normal rat kidneys (lane 1), untreated disease control kidneys (lane 2), and scrambled (lane 3), reverse (lane 4), sense (lane 5) and antisense (lane 6) ODN-transfected nephritic kidneys.

acid-Schiff (PAS) reagent. To quantify mesangial matrix accumulation and the total cell number per glomerulus, all sections were evaluated by an observer with no knowledge of experimental treatments. Thirty glomeruli (80 to 100 μm in diameter) were selected at random in cross sections from each rat. The degree of mesangial matrix accumulation was determined as the percentage of each glomerulus occupied by mesangial matrix according to the

method of Raij, Azar and Keane [24]. To evaluate the effect of antisense ODN transfection on the disease progression in our anti-Thy 1.1 model, the contralateral (right) kidney was also examined as an untreated disease control.

Histologic sections 4 μm thick were processed by an indirect immunoperoxidase technique using a commercial kit (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, CA, USA) with

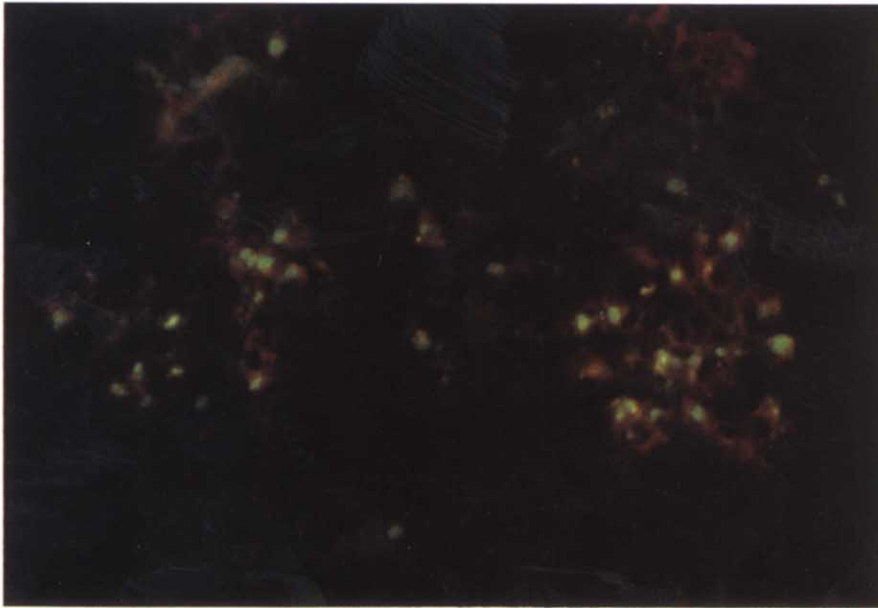


Fig. 2. Glomerular localization of FITC-labeled ODN. To examine the cellular localization of the transfected ODN, OX-7, a specific monoclonal antibody for mesangial cells, and rhodamine-conjugated rabbit anti-mouse IgG were used to stain mesangial cells. FITC (green)-positive nuclei were seen mainly in the mesangial area (red). The color of FITC-positive nuclei thus changed from green to yellow ($\times 300$).

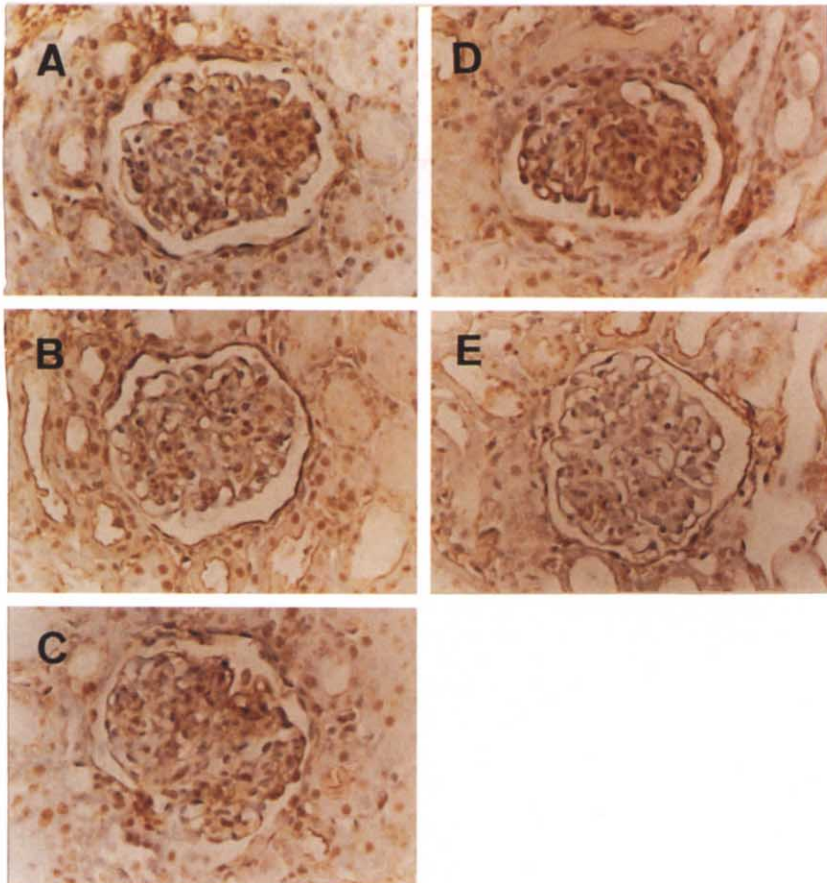


Fig. 4. Inhibition of TGF- $\beta 1$ expression in glomeruli by introduction of antisense ODN. Representative photomicrographs of glomeruli from untreated disease control (A), scrambled (B), reverse (C), sense (D) and antisense (E) ODN-transfected nephritic rats. Kidney sections were stained with an antibody against TGF- $\beta 1$ ($\times 400$).

a chicken polyclonal antibody against TGF- $\beta 1$ (R&D Systems Inc., Minneapolis, MN, USA). Biotinylated goat anti-chicken IgG (Vector Laboratories) was used as a second antibody.

Statistical analysis

All values are expressed as mean \pm SE. Statistical significance (defined as $P < 0.01$) was evaluated using the one-way analysis of variance (ANOVA).

Results

Confirmation of transfer of antisense ODN into the nuclei of glomerular cells

We examined the efficiency of transfer of ODN into the glomerular cells using FITC-labeled ODN. FITC-labeled ODN were introduced into the left kidney of normal rats via renal artery by the HVJ-liposome method, and the rats were sacrificed 20

minutes after transfection. FITC luminescence was then examined in the kidneys on both sides. Transfer of FITC-labeled ODN into glomerular cells was successful in two of three rats in three independent experiments. As shown in Figure 1, FITC-labeled ODN were observed in all glomeruli and accumulated in the nuclei of glomerular cells in the transfected left kidney. No luminescence was observed in the contralateral right kidney. Of note, immunofluorescence staining with the OX-7 antibody confirmed that the majority of FITC-positive nuclei were present in the mesangial area which is colored in red in Figure 2, indicating that the HVJ-liposome-mediated gene transfer method enabled us to introduce ODN mainly into mesangial cells.

Inhibition of TGF- β 1 expression in the glomeruli by introduction of antisense TGF- β 1 ODN

Antisense, sense, reverse or scrambled ODN was introduced selectively into the left kidney two days after injection of the OX-7 antibody. On day 4, we obtained glomerular RNA to examine whether the antisense ODN for TGF- β 1 could decrease the mRNA of TGF- β 1 in glomeruli, because the mRNA level for TGF- β 1 in glomeruli reaches a peak four days after injection of OX-7 in this model. Glomeruli were isolated by the graded sieving technique, and Northern blot analysis was performed to compare the levels of TGF- β 1 mRNA. As shown in Figure 3, TGF- β 1 mRNA level was elevated by more than 15-fold in untreated disease controls four days after OX-7 injection (lane 2). Laser densitometric analysis revealed that TGF- β 1 mRNA expression in glomeruli from nephritic rats treated with antisense ODN was reduced to 30% of that in untreated disease controls. In contrast, there were no differences in the level of TGF- β 1 mRNA in scrambled, reverse or sense ODN-transfected rats. Furthermore, the level of GAPDH mRNA remained unaffected in all transfected kidneys. TGF- β 1 protein expression was also studied with an antibody to TGF- β 1 seven days after transfection (on day 9). Expression of TGF- β 1 was up-regulated in the glomeruli from nephritic rats, and this up-regulation was suppressed in those rats treated with antisense TGF- β 1 ODN. In contrast, the level of expression of TGF- β 1 protein was unchanged in rats treated with scrambled, reverse or sense ODN (Fig. 4).

Suppression of extracellular matrix expansion in experimental glomerulonephritis by antisense TGF- β 1 ODN

Given the effective suppression of TGF- β 1 message production, we then assessed the inhibitory action of antisense ODN on the histological changes in the kidney seven days after transfection. Figure 5 shows the representative appearance of the glomeruli. In the kidneys transfected with scrambled, reverse or sense ODN, glomeruli exhibited marked increases in mesangial ECM. In contrast, introduction of antisense ODN markedly suppressed the increase in mesangial matrix expansion. The results of quantitative analyses of mesangial matrix and cell proliferation indices are summarized in Figure 6. The mesangial matrix indices of glomeruli from the kidneys transfected with antisense, sense, reverse and scrambled ODN, and untreated disease control kidneys were 36.1 ± 2.2 , 55.0 ± 1.7 , 57.8 ± 2.7 , 60.9 ± 2.9 , and $58.0 \pm 1.9\%$, respectively. However, glomerular cell count was less affected. These results demonstrated that a single introduction of antisense TGF- β 1 ODN two days after OX-7 injection suppressed extracellular matrix expansion.

Discussion

We have established an HVJ-liposome-mediated technique for *in vivo* gene transfer into glomerular cells [13, 25]. However, cellular and intracellular localization of the transfected DNA has not been reported. In the present study, we employed FITC-labeled antisense ODN to examine the cellular localization of the transfected DNA. Immunofluorescence staining for Thy 1.1, a specific marker of mesangial cells, combined with FITC-labeled ODN revealed that the transfected ODN were rapidly accumulated in the nuclei of glomerular cells, mainly mesangial area. It is not clearly understood why antisense ODN should be effectively introduced into mesangial cells by the HVJ-liposome method, although HVJ-liposome may theoretically reach mesangial cells through fenestrations. Further study is necessary to clarify the mechanism of the susceptibility of HVJ-liposome to fuse with mesangial cells in the kidney.

Expression of transfected genes was observed in approximately 30% of glomeruli [13, 25], whereas transfected ODN were detected in all glomeruli. The efficacy of expression of transfected genes thus seems to be low. The difference in molecular weight between 15-mer ODN and 7000 bp expression vector for TGF- β 1 may partially explain this difference, since transfection efficiency decreases in proportion to the size of the transfected DNA. In addition, HVJ-liposome DNA complexes are considered to contain similar amounts of DNA. Therefore, HVJ-liposomes containing 15-mer ODN can transfer approximately 500-fold more copies of DNA than those containing 7000 bp expression vector once fusion with the target cell occurs.

There are several lines of evidence that support the pivotal role of TGF- β 1 in the progression of extracellular matrix expansion. The up-regulation of TGF- β 1 in glomerular lesions has been reported in clinical as well as experimental glomerular lesions [1, 2, 10, 11, 14]. In addition, we reported that overexpression of TGF- β 1 in normal rat glomeruli induced marked ECM accumulation with mild mesangial cell proliferation [13]. Suppression of the action of TGF- β 1 by administration of either anti-TGF- β 1 antiserum or decorin, a natural inhibitor of TGF- β 1, reduced ECM accumulation in experimental glomerulonephritis [14, 26]. Here, we tested the possibility of the intervention in the action of TGF- β 1 by antisense ODN in the anti-Thy 1.1 model, an experimental model of mesangioproliferative glomerulonephritis. In this model TGF- β 1 mRNA expression reached a peak on day 4 after disease induction by injection of OX-7, followed by a peak of TGF- β 1 protein expression on day 6. Antisense ODN transfection on day 2 effectively suppressed the ECM accumulation in the nephritic rat through the reductions in levels of TGF- β 1 mRNA and protein, whereas scrambled, reverse and sense ODN-transfected kidneys and untreated disease control kidneys exhibited marked increases in mesangial ECM expansion. The principal mechanism of suppression of protein synthesis by antisense ODN has been reported to center on degradation of the target mRNA by RNase H, although the translational arrest is considered to be another important mechanism [27]. Northern blot and immunohistological analyses revealed that the levels of TGF- β 1 mRNA and protein in the antisense ODN transfected-left kidney were markedly decreased. In contrast, scrambled, reverse and sense ODN failed to affect the expression of TGF- β 1 mRNA or protein in the nephritic kidney. It was reported that the antiproliferative activities of antisense *c-myc* or *c-myb* ODN were not only due to

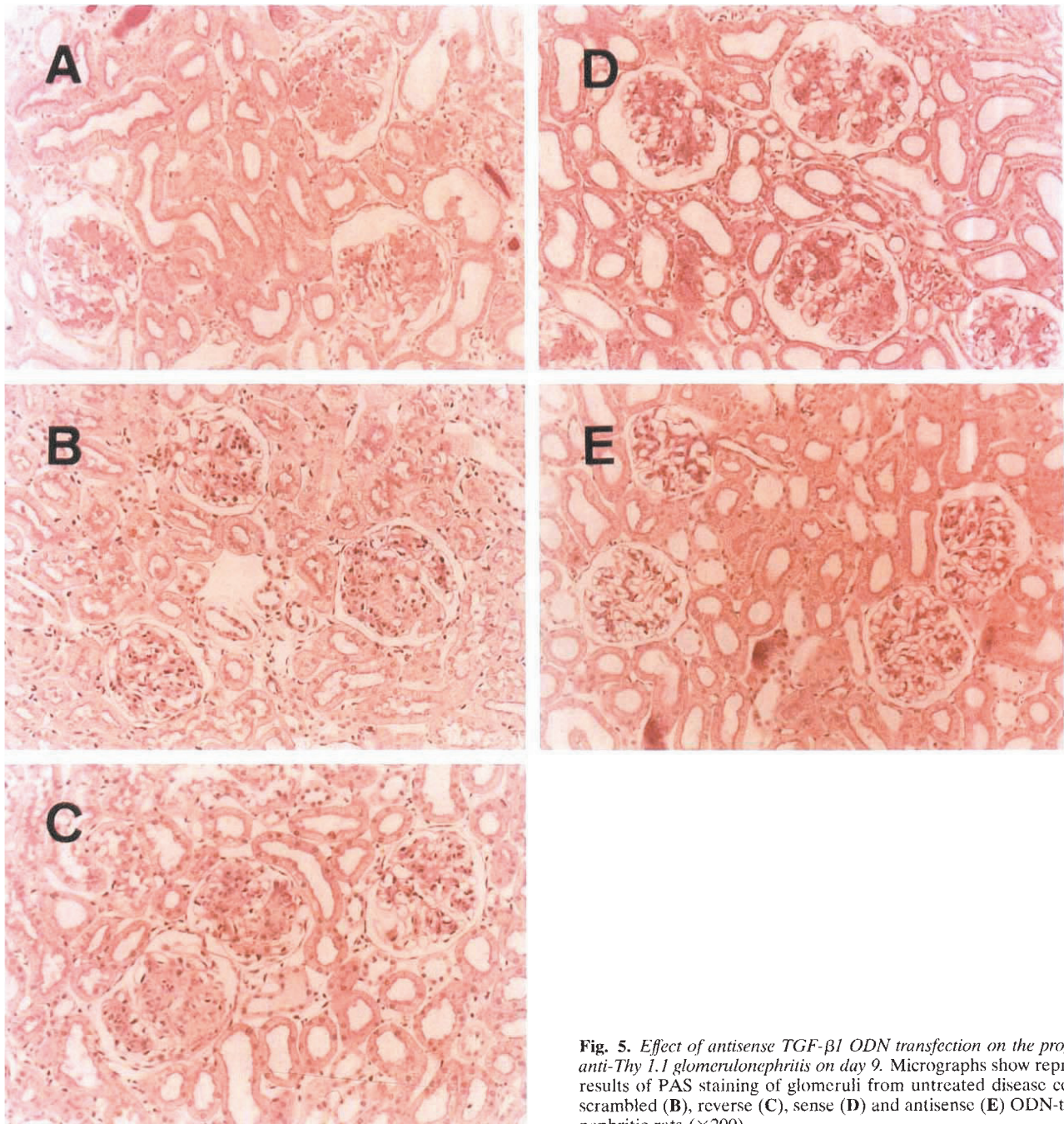


Fig. 5. Effect of antisense TGF- β 1 ODN transfection on the progression of anti-Thy 1.1 glomerulonephritis on day 9. Micrographs show representative results of PAS staining of glomeruli from untreated disease control (A), scrambled (B), reverse (C), sense (D) and antisense (E) ODN-transfected nephritic rats ($\times 200$).

a hybridization-dependent antisense mechanism, but due to four contiguous guanosine (G) residues [28]. In our experiment, we used scrambled ODN, which contained four contiguous G residues, as a control. In the scrambled ODN-transfected kidneys, however, neither antiproliferative effects nor suppression of TGF- β 1 was observed. Suppression was observed only in the antisense ODN-transfected kidneys, suggesting that this inhibition took place via a hybridization-dependent antisense mechanism. In addition, the specificity of suppression of TGF- β 1 by the transfected antisense ODN was also supported by the observation that

there was no alteration in the level of GAPDH mRNA. The present study demonstrated that the disease progression in the anti-Thy 1.1 model was suppressed in association with the decrease in TGF- β 1 mRNA level as well as that in TGF- β 1 protein. Therefore, *in vivo* transfection of antisense TGF- β 1 ODN by the HVJ-liposome method could prevent the TGF- β 1-mediated progression of glomerular disease.

In this Thy1.1 model of glomerulonephritis, following a single episode of immune injury induced by injection of anti-Thy 1.1 antibody, TGF- β 1 mRNA expression in the glomeruli along with

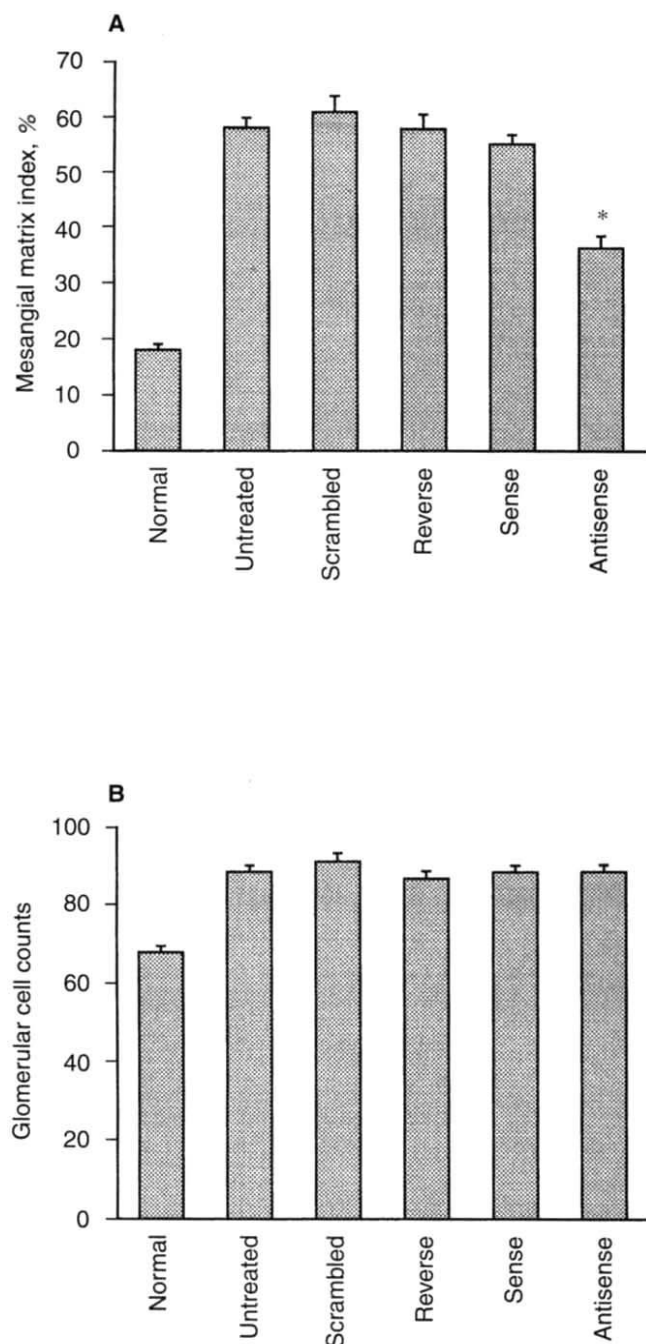


Fig. 6. Quantification of pathological effect of antisense ODN transfection. The severity of glomerular damage of the scrambled, reverse, sense and antisense ODN-transfected kidneys was evaluated by the ECM accumulation (A) and total glomerular cell counts (B). Normal rats were included for comparison. Values are expressed as mean \pm SE. * $P < 0.001$ versus other groups. Each group contained 3 to 8 animals.

increased production of matrix components reached a peak and returned to basal level by four weeks. Subsequently the pathological matrix began to regress and the glomeruli returned to a histologically normal appearance. In contrast to the self-limited and reversible disease observed after single anti-Thy 1.1 antibody injection, repeated injection of anti-Thy 1.1 antibody has been

reported to produce chronic diseases. The treatment of therapeutic effect of transfection of antisense ODN on this Thy 1.1 model, however, may not be applicable to chronic glomerulosclerosis. In the chronic model TGF- β 1 may perpetuate the disease process through continued induction in cells at the glomerular lesion. In this regard, it seemed interesting to attempt to suppress the overproduction of TGF- β 1 in a chronic model of progressive glomerular scarring by the continuous introduction of antisense ODN.

Hence, the marked inhibition of TGF- β 1 expression by antisense ODN in *in vivo* experimental glomerulonephritis might facilitate the development of novel methods for the treatment of glomerulosclerosis. There have been many studies in which antisense ODN were shown to suppress the expression of various mammalian genes *in vitro* [29]. It is of note that successful inhibition of the progression of tumor growth by antisense ODN was reported in animal cancer models [30]. A major problem with antisense ODN was their inability to cross cellular membranes and accumulate in nuclei efficiently. HVJ-liposome-mediated transfection for ODN, which makes good use of fusion mediated by HVJ, permits direct transfer of ODN into the cytoplasm without degradation through the process of endocytosis. HVJ-liposome-mediated gene transfer has been shown to increase efficiency in delivering antisense ODN into target cells and to sustain the stability of the transfected ODN as compared with conventional methods using cationic liposomes [31]. However, before this method can be used clinically, several improvements are necessary: development of selective delivery system into the kidney, improved efficacy of transfection of ODN, regulation of the strength and the duration of gene expression and establishment of toxicological safety. HVJ-liposome-mediated antisense ODN transfer is useful for the investigation of the pathophysiology of renal diseases, and further, this method may be a promising new therapeutic method for blocking the progression of glomerular diseases.

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