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Gene therapy by transforming growth factor-β receptor-IgG Fc chimera suppressed extracellular matrix accumulation in experimental glomerulonephritis

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Background. The evidence that transforming growth factor-β (TGF-β) is a key mediator in the pathogenesis of fibrotic diseases is now supported by several lines of investigation. This evidence provides a certain base for targeting TGF-β as an antifibrotic agent.

Methods. We generated a chimeric cDNA, termed TGFβRII/Fc, encoding an extracellular domain of the TGF-β type II receptor fused to the IgG-Fc domain, and tested whether TGFβRII/Fc could be a novel strategy for treating glomerular diseases.

Results. In cultured BNul-7 cells, recombinant TGFβRII/Fc reversed the antiproliferative response induced by TGF-β1. In addition, TGFβRII/Fc diminished the TGF-β1–induced production of EIIIA-positive fibronectin in cultured normal rat kidney cells. We then introduced the chimeric cDNA into the muscle of the nephritic rats by the hemagglutinating virus of Japan liposome–mediated gene transfer method in order to block the TGF-β activity in nephritic glomeruli through systemic delivery of chimeric molecules. Treatment with TGFβRII/Fc gene transfection could suppress the glomerular TGF-β mRNA in nephritic rats with a comparable effect in the reduction of extracellular matrix accumulation.

Conclusion. TGFβRII/Fc successfully inhibited the action of TGF-β *in vitro* and *in vivo*, and gene therapy by chimeric TGFβRII/Fc might be feasible for the therapy of glomerulosclerosis.

sTransforming growth factor-β (TGF-β), a multifunctional cytokine, plays an important role in regulating tissue repair and remodeling following injury. One of the most important biological actions of TGF-β is the regulation of extracellular matrix deposition [1, 2]. TGF-β up-regulates the synthesis of individual matrix components, including proteoglycans, collagens, and glycoproteins [3]. TGF-β also inhibits matrix degradation by increasing the synthesis of protease inhibitors and decreasing the synthesis of proteases [4]. Finally, TGF-β alters the expression of integrins and modulates their relative proportions on the cell surface in a manner that could facilitate adhesion to the matrix [5]. However, sustained or excessive expression of TGF-β, in response to repeated injury, is considered to be a major rationale of glomerular matrix expansion in human and experimental glomerular disease [2, 6–8].

The TGF-β actions in pathophysiological conditions have now been demonstrated by several lines of evidence. It has been shown that the actions of TGF-β observed *in vivo* can be reproduced in cultured cells by the addition of TGF-β [2]. Intravascular injection of TGF-β into rats or rabbits rapidly caused glomerulosclerosis [9]. Overexpression of TGF-β in normal glomeruli by TGF-β1 gene transfection induced glomerulosclerosis within several days [10]. Hepatic expression of a TGF-β1 transgene under the control of an albumin promoter/enhancer in the transgenic mice resulted not only in hepatic fibrosis, but also in renal lesions characterized by progressive mesangial expansion and tubulointerstitial fibrosis [11, 12]. Finally, fibrosis has been blocked *in vivo* by injection of anti-TGF-β neutralizing antibodies in a number of animal models, including kidney diseases [6]. These results support the hypothesis that modulation of dysregulated expression of TGF-β could preclude the exuberant glomerular matrix deposition.

We reported that overexpressed TGF-β in the normal rat glomeruli by TGF-β gene transfer leads to glomerulosclerosis [10]. In contrast, inhibition of the TGF-β gene

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expression by antisense oligonucleotides could suppress the development of experimental glomerulonephritis [13]. Furthermore, continuous delivery of decorin, which was accomplished by *in vivo* gene transfer into skeletal muscle, also inhibited the extracellular matrix expansion in experimental glomerulonephritis [14]. Combined, this evidence strongly suggests the hypothesis that the inhibition of overexpressed TGF-β can ameliorate the progression of renal diseases.

It has been shown that TGF-β signals are transduced by simultaneously contacting two transmembrane serine/threonine kinases known as type I and type II receptors [15]. The type II receptor recognizes the active TGF-β ligand, whereas their type I receptor does not. Thus, TGF-β binds directly to the type II receptor, which is a constitutively active kinase. The TGF-β–binding type II receptor is then recognized by the type I receptor, which is recruited into the complex and becomes phosphorylated by the type II receptor. Both receptors are required for TGF-β action in mammalian cells [16], because mutations in either receptor type disrupt signaling in each case. Based on the differences between their ligand-binding properties, the type II receptor acts upstream of the type I receptor, and thus, one may think of these components as primary receptor and transducer, respectively [16]. These results motivate us to produce an inactive type II receptor construct to impede the activities of TGF-β. However, the soluble type II receptor was reported to have an approximate 10-fold lower binding affinity for TGF-β than does the cell-surface type II receptor [17]. It may partly depend on the fact that the soluble type II receptor is monomeric, because the type II receptor found on the cell surface is primarily a homodimer and the TGF-β ligand is also a disulfide-linked dimer.

To create an efficient inhibitor with the capacity to block the binding of TGF-β to type II receptor and thereby to prevent the subsequent signal transduction, we have generated a chimeric inhibitor molecule, termed TGFβRII/Fc, in which the extracellular portion of the TGF-β type II receptor was fused to an immunoglobulin heavy-chain Fc fragment. When expressed in mammalian cells, TGFβRII/Fc is expected to be secreted as a disulfide-linked homodimer. The design of this molecule was based on previous findings demonstrating that similar chimeric molecules possess functional binding sites and a markedly longer plasma half-life compared with the soluble extracellular domain [18]. Repeated injection of the purified recombinant TGFβRII/Fc molecule may be applicable for molecular intervention of TGF-β action. However, the harvest of a large amount of purified TGFβRII/Fc is expensive and time consuming. In contrast, gene transfer of TGFβRII/Fc to the skeletal muscle is less expensive, and lasting delivery of TGFβRII/Fc chimeric molecule could be expected.

Here, we have examined the possibility of gene therapy to determine whether TGFβRII/Fc could efficiently neutralize the activities of TGF-β *in vitro* and *in vivo.*

METHODS

Cell lines

The BNul-7 mast cell line was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 5×10^{-5} M of 2-mercaptoethanol, 50 U/ml of penicillin, and 50 μg/ml of streptomycin. Normal rat kidney (NRK) cells were propagated in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 50 U/ml of penicillin, and 50 μg/ml of streptomycin. These cell lines were used for the growth inhibition assay and the fibronectin induction assay for TGF-β, respectively.

Construction of the chimeric gene and purification of the chimeric molecule

The cDNA fragment corresponding to the extracellular domain of the human TGF-β type II receptor gene (codon −7 to 480) was generated by reverse transcription-polymerase chain reaction (RT-PCR) amplification from mRNA of HepG2 cells [19]. The primers used were 5′-CCA*CTCGAG*TCTGCCATGGGTCGGGGGCTG and 5′-CCG*GGATCC*AAGTCAGGATTGCTGGTGT TATATTC as sense and antisense primers, respectively. For cutting out cDNA for the TGF-β type II receptor, Xho I and BamHI restriction sites were added to sense and antisense primers, respectively, as indicated by the italics. The cycling parameters were 30 seconds at 94°C and one minute at 68°C. The amplification was performed for 30 cycles by a GeneAmp PCR system 2400 (Perkin-Elmer Cetus Corp., Norwalk, CT, USA). The fragment was cloned into the pCRII AT cloning vector (Invitrogen, San Diego, CA, USA). The amplified PCR product was digested by Xho I and BamHI and inserted into CD4Rg (a kind gift of Dr. B. Seed) from which the Xho I BamHI fragment containing the CD4 gene was removed, thereby making a fusion gene between the N-terminal half of TGF-βRII and the C-terminal half of human IgG1-Fc.

This construct was transiently transfected by using the diethylaminoethyl (DEAE) dextran method [20] into COS-1 cells, which were then cultured for one week with Dulbecco's modified Eagle's medium/F12 medium (GIBCO Laboratories, Grand Island, NY, USA) containing 1% Nutridoma-NS (Boehringer Mannheim, Mannheim, Germany); the chimeric molecule in the culture supernatant was purified using Prosep-A (Bioprocessing, Princeton, NJ, USA). This purified chimeric molecule was used for the *in vitro* study.

The chimeric molecule was analyzed for purity and dimerization by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified molecule

was resolved under nonreducing or reducing (with 0.715 M of 2-mercaptoethanol) conditions in 7.5% gels. After transfer to nitrocellulose membranes (Hybond-c; Amersham International, Amersham, UK), the membrane was blocked with phosphate-buffered saline (PBS) containing 5% (vol/vol) skim milk and 1% bovine serum albumin, incubated with horseradish peroxidase-conjugated avidin (Bio-Rad, Richmond, CA, USA), and developed using the enhanced chemiluminescence (ECL) detection reagent (Amersham Corp., Arlington Height, IL, USA). Because the extracellular domain of TGF-β type II receptor has three glycosylation sites, the reduced chimeric molecule was also deglycosylated by N-glycosidase to address whether this chimeric molecule could be an expected size. After reducing the chimeric molecule, 10 μg of chimeric molecule were incubated with or without 0.4 units of N-glycosidase F (Boehringer Mannheim) at 37°C overnight. Untreated chimeric molecule and chimeric molecule treated with or without N-glycosidase F were analyzed through 4% to 20% SDS-PAGE. After transfer to nitrocellulose membranes, the membrane was incubated with antihuman TGF-β type II receptor antibody (R&D Systems Inc., Minneapolis, MN, USA) for one hour and then labeled with horseradish peroxidase-conjugated antigoat IgG (Santa Cruz, Biotechnology Inc., Santa Cruz, CA, USA). The membrane was washed and was then developed using the ECL detection reagent (Amersham).

For *in vivo* study, pCAGGS-TGFβRII/Fc was constructed by inserting the Xho I-Xma III fragment containing a fusion gene into the Xho I site of pUC-CAGGS [21], which contains the cytomegalovirus enhancer and the chicken β-actin promoter.

Effects of the chimeric molecule on TGF-β's growth inhibition activity

One $\times 10^3$ of BNul-7 cells in 96-well culture plates were cultured with various concentrations of TGF-β1 (0.01 ng/ml to 1.25 ng/ml) in the presence or the absence of chicken anti–TGF-β antibodies (1.25 μg/ml; R&D Systems) or chimeric TGFβRII/Fc (1.25 μg/ml) for three days. Then, the BNul-7 cell growth was measured by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrozolium bromide (MTT; Sigma Chemical Co., St. Louis, MO, USA) assay. Twenty micrograms of MTT at 5 mg/ml in PBS was added to each 100 μl of culture medium. After incubation for an additional five hours at 37°C, the formazan crystals were dissolved by the addition of 100 μl of 10% SDS in 0.01 N HCl. The plates were then incubated overnight, and the optical density of the wells was determined using Multiskan (Labsystems, Helsinki, Finland) at a wavelength of 620 nm. Within at least one hour, the plates were read on a micro-enzyme-linked immunosorbent assay reader at a wavelength of 570 nm and a reference wavelength of 620 nm. The results of the inhibition studies are presented as a percentage of TGF-β–free proliferation of BNul-7 cells.

Effects of the chimeric molecule on TGF-β's fibronectin synthesis activity

Fibronectins comprise a family of adhesive glycoproteins that are prominent components of the mesangial extracellular matrix and are accumulated during glomerular injury. There are several forms of fibronectins that differ in their primary structure by the alternative splicing of a unique mRNA precursor (pre-mRNA) transcribed from a single gene; three sites of alternative splicing have so far been described—ED-A, ED-B, and IIICS—in human fibronectins and the corresponding regions—EIIIA, EIIIB, and V—in rat fibronectins [22, 23]. Fibronectin mRNA containing EIIIA exon has been reported to increase along with the severity of the disease in response to TGF-β [24].

To study the effects of the chimeric molecule on the alternative splicing of fibronectins pre-mRNA induced by TGF-β, we designed specific probes complementary to the sequence of pre mRNA encompassing the EIIIA exon for RT-PCR (Fig. 3A). The primers used were 5′-AGAACCGGAACGGAGAAAGC-3′ and 5′-GAGGT GCTGTCTGGAGAAAG-3′ as the sense and antisense primers, respectively. The amplified fragments were detected as 484 bp and 214 bp for EIIIA positive and EIIIA negative exons, respectively.

One $\times 10^5$ NRK cells were plated in Dulbecco's modified Eagle's medium with 10% FCS in a 25 cm^2 culture flask and were then grown to the confluency. The confluent NRK cell layer was either left untreated or incubated with 1 ng/ml of TGF-β and/or 10 μg/ml of TGFβRII/Fc by placing them in fresh medium for two days. After 48 hours of exposure, the cells were washed with PBS, and mRNA was extracted by a QuickPrep Micro mRNA Purification kit (Quiagen, Hilden, Germany). The mRNA concentrations were determined using spectrophotometric readings at Absorbance 260. The synthesis of cDNA was carried out in a 20 μl reaction volume containing 0.5 μg RNA from NRK cells, 0.5 mM dNTPs, 1 × RT buffer (GIBCO), 0.5 μg/ml Oligo dT 12–18 random primers, 10 mM dithiothreitol, and 20 U murine leukemia virus (MLV) reverse transcriptase (GIBCO) incubated for one hour at 42°C. One microliter of this mixture was incubated in a 50 μl volume reaction with 50 pmol of primers, 0.2 mol of each dNTP, and 2.5 U Taq polymerase (Perkin-Elmer Cetus Corp.) and subjected to 25 cycles of PCR, each consisting of one minute at 94°C, two minutes at 60°C, and three minutes at 72°C. The amplified fragments (484 bp and 214 bp for EIIIA positive and EIIIA negative, respectively) were analyzed by electrophoresis on agarose gels.

Preparation of hemagglutinating virus of Japan liposome

The chimeric TGFβRII/Fc gene was transfected to the gluteal muscle of nephritic rats by the of hemagglutinat-

ing virus of Japan (HVJ) liposome gene transfer method [10, 13, 14], which has been employed for transient transfection *in vivo*. The HVJ liposomes were prepared as described previously [10, 13, 14]. Briefly, phosphatidylserine, phosphatidylcholine, and cholesterol were mixed in a weight ratio of 1:4.8:2. Dried lipid was hydrated in 200 μl of balanced salt solution (BSS) containing 200 μg of pCAGGS-TGFβIIR/Fc, vortexed vigorously, and sonicated to form liposomes. The liposomes were incubated with purified HVJ inactivated by ultraviolet irradiation (110 erg/mm^2/sec) 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 2 ml BSS. The mixture was incubated at 4°C for 10 minutes and was 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 suspension contained approximately 20 μg of pCAGGS-TGFβRII/Fc in 1 ml BSS and was maintained at 4°C.

In vivo experimental design

On day 0, to induce anti-Thy-1 model of experimental glomerulonephritis, the anti-Thy-1 antibody OX-7 (1.0 mg/kg; a gift from Dr. Kenichi Isobe and Dr. Seiichi Matsuo, Nagoya University, Japan) was administrated intravenously to six-week-old male Sprague-Dawley rats (Japan SLC, Inc., Hamamatsu, Japan). On day 1, nephritic rats were anesthetized by ether, and the left gluteal muscles were exposed. HVJ-liposome solutions encapsulating 0 μg, 5 μg, or 20 μg of pCAGGS-TGFβRII/Fc were then injected into the gluteal muscles of the nephritic rats. Three days after transfection, three untreated nephritic rats and three TGFβRII/Fc gene-transferred nephritic rats were sacrificed, and kidney and skeletal muscle were removed to examine the expression of chimeric gene. On day 7, the kidneys from six untreated nephritic rats and six of the 5 μg or 20 μg of pCAGGS-TGFβRII/Fc gene-transfected nephritic rats were perfused with cold autoclaved PBS, and samples of the cortex were taken for histology. Tissues for light microscopy were fixed with 4% paraformaldehyde overnight and dehydrated through a graded ethanol series and embedded in paraffin. Histologic sections (2 μm) of kidneys were stained with periodic acid-Schiff reagent. To quantitate mesangial matrix accumulation, all sections were evaluated by an observer unaware of the experimental protocol. Thirty glomeruli 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 the mesangial matrix as described previously [13, 14]. For glomerular RNA preparations, glomeruli were isolated from the remaining tissue by a standard sieving method.

Detection of chimeric gene expression

Total RNA was isolated from the skeletal muscle of TGFβRII/Fc gene-transferred nephritic rat, and the isolated RNA was subsequently reversed transcribed into cDNA. To examine the expression of chimeric gene, sense and antisense primers were designed from the cDNA sequences of the extracellular domain of the TGF-β type II receptor and IgG-Fc hinge region, respectively. The primers used were 5′-TCCTGTGGACGCGTATCGC-3′ and 5′-GAGTTTTGTCACAAGATTTGGG-3′ as the sense and antisense primers, respectively. The amplified fragment was detected as 420 bp for spliced TGFβRII/Fc mRNA.

Three days after transfection, untreated nephritic rats and TGFβRII/Fc gene-transfected rats were sacrificed, and then the kidneys were removed and were fixed in methylcarn solution. Glomerular deposition of TGFβRII/Fc molecule was stained with the monoclonal antibody to human IgG-Fc (Caltag Laboratories, San Francisco, CA, USA).

Glomerular RNA preparation and Northern blot analysis

To examine the effects on the level of TGF-β1 and type I collagen mRNA, Northern blot analysis was performed with glomerular RNA extracted from nephritic rats treated with 0 μg, 5 μg, or 20 μg of pCAGGS-TGFβRII/Fc chimeric gene transfection. Six days after transfection (seven days after disease induction), total RNA was prepared from pooled glomeruli that were isolated from the minced kidneys of six rats together in each group by the graded sieving technique as described previously [13, 14]. For the Northern blot analysis, 20 μg aliquots of total RNA were separated on 1% agarose formaldehyde gel and were transferred onto nylon membranes (Hybond™-N; Amersham). TGF-β1, type I collagen, and GAPDH cDNA [13, 25] were labeled by the random priming method using [α-^{32}P] dATP (3000 Ci/mmol; Amersham). Hybridization was carried out at 42°C overnight in 50% formamide, 10 × Denhardt's solution, 1% SDS, 5 × SSC, 50 mM Na phosphate, and 200 μg/ml salmon sperm DNA. The blots were washed three times at 50°C in 0.5 × SSC and 0.1% SDS, and the signals were quantitated by laser densitometry (ScanningImager; Molecular Dynamics, Sunnyvale, CA, USA).

Statistical analysis

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

RESULTS

Purification of TGFβRII/Fc

TGFβRII/Fc was created by cDNA encoding the extracellular domain of the human TGF-β type II receptor

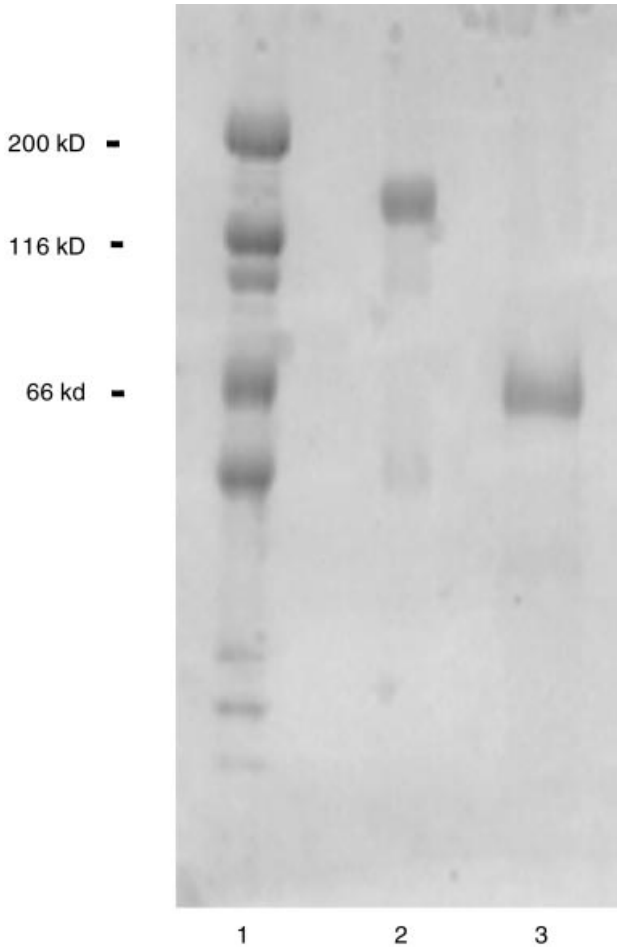

Fig. 1. SDS-PAGE analysis of the chimeric TGFβRII/Fc molecule purified by protein A sepharose chromatography. The proteins were visualized by Coomassie brilliant Blue staining. Molecular weight standards are kilodaltons (lane 1). The purified chimeric TGFβRII/Fc molecule was subjected under nonreducing (lane 2; 2-ME−) or reducing (lane 3; 2-ME+) conditions.

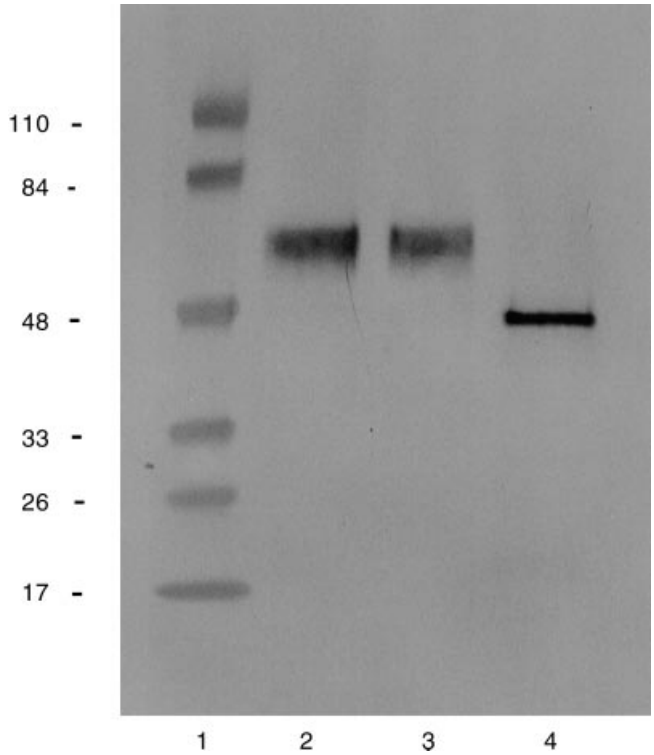

Fig. 2. Western blot analysis of the chimeric TGFβRII/Fc molecule deglycosylated by N-glycosidase F. Molecular weight standards are kilodaltons (lane 1). Untreated reduced chimeric TGFβRII/Fc molecule (lane 2), chimeric TGFβRII/Fc molecule incubated without (lane 3), or with N-Glycosidase F (lane 4) was analyzed by Western blot analysis incubated with antihuman TGF-β type II receptor antibody.

ligated to the cDNA for the Fc portion of the human IgG1 heavy chain. The immunoglobulin portion of this fusion molecule contains the hinge and constant region CH2 and CH3 domains. These fusion molecules lack the TGF-β type II receptor transmembrane domain but contain the IgG heavy-chain hinge region Cys residues and, therefore, should be secreted from cells as a soluble dimer.

Based on the primary structure of the extracellular domain connected with the IgG-Fc molecule, our model predicts that these molecules will behave as homodimers, by virtue of the IgG hinge region. Analysis of the purified molecules by SDS-PAGE under both reducing (with 0.715 M of 2-mercaptoethanol) and nonreducing conditions demonstrates the purity of the preparations and confirms the predicted homodimer structure of the native chimeric molecules (Fig. 1). Under nonreducing conditions, the TGFβRII/Fc chimeric molecule is estimated to be an associated dimer with an apparent molecular weight of approximately 130 kDa. Upon reduction (with 0.715 M of 2-mercaptoethanol), it runs at approximately 65 kDa. Both samples migrate considerably higher on the gels than their predicted molecular weight (45 kDa). However, deglycosylating the chimeric molecule using N-glycosidase altered the molecular weight to 45 kDa, suggesting that this chimeric molecule was secreted after glycosylation (Fig. 2).

Effects of the chimeric molecule on TGF-β's growth-inhibition activity

To characterize the *in vitro* properties of the chimeric molecule consisting of the TGF-β type II receptor fused to the IgG-Fc domain, the recombinant chimeric molecule was purified. Because the TGFβRII/Fc chimeric molecule was expected to act as a competitive antagonist for TGF-β, we assessed whether the recombinant chimeric TGFβRII/Fc molecule could attenuate the effects of TGF-β on the growth-inhibiting action. To test this possibility, BNul-7 cells, which express the native TGF-β receptors, were treated with TGF-β1, and cell growth was measured by an MTT assay in the presence of either TGFβRII/Fc (1.25 μg/ml) or anti–TGF-β antibodies

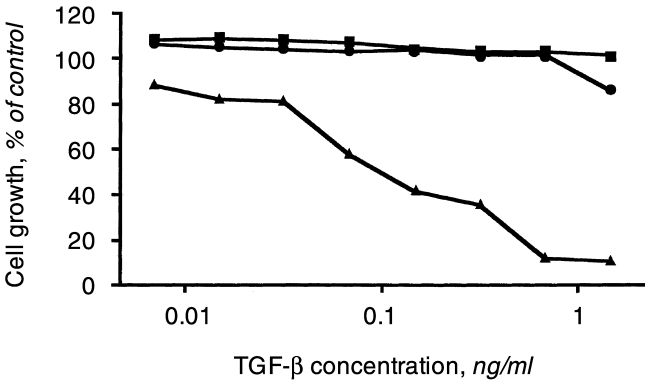

Fig. 3. Effects of the chimeric molecule on TGF-β's growth inhibition activity. One × 10^3 of BNul-7 cells were cultured with TGF-β (0.01 ng/ml to 1.25 ng/ml) in the absence (▲) or presence of chicken anti-TGF-β antibodies (■) or chimeric TGFβRII/Fc (●) for three days. Three days later, the growth of the BNul-7 cells was measured by MTT assay. At concentrations from 0.01 ng/ml to 1.25 ng/ml of TGF-β, the growth of BNul-7 cells was inhibited by TGF-β1 in a dose-dependent manner. Both anti-TGF-β antibodies and chimeric TGFβRII/Fc completely blocked the growth inhibitory action of TGF-β1.

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Effects of the chimeric molecule on TGF-β's matrix-synthesis activity

For an alternative approach to identifying the properties of TGFβRII/Fc as a competitive antagonist for TGF-β1, we addressed whether recombinant chimeric TGFβRII/Fc molecule could manipulate the effects of TGF-β on the extracellular matrix synthesis. NRK cells were treated with TGF-β1 (1 ng/ml), and the expression of fibronectin EIIIA was measured in the presence or absence of TGFβRII/Fc (10 μg/ml). Alternative splicing at EIIIA exon generates two types of fibronectin mRNA that differ in terms of the presence or absence of the EIIIA sequences. The two fibronectin mRNA variants EIIIA+ (with EIIIA exon) and EIIIA− (without EIIIA exon) were examined by the RT-PCR method. EIIIA+ fibronectin is considered to be a specific marker for TGF-β activity and matrix accumulation, because it is almost undetectable in normal adult rat glomeruli and is strongly induced by TGF-β [24].

Exposed to TGF-β1, EIIIA+ fibronectin mRNA increased over control NRK cells, whereas EIIIA− fibronectin mRNA increased slightly. There was a clear reduction in EIIIA+ after treatment with the purified chimeric molecule even though the reduction of EIIIA− was much less dramatic (Fig. 4). Thus, the chimeric TGFβRII/Fc molecule also inhibits the TGF-β–induced extracellular matrix synthesis.

Detection of chimeric gene expression

To show the feasibility of the chimeric gene transfer into muscle, we examined the TGFβRII/Fc gene expression in the transfected muscle by using the specific primers encompassing the extracellular domain of the TGF-β type II receptor and IgG-Fc hinge region (Fig. 5A). Amplification with the specific primer set revealed the presence of transcript for the 420 bp product in the cDNA obtained from TGFβRII/Fc-transfected muscle (lane 1). However, an extract from TGFβRII/Fc transfected muscle (without reverse transcription; lane 2) did not result in a 420 bp product.

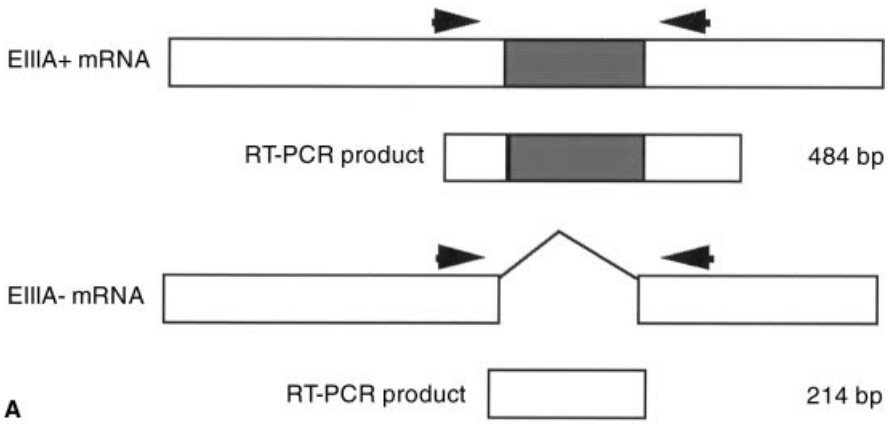
Immunohistochemical staining with the monoclonal antibody to human IgG-Fc unveiled the glomerular deposition of chimeric TGFβRII/Fc molecule in the mesangial area three days after transfection (Fig. 5B), whereas there was no deposition in the glomeruli of untreated disease control rats (Fig. 5C).

Inhibition of TGF-β1 expression in nephritic glomeruli

Given the competitive properties of chimeric TGFβRII/Fc against TGF-β1 *in vitro*, we assessed the feasibility of chimeric gene transfer *in vivo*. HVJ-liposome solution containing 5 μg or 20 μg of pCAGGS-TGFβRII/Fc was introduced into the gluteal muscles of nephritic rats one day after injection of the OX-7 antibody. HVJ-liposome solution without DNA was also examined in nephritic rats as untreated disease controls. On day 7, we obtained glomerular RNA to examine whether the chimeric TGFβRII/Fc gene transfection could reduce the message of TGF-β1 and type I collagen in glomeruli. Glomeruli were isolated by the graded sieving technique, and Northern blot analysis was performed to compare the levels of TGF-β1 and type I collagen mRNA. The TGF-β1 and type I collagen mRNA levels were elevated by more than 10- and 12-fold in untreated disease controls seven days after OX-7 injection, respectively. Laser densitometric analysis revealed that TGF-β1 mRNA expression in glomeruli from nephritic rats transfected with 5 μg and 20 μg of the chimeric gene was reduced to 48% and 39% of that in untreated disease controls, respectively (Fig. 6). The effect of TGFβRII/Fc transfection on type I collagen mRNA expression was observed as a similar pattern. The level of GAPDH mRNA remained unaffected in all transfected kidneys.

Suppression of extracellular matrix expansion in nephritic glomeruli

Given the effective suppression of TGF-β1 message production, we assessed the inhibitory action of chimeric gene transfection on the histologic changes in the kidney on day 7. Figure 7 shows the representative appearance of the glomeruli. In the kidneys from the untreated ne-



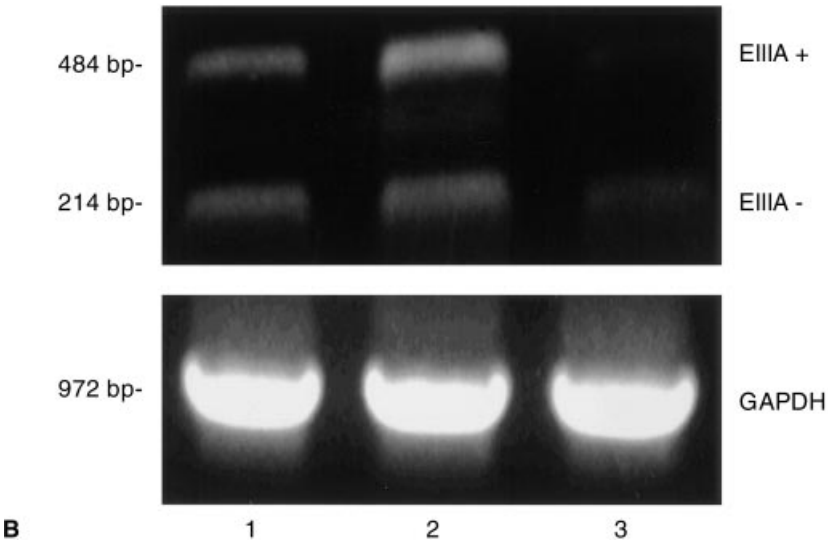


Fig. 4. Effects of the chimeric TGFβRII/Fc on TGF-β's fibronectin synthesis activity. (*A*) Specific probes complementary to mRNA, encompassing EIIIA exon for RT-PCR. The amplified fragments were detected as 484 and 214 bp for EIIIA+ and EIIIA−, respectively. (*B*) The effects of chimeric TGFβRII/Fc on EIIIA expression. NRK cells were incubated with medium alone (lane 1) or 1 ng/ml of TGF-β1 (lane 2). TGF-β1–treated NRK cells were coincubated with 10 μg of TGFβRII/Fc chimeric protein (lane 3). The expression of fibronectin EIIIA was measured by RT-PCR by using specific primers.

phritic rats, the glomeruli exhibited marked increases in mesangial extracellular matrix. In contrast, chimeric gene transfection markedly suppressed the increase in mesangial matrix expansion. Semiquantitative analysis of mesangial matrix indices is summarized in Figure 8. The mesangial matrix indices of glomeruli from the kidneys untransfected, 5 μg transfected, and 20 μg transfected were 3.1 ± 0.4, 2.1 ± 0.5, and 1.9 ± 0.6, respectively. These results demonstrated that chimeric TGFβRII/Fc gene transfer one day after disease induction could suppress extracellular matrix expansion.

DISCUSSION

Transforming growth factor-β is a key cytokine in which its actions in promoting the production and deposition of extracellular matrices are essential for normal tissue repair following injury, and sustained or excessive expression of TGF-β is considered to be clearly causal in the pathogenesis of human and experimental glomerular disease [1, 2, 7, 8, 11, 12]. In conditions characterized by chronic inflammation and fibrosis caused by overproduced TGF-β, blocking the action of TGF-β seems very promising. Therefore, there is considerable interest in potential therapeutic applications to manipulate TGF-β actions in glomerular disease by gene therapy. It seems that there are two clear possible therapeutic strategies for decreasing the action of TGF-β that may be more suitable than antibodies in humans. We have been actively investigating the potential of molecular biological intervention to antagonize the action of TGF-β in glomerular diseases in two ways: One strategy is a direct inhibition of TGF-β synthesis by application of antisense oligodeoxynucleotides [13], and the other is gene transfer of an inhibitory molecule against TGF-β, such as decorin, a small proteoglycan, by competitively blocking the active TGF-β binding to the cognate receptor [14].

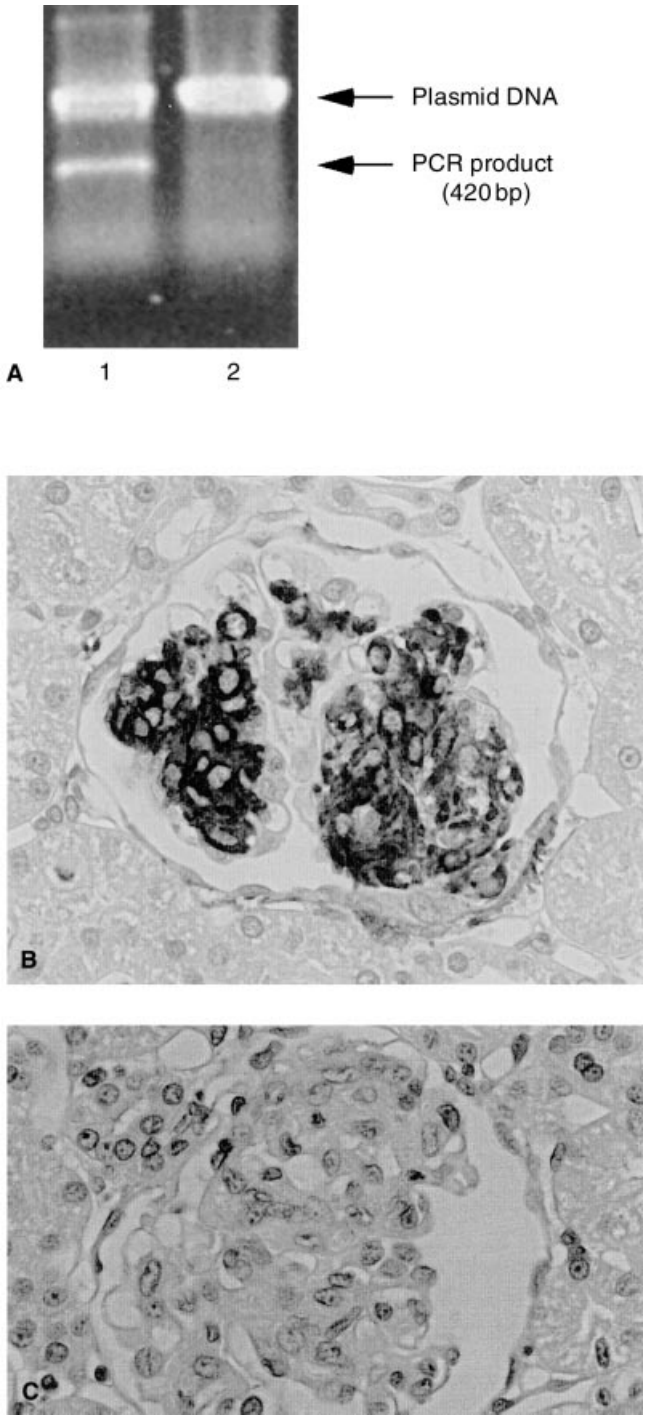


Fig. 5. Detection of chimeric gene expression. (*A*) RT-PCR for chimeric TGFβRII/Fc mRNA expression. Total RNA was isolated from the skeletal muscle of TGFβRII/Fc gene-transferred nephritic rat, and the isolated RNA was subsequently reversed transcribed into cDNA. cDNA obtained from TGFβRII/Fc transfected muscle (lane 1) was amplified by using the specific primers encompassing the extracellular domain of the TGF-β type II receptor and the IgG-Fc hinge region. The amplified fragment was detected as 420 bp for spliced TGFβRII/Fc mRNA. Extracted product obtained from TGFβRII/Fc transfected muscle was also amplified without reverse transcription (lane 2). (*B*) Immunohistochemical staining of a glomerulus from nephritic rat with the monoclonal antibody to human IgG-Fc three days after TGFβRII/Fc gene transfection. (*C*) Untreated disease control kidney was also stained with the monoclonal antibody to human IgG-Fc, demonstrating that there was no deposition in disease control kidney.

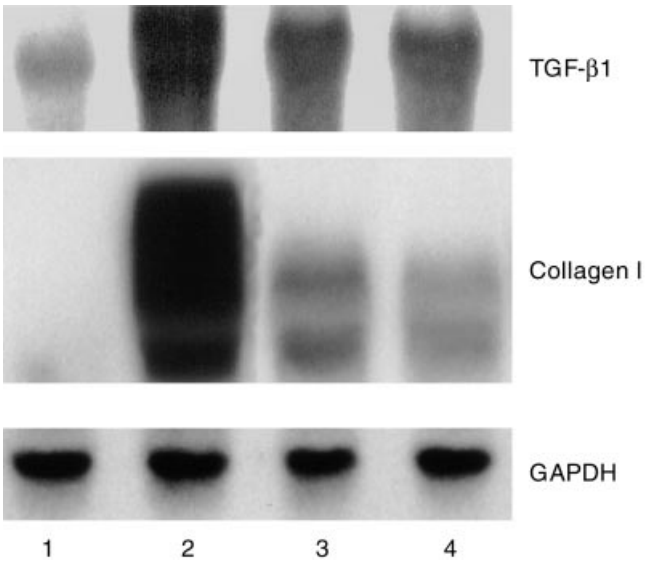


Fig. 6. Northern blot analysis of the chimeric TGFβRII/Fc gene transfection on glomerular TGF-β1 and type I collagen mRNA in nephritic rats. The glomeruli were obtained from age-matched normal control rats (lane 1), untreated disease control kidneys (lane 2), and the kidneys from nephritic rats treated with 5 μg of DNA (lane 3) and 20 μg of DNA (lane 4) transfection.

In this study, we generated a fusion protein in which TGF-β type II receptor cDNA encoding extracellular domain was recombined in frame with the Fc portion of the human immunoglobulin IgG1 heavy-chain cDNA at the hinge region. Because of the T-cell receptor and CD4 immunoadherin fusion proteins [18], receptor-IgG chimeras have been used for structural and functional studies of the Ig-like superfamily of receptors, including c-kit [26] and the platelet-derived growth factor receptor [27]. The ligand-binding properties of other non-Ig-like receptors, including the natriuretic peptide receptors [28], tumor necrosis factor receptor [29], and hepatocyte growth factor receptor [30], have been analyzed by means of secreted immunoglobulin fusion proteins as well. It was reported that dimeric tumor necrosis factor receptor-IgG heavy-chain chimeric protein was quite effective as a tumor necrosis factor inhibitor. In contrast, the monovalent extracellular portion had a greatly reduced tumor necrosis factor inhibitory activity compared with that of the bivalent inhibitor [29]. Therefore, we ligated the variable and CH1 domains from the heavy chain with the extracellular domain of the TGF-β type II receptor to the random structure of the hinge to facilitate dimer secretion and to allow maximal adaptability to the

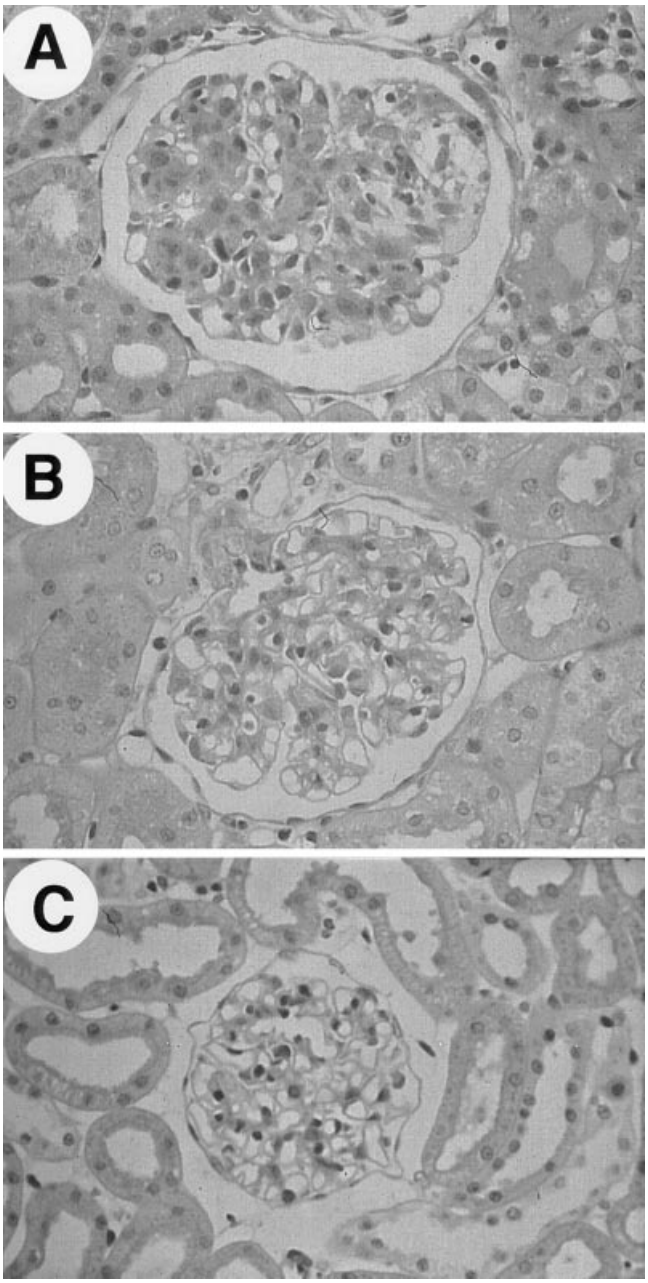


Fig. 7. Effects of chimeric TGFβRII/Fc gene transfection on the progression of anti-Thy-1 glomerulonephritis on day 7. Micrographs show representative results of periodic acid-Schiff staining of glomeruli from untreated disease control kidneys (*A*), 5 μg of DNA transfected (*B*), and 20 μg of DNA transfected (C) nephritic rats (×400).

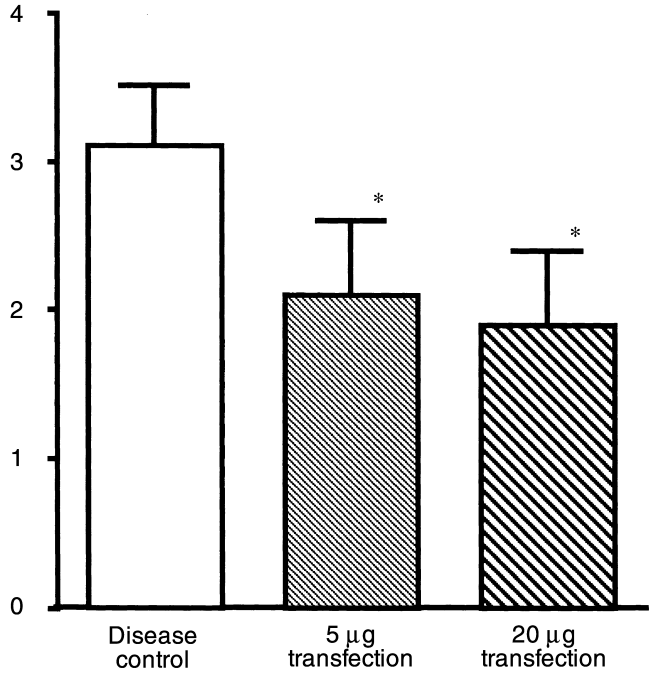


Fig. 8. Semiquantitation of the pathological effects of chimeric TGFβRII/Fc gene transfection. The severity of the glomerular damage of the untreated disease control kidneys and the kidneys from nephritic rats treated with 5 μg of DNA and 20 μg of DNA transfection was evaluated by extracellular matrix accumulation. The values are expressed as mean ± SE. $*P < 0.001$ vs. the other groups. Each group contained six animals.

ligand of TGF-β. In fact, SDS-PAGE analysis demonstrated that TGFβRII/Fc is processed to a two-chain form when expressed in COS cells such as the cell-associated TGF-β type II receptor (Fig. 1). Our data confirmed that indeed, recombinant chimeric TGFβRII/Fc was a competitive antagonist of TGF-β in two ways: (*a*) Recombinant chimeric TGFβRII/Fc (1.25 μg/ml) completely blocked the growth inhibitory action of TGF-β in BNul-7 cells (Fig. 3), and (*b*) recombinant chimeric TGFβRII/Fc protein (10 μg/ml) diminished EIIIA+ mRNA induced by TGF-β (1 ng/ml) below the control level of untreated NRK cells (Fig. 4).

The chimeric-soluble TGF-β receptor is a secreted protein, and when the expression vector is transferred in the skeletal muscle, soluble TGF-β receptors would be expected to increase the secretion and delivery to the kidneys via the bloodstream in the context of a disulfide-linked dimer. RT-PCR analysis of the chimeric gene-transfected muscle demonstrated that processed TGFβRII/Fc mRNA was expressed three days after transfection (Fig. 5A). When transfected into muscle, gene expression was observed until at least 14 days [14]. The chimeric soluble receptor with IgG-Fc was also reported to confer advantage for *in vivo* studies because of its extended plasma half-life [18]. It would thus appear that adding an IgG heavy chain as a partner in chimeric molecules may be a useful strategy for the expression of molecules that would otherwise prove unstable in circulation.

The phenomenon of protein deposition in the glomerulus is well known and has been extensively studied. Deposition of the molecule in the glomerulus depends on the size and charge of the molecule, and the capability of the molecule phagocytosed/endocytosed by mesangial cells. Anionic antigens or antigens with a large size have

a tendency to form deposits in the mesangium. Large particles may enter the mesangium through fenestrated endothelium. It remains unclear why the large size and anionic property of the molecule determine its mesangial localized deposition; however, it may be feasible that a portion of the molecule enables the specific delivery to the mesangium. Employing this concept (delivering a specific molecule into the glomerulus via gene transfer to a nonrenal organ) we have already shown that the decorin secreted from the transfected muscle could target the glomerulus and block the fibrotic actions of TGF-β [14]. Here, it was also shown that the chimeric TGFβRII/Fc molecule was detected in the mesangial area (Fig. 5B), indicating that TGFβRII/Fc was produced in the muscle and delivered to the kidney.

Of interest is that chimeric TGFβRII/Fc treatment reduced the increase in TGF-β1 mRNA levels. This finding strongly supports the hypothesis that a positive feedback system exists for stimulation of TGF-β production by TGF-β, by itself, in glomeruli [31] and that the binding of TGF-β to a chimeric TGFβRII/Fc intercepted the positive feedback pathway. These results suggest that the characteristic features of glomerular lesions are largely mediated by increased endogenous TGF-β activity in the kidney and that these manifestations can also be attenuated by blocking TGF-β.

The HVJ liposome-mediated transfer of a TGF-β inhibitor gene in the muscle represents a means for imposing the blockade of TGF-β activity in nephritic glomeruli and maintaining it for a few weeks. Conceptually, the approach that we used differs from the infusion of recombinant inhibitory proteins, in that the function of the overexpressed TGF-β has been abrogated by the introduction of an artificial gene, the product of which neutralizes TGF-β. Gene therapy has several important advantages over therapy with a recombinant protein. For example, it does not require repeated intravascular injections for administration; this may significantly improve compliance. Sustained production and secretion of the protein by muscle cells may lead to continuously retained concentrations and a greater therapeutic effect than if administered by intravascular injection. Finally, and importantly, gene therapy is advantageous because it is less expensive and overcomes the need to manufacture and purify a recombinant protein.

Blocking TGF-β with antibodies is therapeutic in a number of models of fibrotic disease in other organs. None of the studies reported any adverse effects of treating animals by neutralizing TGF-β antibodies [2]. It was reported that the injection of neutralizing antibody for TGF-β into dermal wounds healed without scar tissue formation, whereas all control wounds with irrelevant antibodies or TGF-β injection healed with scarring [32]. Neutralizing antibody-treated wounds had fewer macrophages and blood vessels, lower collagen and fibronectin contents, but identical tensile strength and more normal dermal architecture than the other wounds. We have seen no such adverse effects in our work with chimeric gene transfer. Extreme reduction of TGF-β levels may be pathological; however, our results show that chimeric gene transfer suppressed TGF-β1 mRNA expression, but the level of TGF-β1 was over the normal level, and it is unlikely that gene transfer renders cells unresponsive to normal signal transduction of TGF-β1. For these reasons, we feel that suppressing TGF-β with chimeric gene therapy will be safe. However, the long-term consequences of reducing TGF-β levels are unknown, and the possibility of adverse effects must be carefully studied.

In conclusion, we constructed a chimeric molecule designed specifically to inhibit TGF-β–mediated glomerular matrix accumulation. The TGFβRII/Fc binds TGF-β1, thereby blocking the biological activities of TGF-β *in vitro*. Moreover, the transfection of the TGFβRII/Fc gene into the muscles of nephritic rats prevented glomerular matrix expansion *in vivo*. These results suggest that TGFβRII/Fc may prove to be clinically useful, given that blocking of TGF-β overexpression in the glomeruli provides a potentially powerful therapeutic approach to the treatment of TGF-β–mediated glomerular diseases.

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