In Vivo Administration of a Nuclear Transcription Factor-κB Decoy Suppresses Experimental Crescentic Glomerulonephritis

NARUYA TOMITA,* RYUICHI MORISHITA, * † HUI Y. LAN, ‡ KEI YAMAMOTO,* MASAHIDE HASHIZUME, † MITSUE NOTAKE, ‡ KAORU TOYOSAWA, ‡ BUICHI FUJITANI, ‡ WEI MU, ‡ DAVID J. NIKOLIC-PATERSON, ‡ ROBERT C. ATKINS, ‡ YASUFUMI KANEDA, † JITSUO HIGAKI,* and TOSHIO OGIHARA*

Departments of *Geriatric Medicine and †Gene Therapy Science, Osaka University Medical School, Suita, Japan; ‡Department of Nephrology, Monash Medical Centre, Clayton, Victoria, Australia; and ‡Dainippon Pharmaceutical Co., Ltd., Suita, Japan.

Abstract. Glomerular expression of cytokines, interleukin-1 (IL-1), and tumor necrosis factor-α (TNF-α), together with leukocytic infiltration, are prominent features in crescentic glomerulonephritis. Because these cytokines are targets for nuclear transcription factor-κB (NF-κB), the use of NF-κB decoy oligodeoxynucleotide (ODN) treatment was evaluated in an experimental disease model. Crescentic glomerulonephritis was induced in primed Wistar rats by injection of sheep antiglomerular basement membrane serum. Thirty minutes after injection, rats were anesthetized and the left kidney was perfused with NF-κB decoy ODN or scrambled ODN control mixed with a virus-liposome complex, and then killed 7 d later. Animals given the scrambled control ODN developed severe glomerulonephritis by day 7 with heavy proteinuria, glomerular crescents and interstitial lesions, marked leukocytic infiltration, and upregulated renal expression of cytokines (IL-1 and TNF-α) and adhesion molecules (intercellular adhesion molecule-1). In contrast, NF-κB decoy ODN treatment substantially inhibited the disease with a 50% reduction in proteinuria, a threefold reduction in histologic damage, a 50% reduction in leukocytic infiltration, and a 50 to 80% reduction in the renal expression of cytokines and leukocyte adhesion molecules. In conclusion, this study has demonstrated that NF-κB plays a key role in cytokine-mediated renal injury and that NF-κB decoy ODN treatment has clear therapeutic potential in rapidly progressive glomerulonephritis.

Crescentic glomerulonephritis is a rapidly progressive form of kidney disease with a poor prognosis (1). The aggressive nature of this disease is attributed to the prominent leukocytic infiltration observed in biopsy samples (2,3). This infiltrate is associated with marked upregulation of renal expression of proinflammatory cytokines (interleukin-1 [IL-1] and tumor necrosis factor-α [TNF-α]), chemokines (monocyte chemoattractant protein-1), and leukocyte adhesion molecules (intercellular adhesion molecule-1 [ICAM-1] and vascular adhesion molecule-1) (4–8). Experimental models of crescentic glomerulonephritis, such as anti-glomerular basement membrane (GBM) disease, have been shown to be leukocyte-dependent (9). Furthermore, blocking the action of IL-1 or TNF-α in animal models of crescentic glomerulonephritis has been shown to inhibit renal T cell and macrophage infiltration and suppress renal injury, including crescent formation (10–14).

The transcription factor nuclear factor-κB (NF-κB) is important in the coordinated expression of various proinflammatory molecules, including IL-1, TNF-α, and ICAM-1 (15,16). Therefore, we postulate that inhibiting the action of NF-κB using a synthetic decoy for the cis-acting element in the promoter region of these genes can block the underlying inflammatory response in crescentic glomerulonephritis. The decoy approach to blocking transcription factor activity has been shown to be an effective strategy for inhibiting specific gene expression in vitro and in vivo (17–19). We have developed a gene therapy approach using oligodeoxynucleotides (ODN), or plasmid DNA, complexed with hemagglutinating virus of Japan (HVJ) liposomes that enables efficient transfection of various organs, such as the liver and the vessel wall (20–23). This method has been refined to allow efficient transfection of glomerular cells via perfusion of the renal artery (24,25). The aim of the current study was to transfect glomerular cells with an NF-κB decoy ODN to determine whether NF-κB is a key regulator of the inflammatory response causing renal injury in an experimental model of crescentic glomerulonephritis.
Materials and Methods

Synthesis of ODN and Selection of Sequence Targets

Sequences of phosphorothioate double-stranded ODN were as follows:

NF-κB decoy ODN containing the NF-κB consensus sequence (underlined):

5′-CTTGAAGGGATTTCCCTCC-3′
3′-GGAACTTCCCTAAAGGGAGG-5′

Scrambled ODN:

5′-TTGCCGTACCTGACTTAGCC-3′
3′-AACGGCATGGACTGAATCGG-5′

The NF-κB decoy ODN have been shown to bind to the NF-κB transcription factor (Figure 1b) (19). Synthetic ODN were washed with 70% ethanol, dried, and dissolved in sterile Tris-ethylenediaminetetra-acetic acid (EDTA) buffer (10 mM Tris, 1 mM EDTA). The supernantant was purified over a Nick column (Pharmacia Biotech, Uppsala, Sweden) and quantified by spectrophotometry. The single-stranded ODN were annealed for 2 h while the temperature decreased from 80 to 25°C (17–19). FITC-labeled ODN were also prepared for the initial studies.

Gel Mobility Shift Assay

The cortex was isolated from the whole kidney, and a nuclear extract was prepared as described previously (17). Tissues were homogenized using a Potter-Elvehjem homogenizer in 4 vol of ice-cold homogenization buffer (10 mM Hepes, pH 7.5, 0.5 M sucrose, 0.5 mM spermidine, 0.5 mM spermine, 5 mM EDTA, 0.25 M ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetra-acetic acid, 7 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride). After centrifugation at 12,000 x g for 30 min at 4°C, the pellet was lysed in 1 vol of ice-cold homogenization buffer containing 0.1% Nonidet P-40 using a Dounce homogenizer. After centrifugation at 12,000 x g for 30 min at 4°C, nuclei were washed twice with ice-cold buffer containing 0.35 M sucrose. After washing, nuclei were preextracted with 1 vol of ice-cold homogenization buffer containing 0.05 M NaCl and 10% glycerol for 15 min at 4°C. The nuclei were then extracted with homogenization buffer containing 0.3 M NaCl and 10% glycerol for 1 h at 4°C, and the concentration of DNA was adjusted to 1 mg/ml. After pelleting the extracted nuclei at 12,000 x g for 30 min at 4°C, the supernatant was added to 45% (NH₄)₆SO₄ and stirred for 30 min at 4°C. The precipitated protein was pelleted at 17,000 x g for 30 min, resuspended in homogenization buffer containing 0.35 M sucrose, and stored in aliquots at −70°C. NF-κB and scrambled ODN probes were labeled with 32P using a 3′ end-labeling kit (Clontech, Palo Alto, CA). Labeled ODN probes were purified using a Nick column (Pharmacia Biotech). Binding reactions (10 μl), including 32P-labeled probe (0.5 to 1 ng; 10,000 to 15,000 cpm), and 1 μg of poly(deoxyinosinic-deoxy cytidylic) acid (Sigma Chemical Co., St. Louis, MO) were incubated with 10 μg of nuclear extract for 30 min at room temperature, and then loaded onto a 5% polyacrylamide gel. The gels were subjected to electrophoresis, drying, and autoradiography.

Preparation of HVJ Liposomes

HVJ liposomes were prepared in a manner identical to that described previously (21–24). Phosphatidylserine, phosphatidylcholine, and cholesterol were mixed in a weight ratio of 1:4.8:2 (21–24). The lipid mixture (10 mg) was deposited on the sides of a flask by removal of tetrahydrofuran in a rotary evaporator. Dried lipid was hydrated in 200 μl of balanced salt solution (137 mM NaCl, 5.4 mM KCl, 10 mM Tris-HCl, pH 7.6) containing ODN. Liposomes were prepared by shaking and sonication. Purified HVJ (Z strain) was inactivated by

![Figure 1. Gel mobility shift assay. (A) Nuclear extracts were prepared from rat kidneys and incubated with 32P-labeled nuclear factor-κB (NF-κB) oligodeoxynucleotide (ODN) probe as follows: lane 1, probe with no nuclear extract; lane 2, probe with extract from normal rat kidney; lane 3, probe with extract from anti-glomerular basement membrane (GBM) disease kidney. The NF-κB-bound complex is indicated by the arrow. (B) To confirm the specificity of NF-κB binding, a competition assay was performed on binding of 32P-labeled NF-κB ODN to the nuclear extract from anti-GBM disease kidney, as follows: lane 1, labeled probe with no extract; lane 2, labeled probe with extract; lanes 3 and 4, labeled probe with extract plus 100-fold (lane 3) or 200-fold (lane 4) excess of unlabeled scrambled ODN; lanes 5 and 6, labeled probe with extract plus 100-fold (lane 5) or 200-fold (lane 6) excess of unlabeled NF-κB ODN.](image-url)
ultraviolet irradiation (110 erg/nm² per s) for 3 min just before use. The liposome suspension (0.5 ml, containing 10 mg of lipids) was mixed with HVJ (30,000 hemagglutinating units) in a total volume of 4 ml of balanced salt solution. The mixture was incubated at 4°C for 5 min and then for 30 min with gentle shaking at 37°C. Free HVJ was removed from the HVJ-liposomes by sucrose density gradient centrifugation. The top layer of the sucrose gradient was collected for use (21–24).

Transfection of ODN-HVJ Liposomes into the Kidney

Male 8-wk-old Wistar rats weighing 150 g were purchased from Charles River Japan (Osaka, Japan). Rats were anesthetized with pentobarbital, and the left renal artery was surgically exposed. A cannula was introduced into the left renal artery via the aorta. The artery was transiently isolated by temporary ligatures, and the ODN-HVJ liposome complex was infused into the kidney over a 5-min period at room temperature. The infusion cannula was then removed, blood flow to the renal artery was restored by release of the ligatures, and the wound was closed. A series of preliminary studies was performed in normal rats in which the left kidney was perfused with FITC-labeled ODN alone or FITC-labeled ODN complexed with HVJ liposomes, and rats were killed after 1 h, 1 d, or 7 d; the tissue was examined by immunofluorescence to determine transfection of glomerular cells.

Rat Model of Crescentic Glomerulonephritis

A well characterized rat model of crescentic glomerulonephritis was used (26). Disease was induced in groups of six inbred male Wistar rats (150 g) by immunization with 5 mg of normal rabbit IgG.
in Freund’s complete adjuvant followed 5 d later (termed day 0) by intravenous injection of rabbit anti-GBM serum (50 μg IgG/100 g body wt). Thirty minutes after the serum injection, the left kidney was perfused with either NF-κB decoy ODN or scrambled ODN complexed with HVJ liposomes, as described above. Blood and 24-h urine collections were taken on days 1, 3, 5, and 7. Animals were killed on day 7 and tissue was collected for examination. A group of rats excluded from experimentation was used as the normal control.

Twenty-four-hour urine collections were obtained from rats that were individually housed in metabolic cages. Rats were fasted during the collection period, but were allowed free access to water. Urine protein excretion was measured by a sulfosalicylic acid method.

**Histologic Analysis and Immunohistochemistry Staining for Leukocytes**

Kidney tissue was fixed in 4% paraformaldehyde in phosphate-buffered saline and embedded in paraffin. Hematoxylin and eosin-stained 4-μm sections were used to evaluate glomerular histology, as described previously (26). Fifty glomeruli per animal were assessed for glomerular cell number and crescent formation. The degree of interstitial damage (fibrosis, tubular atrophy, and leukocytic infiltration) was graded on a scale of 0 to 4 as described previously (26). For detection of leukocytes, snap-frozen tissue sections were fixed in 2% paraformaldehyde and then stained with the OX-1 monoclonal antibody (anti-rat CD45) (27), using a three-layer peroxidase anti-peroxidase method with diaminobenzidine for color development (26). The number of OX-1+ leukocytes in 50 glomeruli per animal was counted under high power. In addition, the number of interstitial OX-1+ cells in 20 high-power cortical fields was counted (26). All scoring was done on coded slides.

**RNA Extraction and Northern Blot Analysis**

Total cellular RNA was extracted from a half kidney using RNAzol (Tel-Test Inc., Friendswood, TX). Samples (30 μg) were electrophoresed on a 1.5% agarose-formaldehyde denaturing gel and transferred to a nitrocellulose membrane (Amersham International, Buckinghamshire, United Kingdom). The filter was baked, prehybridized, and hybridized with 32P-labeled IL-1β and TNF-α oligonucleotide probes (Clontech), GAPDH oligonucleotide probe (Clontech), and ICAM-1 cDNA probe (kindly donated by Dr. Tetsuya Tomita, Osaka University, Osaka, Japan) as described previously (28). Filters were washed stringently and then exposed to x-ray film.

**In Situ Hybridization**

**In situ** hybridization was performed on 4-μm paraffin sections using a microwave-based technology (29). After dewaxing, sections were placed in 0.01 M sodium citrate buffer, pH 6.0, and heated for 2 × 5 min in a microwave oven at 2450 MHz and a power output of 800 W. Sections were then treated sequentially with 0.2 M HCl for 15 min, 1% Triton X-100 for 15 min, and digested for 20 min with 10 μg/ml proteinase K at 37°C (Boehringer Mannheim, Mannheim, Germany). Sections were then washed in 2× SSC, prehybridized, and then hybridized with 0.3 ng/ml digoxigenin-labeled sense or antisense IL-1β and TNF-α cRNA probe overnight at 37°C in a hybridization buffer containing 50% deionized formamide, 4× SSC, 2× Denhardt’s solution, 1 mg/ml salmon sperm DNA, and 1 mg/ml yeast tRNA. Sections were then washed in 0.1× SSC at 37°C, and the hybridized probe was detected using sheep anti-digoxigenin antibody conjugated with alkaline phosphatase and color development with nitroblue tetrazolium/X-phosphate (Boehringer Mannheim).

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**Table 1. NF-κB decoy ODN treatment inhibits histologic damage in rat anti-GBM disease**

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Normal Kidney</th>
<th>Scrambled ODN-Treated Anti-GBM Disease</th>
<th>NF-κB Decoy ODN-Treated Anti-GBM Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerular cellularity (cells/gcs)</td>
<td>56.3 ± 8.6</td>
<td>100.3 ± 12.9b</td>
<td>78 ± 6c</td>
</tr>
<tr>
<td>Focal glomerular sclerosis (%)</td>
<td>0</td>
<td>28.5 ± 9.0</td>
<td>9.6 ± 5.9d</td>
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<td>Glomerular crescents (%)</td>
<td>0</td>
<td>20.5 ± 9.9</td>
<td>4.4 ± 2.0d</td>
</tr>
<tr>
<td>Interstitial lesions (%)</td>
<td>0</td>
<td>32.9 ± 21.7</td>
<td>8.4 ± 2.7d</td>
</tr>
</tbody>
</table>

*a* NF-κB, nuclear factor-κB; ODN, oligodeoxynucleotide; GBM, glomerular basement membrane; gcs, glomerular cross section.

*b* *p* < 0.001 versus normal by ANOVA.

*c* *p* < 0.01 versus scrambled by ANOVA.

*d* *p* < 0.05 versus scrambled by t test.

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**Figure 5.** Northern blot analysis of cytokine and adhesion molecule expression in anti-GBM disease. Thirty micrograms of total cellular RNA was examined per lane for interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and intercellular adhesion molecule-1 (ICAM-1). Blots were reprobed for GAPDH as a control. Lane 1, normal rat kidney; lane 2, NF-κB decoy ODN-treated anti-GBM disease; lane 3, scrambled ODN-treated anti-GBM disease.
Figure 6. In situ hybridization of IL-1β and TNF-α mRNA expression in anti-GBM disease. (a and b) Normal rat kidney. (c and d) Scrambled ODN-treated anti-GBM disease. (e through h) NF-κB decoy ODN-treated anti-GBM disease. Sections were hybridized with the following probes: antisense IL-1β (a, c, and e), sense IL-1β (g), antisense TNF-α (b, d, and f), and sense TNF-α (h). Constitutive IL-1β mRNA expression was seen in normal rat kidney (a), which was substantially upregulated in both glomeruli and tubules in scrambled ODN-treated anti-GBM disease (c). Treatment of anti-GBM disease with the NF-κB decoy ODN largely abrogated the increase in glomerular IL-1β signal and partially reduced the percentage of tubules expressing IL-1β (e). Constitutive TNF-α mRNA expression was also evident in normal rat kidney (b), which was increased markedly in scrambled ODN-treated anti-GBM disease (d). NF-κB decoy ODN treatment substantially reduced TNF-α expression in anti-GBM disease (f). No signal was seen in sections hybridized with sense probes (g and h). Magnification, ×100.
Statistical Analyses
All values are expressed as the mean ± SEM. ANOVA with subsequent Dunnett test was used to determine significant differences in multiple comparisons. \( P < 0.05 \) was considered significant.

Results
Specificity of NF-κB Decoy ODN
The presence of active NF-κB protein was easily detected in nuclear extracts prepared from anti-GBM disease kidney, but was barely detectable in normal rat kidney (Figure 1a). The addition of excess unlabeled scrambled ODN had no effect on the binding of labeled NF-κB ODN to the extract from anti-GBM disease kidney, but this binding was completely blocked by the addition of excess unlabeled NF-κB ODN (Figure 1b).

Transfection of Glomerular Cells using the HVJ Liposome Technique
In preliminary experiments, the left kidney of normal rats was infused with FITC-conjugated NF-κB ODN complexed with HVJ liposomes. Animals were killed at different times and the presence of ODN within the kidney was assessed by immunofluorescence microscopy. As early as 1 h after infusion, 40 to 50% of glomeruli in the left kidney contained FITC-ODN. The ODN were still present at 24 h (Figure 2), and remained for 7 d (not shown). Glomerular staining was seen in the right kidney, although some tubular uptake of FITC-ODN was evident (Figure 2). Infusion of FITC-ODN without HVJ liposomes was unable to transfect glomerular cells (not shown).

NF-κB Decoy ODN Inhibits Renal Injury in Crescentic Glomerulonephritis
Rats infused with the scrambled ODN control developed crescentic glomerulonephritis in terms of severe renal injury, as demonstrated by heavy urinary protein excretion (Figure 3), and marked histologic damage, as shown by the presence of glomerular hypercellularity, focal glomerular sclerosis, glomerular crescent formation, and interstitial lesions (Figure 4 and Table 1). In contrast, infusion with the NF-κB decoy ODN caused an approximately 50% reduction in urinary protein excretion over the 7-d period. There was also a substantial reduction in histologic damage in the NF-κB decoy ODN-treated kidney compared with the scrambled ODN-treated kidney, including a fivefold reduction in glomerular crescent formation (Figure 4 and Table 1).

NF-κB Decoy ODN Inhibits Cytokine Production and Leukocytic Infiltration in Crescentic Glomerulonephritis
Northern blot analysis showed that expression of IL-1\( \beta \), TNF-α, and ICAM-1 is increased in scrambled ODN-treated anti-GBM disease compared with normal kidney, and reduced by treatment with the NF-κB decoy ODN (Figure 5). A more detailed analysis of IL-1\( \beta \) and TNF-α expression was performed by in situ hybridization. Constitutive expression of IL-1\( \beta \) mRNA in normal kidney was restricted to a small number of glomerular cells (mostly podocytes) and a minority of tubules (Figures 6 and 7). There was a dramatic increase in

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Infusion of the renal artery with the NF-κB decoy ODN complex produced efficient transfection of glomerular cells, but relatively little transfection of tubular epithelial cells. However, NF-κB decoy ODN treatment not only suppressed cytokine production and inflammation within the glomerulus, but it also markedly inhibited tubular IL-1β and TNF-α expression, and the associated interstitial leukocytic infiltrate and interstitial damage. This finding lends support to the hypothesis that interstitial inflammation and damage in this disease model are secondary to the release of glomerular cytokines (34).

An interesting observation is that intrinsic renal cells, rather than just leukocytes, are the major source of IL-1 and TNF-α mRNA expression in the kidney. This is consistent with studies of human crescentic glomerulonephritis, in which IL-1 and TNF-α have been shown to be expressed by glomerular and tubular epithelial cells (4–6). This emphasizes the importance of direct transfection of resident glomerular cells.

This study has shown that the NF-κB decoy ODN is a powerful inhibitor of the proinflammatory response in experimental crescentic glomerulonephritis. This is a major step forward in the potential development of molecular therapies for renal diseases. The kidney has proven to be a difficult organ in which to achieve efficient cell transfection. Previous attempts to transfect glomerular cells using adenoviral vectors have been relatively unsuccessful (35). Similarly, administration of naked ODN is unable to transfec glomerular cells, resulting only in nonspecific tubular uptake. The use of HVJ liposomes together with renal artery infusion has proven to be an effective means to transfect glomerular cells with ODN, or plasmids, in this and previous studies (24,25). The next issues to address in developing molecular therapy for renal diseases will be to prolong the stability of ODN within the transfected cells and to enable repeated NF-κB decoy ODN treatments.

The results of this study have implications for immune-mediated diseases generally. Given that blockade of IL-1 or TNF-α is an effective treatment for a variety of different models of immune-mediated disease (36), it is likely that NF-κB decoy ODN treatment will also be effective in such diseases. This postulate is supported by the ability of antisense oligonucleotides to the p65 subunit of NF-κB to suppress experimental inflammatory bowel disease (37).

In summary, this study has demonstrated that in vivo treatment with NF-κB decoy ODN is both a feasible and effective treatment to suppress renal damage in a model of rapidly progressive crescentic glomerulonephritis. Furthermore, the study provides direct in vivo evidence that activation of NF-κB is a common mechanism underlying inflammatory renal damage.

**Acknowledgments**

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Figure 8. NF-κB decoy ODN treatment inhibits renal leukocytic infiltration in anti-GBM disease. The number of OX-1+ leukocytes in glomeruli (A) and the interstitium (B) was quantified on antibody-stained tissue sections. □, normal rat kidney; ■, scrambled (SD) ODN-treated anti-GBM disease; □, NF-κB decoy ODN-treated anti-GBM disease. Data are given as mean ± SEM. *P < 0.01, **P < 0.001 versus normal rat control; *P < 0.05, **P < 0.01 versus scrambled ODN-treated anti-GBM disease, by ANOVA.

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References


Access to UpToDate on-line is available for additional clinical information at http://www.lww.com/JASN.