# Synergistic Effects of Combining Anti-Midkine and Hepatocyte Growth Factor Therapies Against Diabetic Nephropathy in Rats

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**Abstract:** *Purpose:* This study aimed to assess whether synergism could be achieved when combining midkine (MK) antisense oligodeoxynucleotides (anti-MK ODN) and recombinant human hepatocyte growth factor (HGF) in diabetic nephropathy (DN) rat models. *Methods:* Rats were randomized into 6 groups: control, DN rats without treatment, DN rats treated with scrambled ODN, DN rats treated with anti-MK ODN, DN rats treated with HGF and DN rats treated with anti-MK ODN plus HGF. DN models were created by intraperitoneal injection of streptozotocin. Two weeks later, treatments commenced. ODN (1 mg/kg) was intravenously injected weekly for 4 weeks. HGF (500 μg/kg) was subcutaneously injected daily for 4 weeks. Eight weeks later, rats were euthanized. Serum and urine parameters, kidney histopathological injury scores, immunohistochemistry and protein expressions were measured. *Results:* Blood glucose, creatinine, blood urea nitrogen and urine albumin were significantly elevated in DN rats. Any single treatment markedly reduced their levels, yet combined treatment decreased them significantly further. Any monotherapy could decrease renal injury score and immunohistochemistry positive percentage, although the most prominent change was displayed in combinational therapy. Western blot showed the expression of MK was significantly elevated in DN rats. Anti-MK ODN suppressed MK significantly. The protein expressions and serum concentrations of transforming growth factor-β1 and connective tissue growth factor between monotherapy and the combined therapy were significant. *Conclusions:* This study demonstrated that combining MK gene suppressing ODN and HGF protein synergistically attenuates renal injury in DN rats. This study may provide a novel avenue for designing future therapeutic regimens against DN.

**Key Indexing Terms:** Diabetic nephropathy; Midkine; Hepatocyte growth factor; Transforming growth factor-β1; Connective tissue growth factor; Synergism. **[Am J Med Sci 2015;350(1):47–54.]**

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Diabetic nephropathy (DN) is a major complication of diabetes mellitus, which is also the principle cause of end-stage renal failure in many countries.[1,2] With the worldwide increase in the prevalence of diabetes mellitus, DN has become a threat to human health and mortality. Approximately one-third of all diabetic individuals (either type 1 or type 2) are affected by DN, which produces significant social and economic burdens. Advances in understanding of the pathogenesis and etiology of DN (especially inflammatory status and oxidative stress) have identified additional risk factors for nephropathy, and novel therapeutic options are being explored.[3–5]

It is proven that longstanding hyperglycemia induces severe endothelial dysfunction, oxidative stress and inflammation, leading to changes in renal morphology and function. In diabetic rodent models and in diabetic patients, increased levels of transforming growth factor-β1 (TGF-β1)[6–9] and connective tissue growth factor (CTGF)[10–12] have been demonstrated as key factors during the process of DN. These growth factors, along with albuminuria and pathologic changes (glomerular hypertrophy, tubular dilation, tubular degeneration, interstitial expansion, extracellular matrix [ECM] accumulation and thickening of the glomerular basement membrane), are used as characteristic indicators of DN.

The growth factor midkine (MK) has been implicated in neuronal survival and differentiation, cancer development and inflammation-related diseases.[13] Recently, MK was identified as a key molecule not only in mesangial-mediated nephropathy but also as a direct activator of the tubulointerstitial inflammatory process associated with DN.[14,15] MK antisense oligodeoxynucleotides (anti-MK ODN) can suppress tumor growth in nude mice,[16] ameliorate arterial restenosis, ischemic reperfusion-induced renal damage and cisplatin-induced nephropathy.[17–19]

Hepatocyte growth factor (HGF) is a mesenchyme-derived cytokine with antifibrotic and regenerative properties in experimental models of chronic renal damage.[20–25] HGF inhibition exacerbates renal fibrosis, whereas HGF supplementation reverses this progression.[20–23] The antifibrotic activity of HGF was reported to work through the antagonistic action on TGF-β1.[26–28] The beneficial effect of a long-term treatment with recombinant HGF on streptozotocin-induced DN in mice has also been observed.[29]

In this study, the authors aimed to assess the effects of combining anti-MK ODN and HGF against DN in rat models, and tried to determine whether synergism can be achieved.

## MATERIALS AND METHODS

### Chemicals, Reagents and Animals

MK antisense ODN 5′-AGGGCGAGAAGGAAGAAG-3′ corresponded to bases 15 to 32 in MK cDNA; scrambled ODN 5′-GGGAAAAGAAACGGGAGG-3′ was used as a

control of the antisense DNA, as described before.[16–18] Phosphorothioate-modified ODNs were synthesized from SBS Genetech Co (Beijing, China). These ODNs have been tested previously in murine models as effective in suppressing MK.[16–18] Recombinant human HGF was purchased from R&D Systems (Minneapolis, MN).

One hundred twenty male Sprague Dawley rats weighing (180 ± 20) g, purchased from the Animal Center of Science Institute of China (Beijing, China), were used in this study. All the animals were kept in individual cages at a temperature of 18 to 20°C, humidity of 65% to 69%. The experimental procedures were approved by the animal ethics committee of Tianjin Medical University General Hospital.

### Experimental Protocol

Rats were randomly divided into 6 groups (20 per group): control rats (group 1), DN rats without treatment (group 2), DN rats treated with scrambled ODN (group 3), DN rats treated with anti-MK ODN (group 4), DN rats treated with HGF (group 5) and DN rats treated with anti-MK ODN plus HGF (group 6). All DN rats were 1st intraperitoneally injected with streptozotocin (Sigma, St. Louis, MO) at a dose of 50 mg/kg diluted in citrate buffer (0.1 mol/L, pH 4.0), whereas rats in control group only received equal volume of citrate buffer. During the experimental period, body weight (BW) and fast blood glucose (FG) of rats were measured weekly, and all the diabetic rats were subcutaneously given long-acting insulin (Glargine; Sanofi-Aventis, Frankfurt-Höchst, Germany) to maintain the FG at about 30 mmol/L to avoid ketonemia and promote the well-being of animals. Two weeks after streptozotocin injection, different treatments commenced. One milligram per kilogram of ODN was intravenously injected through the tail vein every week for 4 weeks. Five hundred micrograms per kilogram of HGF was subcutaneously injected daily for 4 weeks. The dosages of ODN[16–18] and HGF[25–27] have already been tested in murine models as optimal when used as monotherapeutic modalities.

Rats were euthanized at the end of 8 weeks after therapy initiation. Before the rats were euthanized, 24-hour urine samples were obtained by using metabolic cages and were centrifuged at 3,000 rpm for 10 minutes and stored at −80°C for subsequent examinations. Urine albumin (UAlb) concentrations were measured by Turbox microalbuminuria assay (Orion Diagnostica, Espoo, Finland). While the rats were euthanized, blood was collected in centrifuge tubes from the inguinal vein. Serum was isolated and stored at −80°C for subsequent examinations. Serum was assayed for values of FG, blood creatinine (Cr) and blood urea nitrogen (BUN) by an automatic biochemistry analyzer (Hitachi Model 7170 analyzer; Hitachi, Tokyo, Japan). Serum was also used for concentration measurements of MK, TGF-β1 and CTGF. The kidneys were quickly removed, 1 part of the tissue was fixed in neutral formalin for histological sectioning and the other part of the tissue was snap frozen into liquid nitrogen for protein extraction.

### Histopathology, Immunohistochemistry and Semiquantification

Removed kidney samples were fixed in neutral buffered formalin (pH 7.4) and embedded in paraffin. Tissue sections from the rats were prepared at 4-μm thickness by a routine procedure. Sections were stained with hematoxylin and eosin for general histology. Kidney lesions, characterized by glomerular hypertrophy, tubular dilation, tubular degeneration, interstitial expansion, ECM accumulation and glomerular basement membrane thickening, were graded according to the extent of cortical involvement on a scale from 0 to 3: 0 = normal; 1 = involvement of less than 30%; 2 = involvement up to 30% to 70%; 3 = extensive damage involving more than 70% of the pertinent area. And they were expressed as injury scores.[17,18]

Immunohistochemistry was performed on paraffin-embedded sections, as described previously.[30] The measurement of positive staining percentage was conducted by counting average positive cells in 3 field areas.

### Protein Levels of MK, TGF-β1 and CTGF in Kidneys Assessed by Western Blot

Protein extraction and Western blot were performed as described before.[18,31,32] Briefly, kidney samples were pulverized in liquid nitrogen and resuspended in tissue lysis buffer on ice, and the supernatants were collected after centrifugation at 13,000*g* at 4°C for 20 minutes. Protein concentration was determined and then stored at −80°C until use Tissue lysates were 1st mixed with an equal amount of 2× SDS loading buffer. Then, equal amounts of protein were electrophoresed by 15% SDS-polyacrylamide gel electrophoresis. Semiquantification was performed by Quantity One Software Version 4.6.2 (Bio-Rad, Hercules, CA). The intensity of the Western blot bands was quantified as the ratio of intensity of the protein of interest to that of β-actin × 100%.[33,34] Antibodies for MK, TGF-β1 and CTGF and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA).

### Serum Levels of MK, TGF-β1 and CTGF Measured by Enzyme-Linked Immunosorbent Assay

Serum levels of MK, TGF-β1 and CTGF were measured by enzyme-linked immunosorbent assay method (Uscn Life Science Inc, Houston, TX). The assays were conducted according to the manufacturer's instructions. Briefly, 100 μL of each serum sample or standard was incubated in precoated 96-well microplate 1st. Then, biotinylated anti-MK, TGF-β1 or CTGF antibodies were added and incubated. Afterward, streptavidin-conjugated horseradish peroxidase was added and incubated. Later, substrate solutions (tetramethylbenzidine) were added. Finally, stop solutions ($H_2SO_4$) were added, and the optical densities of the wells were measured at 450 nm with a Multiskan MS Plate Reader (Labsystems, Helsinki, Finland). After creating a standard curve on log-log graph paper, concentrations of the samples were determined.

### Statistical Analysis

All data were presented as mean ± SD. Statistics were performed with SPSS 17.0 (SPSS Inc, Chicago, IL). Differences between groups were analyzed by 1-way analysis of variance (ANOVA). Least significant difference (LSD) test was used for multiple comparisons among groups. $P$ value not exceeding 0.05 was considered statistically significant.

## RESULTS

### Effects of Different Treatments on Physical Behaviors, BW, Serum Indices and Urine Protein

In the experiments, rats in group 1 had normal growth and behavior; rats in group 2 and group 3 had hypopraxia, cachexia, polyuria/polydipsia, yellowish and damp fur and kyphosis; rats in group 4 and group 5 were relatively vibrant, had gray to white fur; rats in group 6 had almost normal vibrancy and white fur. BWs of rats were different (Table 1), rats in group 2 and group 3 had the lowest BWs, after monotherapy BW was significantly elevated in group 4 and group 5 ($P < 0.01$), and significant weight gain was also