Original Article

Direct transfer of hepatocyte growth factor gene into kidney suppresses cyclosporin A nephrotoxicity in rats

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Abstract

Background. The clinical utility of cyclosporin A (CsA) has been limited by its nephrotoxicity, which is characterized by tubular atrophy, interstitial fibrosis and progressive renal impairment. Hepatocyte growth factor (HGF), which plays diverse roles in the regeneration of the kidney following acute renal failure, has been reported to protect against and salvage renal injury by acting as a renotropic and anti-fibrotic factor. Here, we investigated protective effects of HGF gene therapy on CsA-induced nephrotoxicity by using an electroporation-mediated gene transfer method.

Methods. CsA was orally administered as a daily dose of 30 mg/kg in male Sprague–Dawley rats receiving a low sodium diet (0.03% sodium). Plasmid vector encoding HGF (200 μg) was transferred into the kidney by electroporation.

Results. HGF gene transfer resulted in significant increases in plasma HGF levels. Morphological assessment revealed that HGF gene transfer reduced CsA-induced initial tubular injury and inhibited interstitial infiltration of ED-1-positive macrophages. In addition, northern blot analysis demonstrated that cortical mRNA levels of TGF-β and type I collagen were suppressed in the HGF group. Finally, HGF gene transfer significantly reduced striped interstitial phenotypic alterations and fibrosis in CsA-treated rats, as assessed by α-smooth muscle actin expression and Masson’s trichrome staining.

Conclusions. These results suggest that HGF may prevent CsA-induced tubulointerstitial fibrosis, indicating that HGF gene transfer may provide a potential strategy for preventing renal fibrosis.

Keywords: acute renal failure; cyclosporin A; hepatocyte growth factor gene; kidney; nephrotoxicity

Introduction

The introduction of cyclosporin A (CsA) into clinical practice has resulted in marked improvement in the short-term outcome of organ transplantation, including significant extension in the 1 year survival of renal allografts [1]. However, CsA-induced nephrotoxicity results in long-term graft loss, which limits the clinical utility of this drug. Nephrotoxicity caused by CsA is characterized by tubular atrophy, interstitial fibrosis, hyalinosis of the afferent arteriole and progressive renal impairment [2,3]. Recent studies have shown that CsA-induced nephrotoxicity is associated with an up-regulation of transforming growth factor-β1 (TGF-β1) in type I collagen, and that TGF-β1 is important for the progression of the nephrotoxicity.

Hepatocyte growth factor (HGF), a multifunctional polypeptide originally characterized as a potent mitogen for mature hepatocytes, plays an important role in renal development and in the maintenance of normal adult kidney structure. HGF functions as a potent mitogenic, motogenic, morphogenic and anti-apoptotic factor in renal tubular epithelial cells [4]. Recent studies have suggested that both endogenous and exogenous HGF are protective against the onset and progression of chronic renal diseases in a variety of animal models. Treatment with exogenous HGF protein effectively suppressed phenotypic changes into myofibroblasts, and thus attenuated ECM deposition and interstitial fibrosis by inhibiting TGF-β1 and its receptor expression in vivo [5]. These findings suggest HGF may be a candidate for prevention of CsA-induced nephrotoxicity.

Because HGF is rapidly cleared by the liver causing a reduction in its activity [6], intravenous injections exert

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only short-term actions on target organs and do not produce continuous effects. Recently, we developed a new gene transfer system for electroporation in vivo. We infused DNA solutions via renal artery followed by electric pulses using a tweezers type of electrode to introduce genes into mesangial cells in nearly all of the glomeruli [7]. The electroporation process is free from oncogenicity, immunogenicity and cytotoxicity from viral vectors. We have shown that this electroporation-mediated gene transfer technique produced significantly higher transfection efficiency than the HVJ liposome method [7]. In addition, kidney-targeted gene transfer methods may concentrate actions on the kidney without causing systemic effect. In the present study, we investigated the effects of HGF gene transfer on CsA-induced nephrotoxicity in rat.

Materials and methods

Experimental design

Six-week-old male Sprague–Dawley rats (SLC Japan, Hamamatsu, Japan), weighing 180–190 g on a low-salt diet (0.03% sodium; Test Diet, Richmond, IN, USA) received daily oral doses of CsA at 30 mg/kg (Neoral, Novartis, Japan). In all the following procedures, rats were anaesthetized with pentobarbital. On day 0, the left kidney and renal artery were surgically exposed by a mid-line incision, and a 24-gauge catheter (Terumo, Tokyo, Japan) was inserted into the left renal artery. After clamping the proximal site of the abdominal aorta, the kidney was perfused with PBS via the renal artery, and HGF plasmid DNA (200 μg in 1 ml of PBS) was then injected into the left kidney using a single shot while clamping the renal vein. The kidney was sandwiched with a tweezers-type oval-shaped stainless electrode, and electric pulses were delivered using an electric pulse generator (CUY-21; NEPA GENE, Chiba, Japan). The pulses were square waves and the voltage (75 V) was held constant during the pulse duration. Three pulses of the indicated voltage followed by three additional pulses of the opposite polarity were administrated to the kidney. Intra-pulse delay was 1 s and the duration of the pulse was fixed at 100 ms. In separate groups of rats (n=6 per group) on days 7, 14 and 21, plasma samples were collected and kidneys were removed following perfusion with 20 ml of cold PBS from the aorta. CsA-treated rats with sham operations were also used as untreated disease controls (six animals in each group). In all animals, the cortex was carefully dissected from the medulla and was then processed for evaluation by light microscopy, RNA analysis and immunohistochemistry.

Analysis of plasma samples

Blood samples were collected from the aorta into plastic syringes, transferred to metal-free tubes containing potassium-ethylenediaminetetraacetic acid (EDTA), and then chilled on ice. The samples were immediately centrifuged at 4°C and plasma was stored at −80°C until further determination. Plasma HGF concentration was measured by the enzyme immunogen assay method (Institute of Immunology, Japan).

Morphology

Tissue samples were fixed in 4% buffered paraformaldehyde for 12 h and embedded in paraffin. Samples were cut at 2–4 μm thickness and stained with periodic acid-Schiff (PAS) and Masson’s trichrome. Interstitial fibrosis was stained blue with Masson’s trichrome and the sections were quantified by a colour image analyser. We selected at random 10 non-overlapping fields from the cortical region for analysis. The fibrotic area relative to the total area of the field was calculated as a percentage by a computer-aided manipulator. Glomeruli and large vessels were not included in the microscopic fields for image analysis. Scores from 10 fields per kidney were averaged, and mean scores from six separate animals per group were then averaged.

Immunohistochemical stainings

Renal tissues were fixed in cold melyn Carnoy’s solution for 6 h, placed in 70% ethanol, and then embedded in paraffin. Tissue sections were cut at 4 μm thickness and were dewaxed and stained with anti-rat ED-1 antibody to identify macrophage infiltration, followed by a second reaction with biotin-labelled anti-rat IgG goat IgG (Vector, Bulingame, CA, USA). Finally, an avidin-biotin coupling reaction was performed on the sections (Vectastain Elite; Vector). To identify myofibroblasts, we used monoclonal IgG against human α-smooth muscle actin (SMαA) (EPOSS System; Dako). The SMαA-positive area relative to the total area of the field was calculated as a percentage by a computer-aided manipulator. Glomeruli and large vessels were not included in the microscopic fields for image analysis. The scores of 10 fields per kidney were averaged, and mean scores from six separate animals per group were then averaged.

Northern blot analysis

Renal tissue was finely minced with an autoclaved cutter, was immediately immersed in liquid nitrogen, and then homogenized in TRIzol reagent (Gibco BRL, Grand Island, NY, USA). RNA extraction was performed according to manufacturer instructions. After resuspension in Tris–EDTA buffer, 15 μg of RNA were electrophoresed in each lane in 1% agarose gels containing 2.2 M formaldehyde and 0.2 M MOPS (pH 7.0), and transferred to a nylon membrane (Hybaid N). The membranes were prehybridized for 1 h at 42°C with 50% formamide, 10% Denhardt’s solution, 0.1% sodium phosphate, 5× standard saline citrate (SSC) and 180 μg/ml denatured salmon sperm DNA. They were hybridized overnight at 42°C with cDNA probes labelled with [32P]dCTP by random oligonucleotide priming (RediPrime). The blots were washed twice in 2× SSC, 0.1% SDS at room temperature for 15 min each, and twice in 0.2× SSC, 0.1% SDS at 60°C for 10 min each. Films were exposed at −80°C for ~24 h. Autoradiographs were scanned on an imaging densitometer. The density of bands for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA was used to control for differences in the total amount of RNA loaded onto each gel line.
Statistical analysis

All values are expressed as means ± SD. Statistical significance, defined as \( P < 0.01 \), was evaluated using one-way analysis of variance.

Results

Physiological studies

Plasma concentrations of HGF were measured by the ELIZA method. In normal control rats, plasma HGF was undetectable (<0.5 ng/ml). CsA treatment increased plasma HGF levels to 48.7 ± 18.8 ng/ml on day 7, which may reflect up-regulation of endogenous HGF following renal injury. Increased plasma HGF levels persisted throughout the experiment (61.1 ± 11.7 and 56.4 ± 24.6 ng/ml at 2 and 3 weeks, respectively). The HGF gene transfer method caused a further increase in plasma HGF levels on day 7 (106.5 ± 50.5 ng/ml; \( P < 0.05 \) vs CsA group). Thereafter, plasma HGF levels declined in HGF-treated rats (67.9 ± 48.5 and 71.3 ± 25.6 ng/ml at 2 and 3 weeks, respectively), but remained higher than in untreated rats.

Light microscopy

PAS staining revealed that CsA treatment induced characteristic histological changes, including tubular atrophy, early striped fibrosis and inflammatory cell infiltration. Importantly, gene transfer of HGF suppressed these tubulointerstitial injuries.

We used Masson’s trichrome to cause blue staining of interstitial fibrosis (Figure 1), and used a colour image analyser to semi-quantitatively estimate fibrosis area. The percentage of injured areas per field of cortex was counted at a \( \times 400 \) magnification in a minimum of 10 fields. In parallel with the tubulointerstitial injury findings, CsA treatment induced progressive fibrosis (5.3 ± 1.2 and 7.2 ± 1.5% at 2 and 3 weeks, respectively), and HGF gene transfer significantly suppressed the development of interstitial fibrosis (3.4 ± 1.2 and 5.2 ± 1.4% at 2 and 3 weeks, respectively; \( P < 0.01 \) vs CsA group).

Immunohistochemical staining

The phenotypic transformation into myofibroblasts, estimated by immunohistochemical staining of SM\( \alpha \)A, leads to extracellular matrix (ECM) accumulation. There was strong SM\( \alpha \)A expression in CsA-treated kidneys (5.5 ± 1.7% at 3 weeks) with the most pronounced increases around the thickened basement membrane of the Bowman’s capsule and degenerated tubules. This strong SM\( \alpha \)A expression was inhibited by treatment with the HGF gene transfer method (3.9 ± 1.6%; \( P < 0.01 \) vs CsA group) (Figure 2).

In normal control rats, the number of ED-1-positive macrophages was 4.2 ± 1.2 cells per high power field. CsA administration induced marked and continuous infiltration of ED-1-positive macrophages around the damaged tubules and within the interstitium (52.9 ± 17.3 cells per high power field at 3 weeks). The HGF gene transfer procedure decreased this macrophage accumulation (39.2 ± 13.2 cells; \( P < 0.01 \) vs CsA group) (Figure 3).

Northern blot

Northern blot analysis was performed on renal cortices from the three groups. CsA treatment caused an up-regulation of cortical mRNA expression of TGF-\( \beta \) and type I collagen at 3 weeks compared with the normal control group, and HGF treatment reduced the expression of both TGF-\( \beta \) and type I collagen mRNA expression (Figure 4). HGF treatment significantly reduced TGF-\( \beta \) mRNA expression (ratios of TGF-\( \beta \) signal to GAPDH signal: 1.68 ± 0.27 in untreated vs 0.81 ± 0.22 in HGF group, \( P < 0.01 \)). In parallel with

![Fig. 1. Representative photomicrographs showing renal morphological changes using Masson’s trichrome staining in the CsA (A) and HGF groups (B) at day 21.](image-url)
In the present study, we found that HGF gene transfer into kidneys using electroporation had a protective effect on chronic CsA-induced nephrotoxicity. Specifically, we demonstrated that HGF gene transfer reduced CsA-induced tubulointerstitial injury, and this included reduced macrophage infiltration, lowered phenotypic alterations of interstitial myofibroblasts and restricted interstitial fibrosis.

Several reports have indicated that HGF is involved in renal regeneration [4]. For example, rapid increases in HGF mRNA and/or protein levels in kidneys and plasma were observed in various types of renal injury induced by nephrotoxins, renal ischaemia and ureteral obstruction [4]. Recently, it was reported that endogenous as well as exogenous HGF prevents renal fibrosis in a mouse model of nephritic syndrome [8]. The present study demonstrated that HGF gene transfection suppressed the expression of TGF-β and reduced interstitial fibrosis.

TGF-β is a fibrotic cytokine and plays an important role in CsA-induced accumulation of ECM protein. The role of TGF-β in mediating CsA nephrotoxicity has been evaluated in several studies. CsA has been shown to up-regulate TGF-β expression in murine tubular cells and tubulointerstitial fibroblasts [9]. In this, type I collagen mRNA was also decreased in HGF-treated kidneys (ratios of type I collagen signal to GAPDH signal: 2.47 ± 0.47 in untreated vs 0.69 ± 0.34 in HGF group, \( P < 0.001 \)).
addition, there is increased TGF-β production in patients with chronic allograft nephropathy and CsA nephrotoxicity [10]. Furthermore, TGF-β antibodies prevent matrix synthesis and attenuate renal injury and renal function in CsA nephrotoxicity [11]. Reciprocal changes in the expressions of TGF-β and HGF were noted during the onset of tubulointerstitial fibrosis caused by unilateral ureter-ligated obstruction in mice [5]. We observed an increase in plasma HGF following CsA treatment (48.7 ± 18.8 ng/ml at day 7), however, continuous renal tissue injury and persistent expression of TGF-β may lead to decreases in endogenous HGF and result in irreversible renal insufficiency. We also found that HGF gene transfer caused further elevations in plasma HGF levels (106.5 ± 50.5 ng/ml), indicating that it may be protective against renal damage at several levels beyond the physiological level. Because HGF is rapidly cleared by the liver which reduces its activity [6], intravenous injections of HGF cause only short-term effects on target organs and do not exert continuous effects. HGF that is expressed in mesangial cells can affect renal cells in a paracrine fashion, especially in tubular cells and in interstitial cells. In addition, HGF gene transfer decreased TGF-β mRNA levels in CsA-treated kidneys. HGF prevents epithelial cell death and the remodelling of renal tissue that occurs with injury or fibrosis, an effect which is opposite to the role of TGF-β. Therefore, exogenous HGF supplementation may prove to be a therapeutic strategy against renal injury. In fact, several studies have demonstrated that HGF has preventive and therapeutic effects in acute and chronic renal failure/renal fibrosis models in laboratory animals. Although it is not known whether HGF causes a direct or indirect suppression of TGF-β expression, this treatment clearly suppresses TGF-β expression and enhances remodelling of renal tissues. These results support the hypothesis that a counterbalance between TGF-β and HGF plays a determinant role in the pathogenesis and therapeutics of fibrosis-related diseases.

Alterations in the phenotype and behaviour of renal fibroblasts may be one of the most important events during the development of interstitial fibrosis. The enhanced expression of SMαA provides a marker of interstitial phenotypic changes, and increased numbers of activated fibroblasts, which are called myofibroblasts and have features of both fibroblasts and smooth muscle cells, accompanies interstitial matrix accumulation. We assessed SMαA expression by immunohistochemistry. Immunostaining of SMαA was strong in the interstitial areas of CsA-treated kidneys, however, immunostaining was weak in the HGF gene-transferred kidneys. Although the precise mechanism of how HGF reduced interstitial SMαA expression is unclear, HGF transfection reduced tubular atrophy induced by CsA treatment. Therefore, HGF appears to suppress the epithelial–mesenchymal transition process.

We used the gene transfer approach instead of recombinant proteins for several reasons. First, since the half life of HGF is quite short, recombinant HGF treatment requires very large doses and frequent injections of recombinant protein. In addition, administrations of high-dose HGF protein may cause adverse effects, and finally, recombinant protein is costly. In contrast, gene transfer is simple, safe, cheap and requires less frequent injections. In the present study, we adopted electroporation-mediated gene transfer into glomeruli.

In summary, we demonstrated that HGF gene transfer reduced CsA-induced interstitial fibrosis by inhibiting TGF-β expression. We speculate that electroporation-mediated HGF gene transfer may be of value in treating chronic CsA-induced nephrotoxicity.

Conflict of interest statement. None declared.

References


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