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Effect of hepatocyte growth factor on tacrolimus-induced nephrotoxicity in spontaneously hypertensive rats

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Abstract Renal toxicity is a serious side effect of therapy with tacrolimus (FK506), an immunosuppressive agent administered to renal transplant recipients. We investigated the effect of hepatocyte growth factor (HGF) on tacrolimus-induced nephrotoxicity in spontaneously hypertensive rats (SHR). After a right nephrectomy, rats received a continuous perfusion of either HGF in a dose of 5 μg/kg daily (tacrolimus + HGF group) or normal saline (tacrolimus group) into the left renal artery at a rate of 1 μl/h for 7 days after surgery. Tacrolimus was injected intramuscularly in a dose of 4 mg/kg daily for 10 days after surgery. HGF significantly inhibited the tacrolimus-induced increase in the serum creatinine (SCr) level ($P < 0.05$). HGF also prevented the tacrolimus-induced loss in body weight. The bromodeoxyuridine (BrdU) index was significantly higher in kidney specimens from the tacrolimus + HGF group. These findings suggest that HGF induces the regeneration of renal tubular cells and suppresses tacrolimus-induced renal toxicity in SHR.

Key words Hepatocyte growth factor · Tacrolimus · Renal toxicity · Renal regeneration

Introduction

Hepatocyte growth factor (HGF), originally purified and cloned as a potent mitogen for mature hepatocytes, is a heterodimetric molecule composed of a 69 kDa kringle-containing α-chain and a 34 kDa β-chain [11, 13, 14]. Studies done in the past several years have revealed that HGF is a mesenchymal- or stromal-derived factor that has mitogenic, motogenic, and morphogenic activities for a wide variety of cells [2, 5, 7, 17, 23]. Recent evidence suggests that it is also a renotropic factor. The expression of HGF is rapidly and markedly induced following the experimental induction of acute renal failure and unilateral nephrectomy [12]. Intravenous injection of recombinant HGF in mice significantly suppressed the onset of severe renal dysfunction and stimulated functional recovery after acute renal failure caused by cisplatin, $HgCl_2$,and renal ischemia [3, 6, 9]. These observations suggest that HGF may prevent acute renal failure and induce renal regeneration.

Tacrolimus (FK506) is a new macrolide entity consisting of 23 aliphatic heterocyclic rings that was isolated from the culture broth of *Streptomyces tsukubaensis*. It possesses immunosuppressive properties that are similar to, but more potent than, those of cyclosporin A

(CyA), and it inhibits cell-mediated and humoral immune responses [19]. Tacrolimus is a clinically effective treatment for allograft rejection of the liver, kidney, and pancreas; however, nephrotoxicity in the acute phase after renal transplantation is a major side effect. A recent report suggests that HGF reduces CyA-induced organ injury [1]. Intramuscular administration of tacrolimus induces nephrotoxicity in spontaneously hypertensive rats (SHR) [10]. In the present study, we investigated the effect of HGF on tacrolimus-induced nephrotoxicity in SHR.

Materials and methods

Animals

Ten-week-old SHR, weighing 240–270 g were purchased from Charles River Japan (Kanagawa, Japan) and were housed at the Institute of Experimental Animal Sciences, Osaka University Medical School. Animals were kept under controlled conditions at a constant temperature and were maintained on a normal day and night cycle. Their blood pressure ranged from 160 to 200 mmHg. Rats were weighed every 3 days during the experiment. The principles and regulations of the Institute of Experimental Animal Sciences, Osaka University Medical School, were followed in all of the animal experiments.

Test substances

Human recombinant HGF was purified from the culture medium of Chinese hamster ovary cells transfected with an expression vector containing HGF cDNA. HGF was over 98% pure, as determined by SDS-PAGE, with a 5 amino acid deletion in the first kringle domain [18]. Vials of tacrolimus for intramuscular injection (Fujisawa Pharmaceutical, Osaka, Japan) contained 20 mg of tacrolimus hydrate, 4 mg of polyoxyethylene hydrogenated oil, and 50 mg of mannitol. Tacrolimus was dissolved in sterile normal saline in a volume of 0.4 mg/ml for use.

Experimental protocol

Rats were anesthetized with diethyl ether, an incision was made in the middle of the abdominal cavity, and the right renal artery was identified and ligated. After resection of the right kidney, the left artery was also isolated. The aorta was clamped proximal to the right renal artery and distal to the left renal artery. A siliconized tube with a diameter of 0.37 mm was inserted through the right renal artery into the left artery and connected to an Alzet osmotic pump (Type 2001, Alza, Palo Alto, Calif., USA). The pump was placed in the retroperitoneal space and the wound was closed. HGF in a dose of 5 μg/kg daily (tacrolimus + HGF group) or normal saline (tacrolimus group) was perfused continuously through the pump at a rate of 1 μl/h for 7 days after surgery. Control rats either had no surgery (no operation group) or underwent only resection of the right kidney (nephrectomy group). Tacrolimus was administered intramuscularly at a daily dose of 4 mg/kg for 10 days in the tacrolimus + HGF and tacrolimus group.

Blood samples were obtained from the tail vein every 3 days and from the central vein of anesthetized rats 24 h after the administration of the last dose of tacrolimus and immediately centrifuged. Ten days after surgery, the rats received an intraperitoneal injection of 5-bromo-2′deoxyuridine (BrdU), 50 mg/kg (Dako Japan, Tokyo, Japan). Within 1 h, they were anesthetized with diethyl ether and exsanguinated. After blood was collected in tubes, the kidney was immediately removed and prepared for histopathological examination. One portion of the blood sample was used to measure the blood concentration of tacrolimus.

Biochemical assays

Levels of serum creatinine (SCr), alanine aminotransferase(ALT), amylase, and glucose were measured with an autoanalyzer (Hitachi 7150, Tokyo, Japan). The serum HGF level was determined by an enzyme-linked immunosorbent assay (ELISA) using an HGF test kit (Institute of Immunology, Tokyo, Japan). The blood tacrolimus concentration was measured by ELISA using an anti-tacrolimus monoclonal antibody, according to a previously described method [21].

Histopathology

The kidneys were fixed in phosphate-buffered 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin. Each sample was examined by several histopathologists.

Cell growth analysis

One portion of the kidney was fixed in 70% ethanol for 12 h and embedded in paraffin. For histopathological observation, tissue sections were deparaffinized. To evaluate DNA synthesis, sections were immunohistochemically stained with an anti-BrdU monoclonal antibody (Dako Japan, Tokyo, Japan) as follows. After sections were deparaffinized, they were incubated in 0.3% H_2O_2 in absolute methanol to inactivate endogenous peroxidase. Tissue sections were washed in 0.5% Tween 20 in phosphate-buffered saline, and DNA was denatured in 2 M HCl for 1 h. The sections were neutralized in 0.1 M borate buffer (pH 8.5) and then incubated with the anti-BrdU antibody for 1 h. After being washed, tissue sections were incubated with peroxidase-conjugated rabbit anti-mouse IgG (Dako LSAB Kit, Dako Japan, Tokyo, Japan) and then washed again. The enzyme reaction was initiated by the addition of a substrate solution containing diaminobenzidine. The BrdU labeling index was determined by counting the number of labeled nuclei in randomly selected microscopic fields containing more than 1000 nuclei with NIH image version 1.55, according to published methods [3, 6].

Mixed lymphocyte reaction (MLR)

To investigate the influence of HGF on the immunosuppressive effect of tacrolimus, a one-way MLR was performed using spleen cells from SHR as responder cells and spleen cells from Buffalo rats (Charles River Japan, Kanagawa, Japan) as stimulator cells. Responder cells (2×10^5/well) were co-cultured with 2000-rad-irradiated stimulator cells (4×10^5/well) in 96-well tissue culture plates in RPMI 1640 complete medium. HGF or tacrolimus was then added to the wells in final concentrations of 4 and 20 ng/ml for HGF and 1 and 100 ng/ml for tacrolimus. The cells were incubated at 37 °C in a humidified atmosphere of 0.5% CO_2 for 4 days and then treated with an 18-h tritiated thymidine (^{3}H-TdR) pulse. Cells

Table 1 Laboratory data. Values are expressed as mean ± SD (*Nx* nephrectomy)

	No operation	Nx only	Tacrolimus	Tacrolimus + HGF
Creatinine (mg/dl)	0.56 ± 0.08	0.72 ± 0.06	1.00 ± 0.15	0.77 ± 0.14[a]
ALT (U/l)	51.6 ± 8.68	44.5 ± 7.19	27.0 ± 10.9	22.2 ± 6.34
Glucose (mg/dl)	189 ± 18.9	187 ± 12.4	173 ± 48.1	149 ± 10.2
Amylase (U/l)	880 ± 52.3	822 ± 69.9	580 ± 194	606 ± 95.1
HGF (ng/ml)	–	–	< 0.1	0.91 ± 0.52
Tacrolimus (ng/ml)	–	–	52.0 ± 17.1	58.8 ± 23.3

[a] $P < 0.05$ compared to tacrolimus

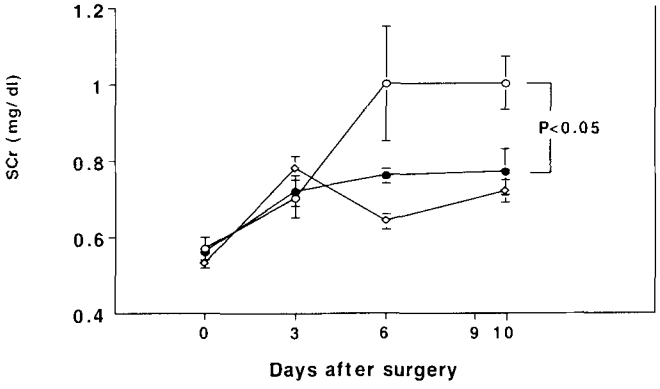

Fig. 1 Serum creatinine (SCr) levels after surgery. Data represent the mean ± SE [*Nx* nephrectomy, —○— tacrolimus ($n = 5$), —●— tacrolimus + HGF ($n = 6$), - · - · ◇ - · - · Nx only ($n = 4$)]

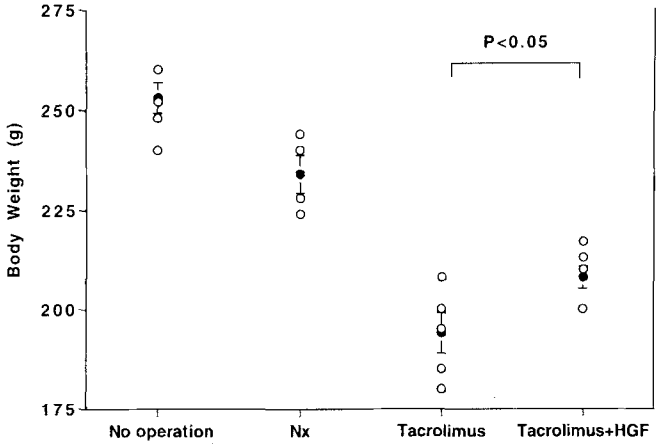

Fig. 2 Body weight in tacrolimus and tacrolimus + HGF groups 10 days after surgery. Data represent the mean ± SE

were harvested onto fiberglass filter disks (1205 Betaplate, Skatron, Lier, Norway) and ^{3}H-TdR incorporation was measured with a liquid scintillation counter (1205 Betaplate).

Statistical analysis

Data represent the mean ± SD. Differences among groups were analyzed by the Mann-Whitney U-test. A P level below 0.05 was accepted as statistically significant.

Results

Effects of tacrolimus and HGF on renal function and physical condition

SCr levels in the tacrolimus-treated animals were 39% (0.72 ± 0.06 to 1.00 ± 0.15 mg/dl) higher than those in the nephrectomy group 10 days after surgery (Table 1). The SCr level had increased significantly by day 6 in the tacrolimus group, indicating the development of renal toxicity (Fig. 1). HGF significantly inhibited the tacrolimus-induced increase in the SCr level by day 6 in the tacrolimus + HGF group. There were no significant differences between the tacrolimus + HGF and tacrolimus groups in the serum concentration of tacrolimus, ALT, glucose, or amylase, indicating that HGF did not affect the metabolism of tacrolimus (Table 1). Body weight was significantly lower in the tacrolimus group than in the no operation and nephrectomy groups. HGF partly inhibited the weight loss induced by tacrolimus (Fig. 2). The weights of the kidney, liver, and lung did not differ between the HGF and tacrolimus groups (data not shown).

Histopathological findings

Vacuolization and collapse of the renal proximal tubules were observed more in the tacrolimus group than in the tacrolimus + HGF group (Fig. 3).

Renal regeneration

The BrdU labeling index was significantly higher ($P < 0.05$) in the tacrolimus + HGF group (2.0%) than in the tacrolimus group (0.45%), especially in the outer medullary region, indicating renal regeneration accelerated by HGF after the onset of toxicity (Figs. 4, 5).

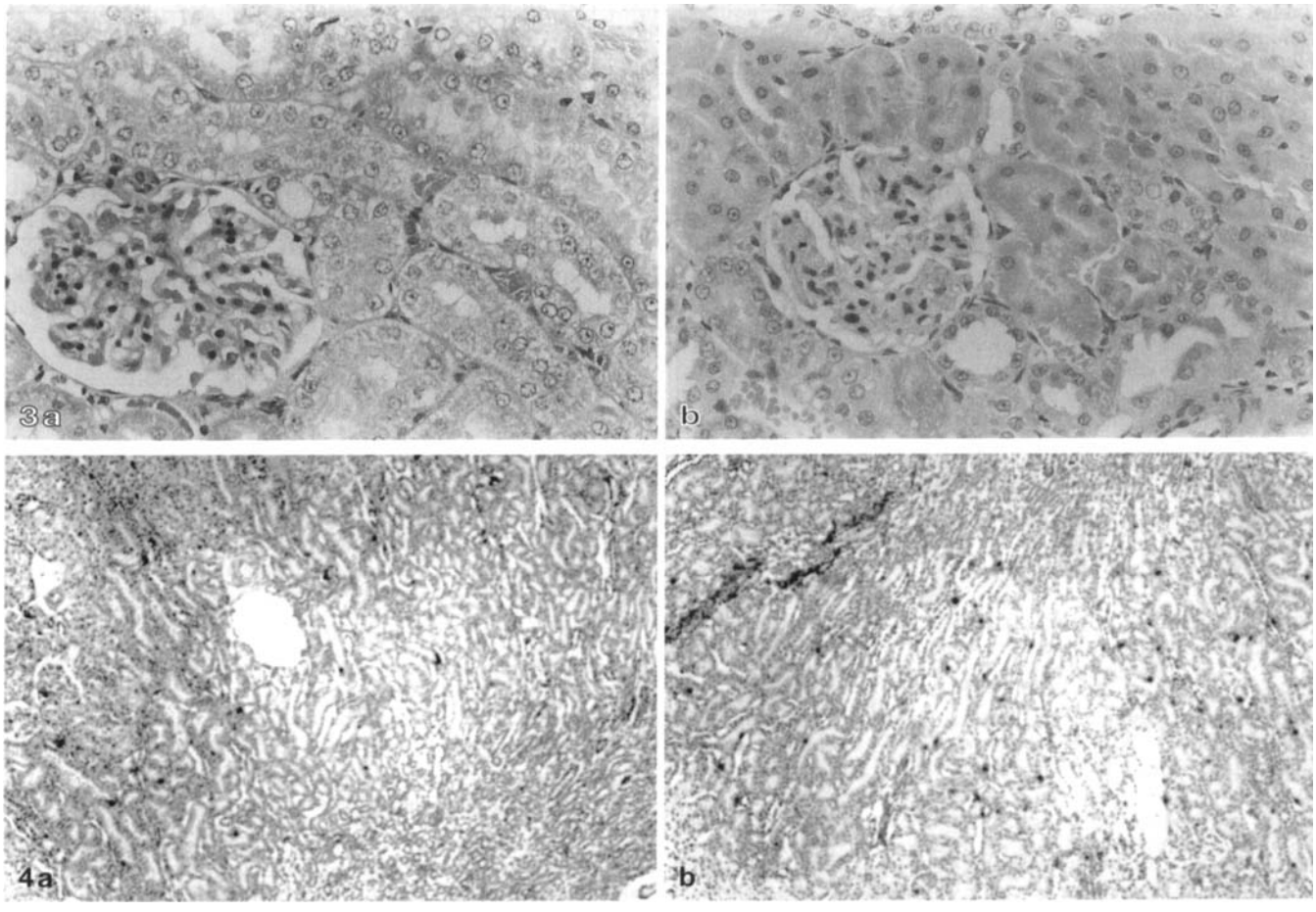

Fig. 3a, b Kidney specimens stained with hematoxylin and eosin. Vacuolization and collapse of the renal proximal tubules were observed more in **a** the tacrolimus group than in **b** the tacrolimus + HGF group (magnification × 400)

Fig. 4a, b Immunostaining of the outer medulla of kidneys with anti-BrdU antibody in representative rats from **a** the tacrolimus group and **b** the tacrolimus + HGF group. Anti-BrdU antibody-positive cells were present in both groups of rats, but they were more prominent in HGF-treated rats (magnification × 40)

Mixed lymphocyte reaction

HGF did not influence the immunosuppressive effect of tacrolimus on effector cells (Fig. 6). Tacrolimus suppressed the MLR whether or not it was administered together with HGF.

Discussion

Tacrolimus is a new macrolide entity consisting of 23 aliphatic heterocyclic rings that was isolated from the culture broth of *Streptomyces tsukubaensis*. It binds FK-binding protein and inhibits the phosphatase activity of calcineurin in T lymphocytes. It is a potent immunosuppressive agent that is used to treat solid organ transplant recipients, and it has played as large a role in the improvement in graft survival rates as CyA. However, especially in high doses, it can induce renal toxicity that is difficult to distinguish from the acute rejection reaction. Recent studies suggest that renal toxicity is more common with tacrolimus than with CyA [15, 16]. Tacrolimus-induced nephrotoxicity can lead to renal dysfunction, and the renal histology shows collapse and vacuolization in proximal tubuli. If this acute renal toxicity could be avoided and the dosage of tacrolimus could be increased, rejection episodes would decrease and successful organ transplantation could be achieved more easily, especially when heavy immunosuppression is required. Since tacrolimus readily induces nephrotoxicity in SHR, we examined whether the direct administration of HGF to the kidney of SHR would reduce the nephrotoxicity.

HGF, a ligand for c-met receptor tyrosine kinase, is a multipotent growth factor that targets not only the liver, but also a wide variety of tissues and organs including the kidney. It regulates cell growth, migration, and mor-

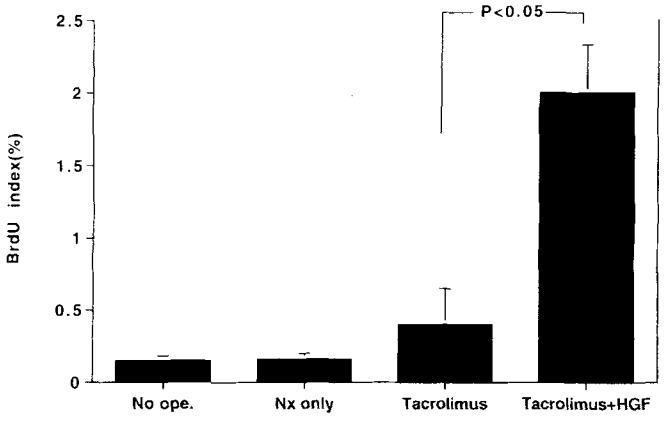

Fig. 5 Mitogenic effect of rhHGF on renal tubular cells 10 days after surgery. Data represent the mean ± SD of the BrdU index

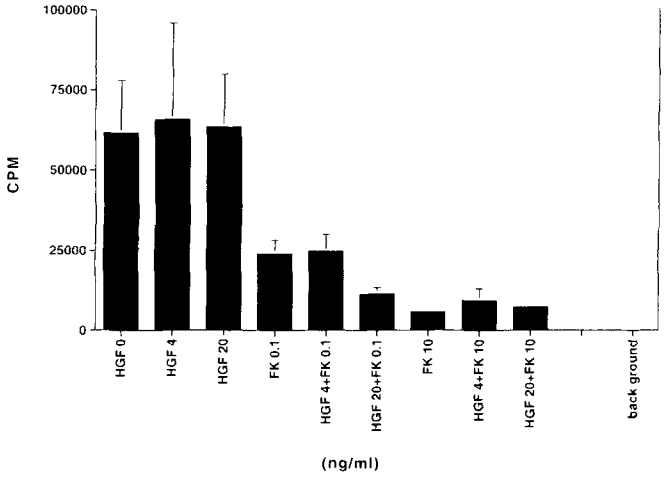

Fig. 6 Immunological effect of HGF as determined by the mixed lymphocyte reaction (MLR). Data represent the mean ± SD of the counts per minute (CPM). Doses are given in nanograms per milliliter

phogenesis of a wide variety of cells, and it plays important roles in the regeneration of organs [7]. An intravenous injection of recombinant HGF in mice significantly suppressed the onset of severe renal dysfunction and stimulated functional recovery after acute renal failure caused by cisplatin, $HgCl_2$ and renal ischemia [3, 6, 9]. Exogenous HGF was found to stimulate mitogenesis of renal tubular cells and to induce rapid reconstruction of normal renal tissue structure after acute renal failure and after unilateral nephrectomy in rats [6, 12].

In the present study, the HGF + tacrolimus group had a lower SCr level even though the tacrolimus group had a lower body weight, suggesting that HGF significantly inhibited the tacrolimus-induced increase in SCr by day 6. Renal cortical tubules showing slight to moderate staining with hematoxylin, which indicates an acceleration of cell proliferation, were much fewer in number in the tacrolimus group than in the tacrolimus + HGF group. Just how HGF reduces renal damage by tacrolimus is not yet clear. Yet, the fact that HGF was still effective, even when injected after the onset of renal failure caused by the administration of cisplatin or $HgCl_2$, suggests that it accelerates the recovery of renal function rather than reduces the toxic effect of the drug [6]. The BrdU labeling index was significantly higher in the tacrolimus + HGF group than in the tacrolimus group, especially in the outer medullary region; the same finding has been reported using $HgCl_2$ [6]. This would suggest that the labeled epithelial cells undergoing DNA synthesis were located in the outer medulla, where tubular vacuolization and collapse are typical indications of renal damage by tacrolimus, and that HGF may enhance renal regeneration. The effect of HGF on glomerular hemodynamics is not yet clear, although drug-induced nephrotoxicity often accompanies any change in glomerular hemodynamics. Since recent reports have shown that tacrolimus can also induce renal vasoconstriction and a decrease in the glomerular filtration rate, further studies are needed to establish exactly how HGF facilitates the recovery of renal function from tacrolimus-induced nephrotoxicity [22].

HGF prevented the loss of body weight that is induced by tacrolimus, suggesting a systemic effect of HGF that maintains homeostasis. There were no significant differences between the tacrolimus and tacrolimus + HGF groups in the serum concentration of tacrolimus, indicating that HGF did not affect the metabolism of tacrolimus. Moreover, HGF had no effect on the MLR in the present study. A recent study showed that HGF did not affect the CyA-induced suppression of IL-2 mRNA [1]. These observations suggest that HGF may prevent the adverse effects associated with tacrolimus in renal transplant recipients and that it may be used safely in conjunction with immunosuppressive agents.

The optimal mode of administration has not been determined for HGF. We injected this agent continuously and locally in the present study, and the serum concentration of rhHGF was approximately 1 ng/ml, which is consistent with that from our previous study of renal transplant recipients [20]. Additional studies are needed to determine the most effective method for administering HGF to prevent chronic renal injury and to preserve the function of the transplant kidney.

HGF is also known to enhance tumorigenicity and/or to promote migration and invasion of cancer cells in vivo [4, 8]. However, it is not yet certain how low doses of HGF in vivo would work. Clinical study has just started for the use of HGF. In vivo data may reveal negative side effects of its administration.

In our investigation, SCr was decreased and the BrdU labeling index was markedly increased in HGF-treated rats compared with those in the tacrolimus groups. HGF had no effect on the immunosuppressive action of tacrolimus, as determined by the MLR. Thus, the present findings show that HGF induces the regeneration of renal tubular cells and suppresses the nephrotoxicity induced by tacrolimus without influencing its immunosuppressive effect.

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