Hepatocyte growth factor upregulates interferon signaling in human hepatocytes: Possible implications for interferon therapy after liver transplantation

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Abstract: *Background/Aim*: Although a recurrent hepatitis C virus (HCV) infection is the leading cause of graft loss in liver transplant recipients, the optimal timing to begin interferon (IFN) therapy after LTx is still unknown. The purpose of this study is to analyze the relationships, between signaling by PEGylated IFN in human hepatocytes, with regard to hepatocyte proliferation, and immunosuppressive drugs *in vitro*. *Methods*: Experiment 1 – Normal human hepatocytes (NhHeps) were cultured with/without recombinant human hepatocyte growth factor (r-hHGF) for 48 h, and then treated with 100 IU/mL IFN at the indicated time. The expressions of double-stranded RNA-dependent protein kinase (PKR) and IFN-α-induced antiviral protein were analyzed using Western blotting for the extracted lysates from these cells. Experiment 2 – The NhHeps were cultured in 10% medium containing varying concentrations of tacrolims (Tac), cyclosporine A (CyA), and methylprednisolone (PLS), and the cells were treated with 100 IU/mL IFN at the indicated time. Subsequently, the density of PKR was examined. *Results*: The expression of PKR was enhanced by HGF. PKR induction by IFN was suppressed by Tac > CyA > PLS. *Conclusion*: Hepatocyte proliferation induced by HGF did not interfere with the signaling by IFN. The presence of immunosuppressive drugs was therefore found to negatively affect IFN signaling.

Keywords: HCV infection, antiviral therapy, immunosuppressive drugs, liver regeneration

Introduction

Chronic hepatitis C virus (HCV) infection is a major public health problem, infecting 3.3% of the world's population, and now HCV is the leading indication for liver transplantation (LTx) worldwide [1]. If HCV infection is not eradicated before LTx, reinfection occurs in 100% of patients, and recurrent disease affects the long-term graft survival. Recurrent infection leading to cirrhosis occurs in 10–25% of transplant recipients within 5–10 years of LTx, and once cirrhosis occurs, the 1-year actual risk of hepatic decompensation is ~40% [2].

Therefore, it seems reasonable to treat HCV reinfection after LTx, particularly since the introduction of interferon (IFN) and ribavirin (RBV) has resulted in high rates of sustained virological response (SVR) in the non-transplanted population. The SVR rates of PEGylated IFN monotherapy are as low as 0–17%. The addition of RBV appears to increase the SVR rate to 50–80% [3, 4]. Furthermore, the patients with SVR after LTx show no progression of liver fibrosis [5]. These reports suggest that the combination therapy of PEGylated IFN and RBV may contribute to improve the outcome in HCV-related transplantation. The optimal time to begin therapy after LTx is still unknown. Some authors start when the chronic lesion is already established, while others start in a pre-emptive fashion. Therefore, this study attempts to evaluate the influences of liver regenerative stimulating on IFN signaling in human hepatocytes. After binding to their receptors, IFN stimulates the intracellular IFN-signaling cascade including the Janus kinase-signal transducers and activators of transcription (Jak-STAT)-1 tyrosine kinases, the phospholylation of STAT-1 and -2, and the formation of IFN-stimulated gene factor 3 (ISGF-3), which consists of STAT-1, STAT-2 and p48 [6].

The aim of this study is to analyze the relationships between signaling by IFN, liver regeneration, and immunosuppressive drugs in human hepatocytes *in vitro*.

Materials and Methods

Reagents and cell culture

NhHeps (Lonza, Switzerland), which were isolated from single donors. Each donor was tested and found to be nonreactive by a Food and Drug Administration-approved method to detect the presence of Human Immunodeficiency Virus-I, Hepatitis B virus and HCV. NhHeps were cultured in Roswell Park Memorial Institute (RPMI) (Invitrogen, Grand Island, NY), supplemented with 10% fatal bovine serum and r-hHGF (Acris Antibodies GmbH, Germany) for 48 h. The cells were cultured in 10% RPMI containing varying concentrations of Tac, CyA and PLS for 16 h, and then the medium was exchanged and the cells were treated with 100 IU/mL IFN in 24 h, to determine the effect of calcineurin inhibitors.

Recombinant human IFN-α2b, Tac and CyA were provided by Schering Plough KK (Tokyo, Japan), Astellas Co. (Tokyo, Japan) and Novartis Pharma Co. (Basel, Switzerland), respectively.

Bromodeoxyuridine cell proliferation assay

A bromodeoxyuridine (BrdU) (Exalpha Biologicals, MA) cell proliferation assay was performed to determine whether the stimulation by HGF affects hNHeps or not. BrdU is incorporated into newly synthesized DNA strands of actively proliferating cells. Various concentrations of the NhHeps and HGF were plated and cultured with 10% RPMI and BrdU label for 48 h. Subsequently, the amount of BrdU incorporation in the proliferating cells was measured by using a spectrophotometric microtiter plate reader set at a dual wavelength of 450/550 nm.

Western blotting

Western blotting with anti-PKR (Santa Cruz Biotechnology, Santa Cruz, CA) was performed as described previously [7]. Cells were briefly incubated with 10% RPMI containing 5 μg/mL HGF for 48 h. Then cells were removed by centrifugation at 14,000 rpm for 30 min at 4°C. The same amount of protein from each lysate (20 μg per well) was analyzed by electrophoresis on 8 – 12% sodium dodecyl sulfate buffer polyacrylamide gel. Protein were transferred onto nitrocellulose membranes which were then blocked for 1.5 h using 5% nonfat dried milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS-T), washed with PBS-T and incubated at 4°C overnight in the presence of each primary antibody. The membranes were washed with PBS-T and incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G, and the immunoreactive bands were visualized by the ECL[AU1] chemiluminescence system (Amersham Life Science, Buckinghamshire, England). The density of each band was quantified using the National Institutes of Health image analysis software program.

Statistical analysis

Categorical variables were expressed as the value (%) and compared with the Mann-Whitney's *U* test. Continuous data are presented as the mean ± SD or median and analyzed with a 1-way analysis of variance. Statistical significance was set as $P < 0.05$ for all analyses. The statistical package used was StatMate III (ATMS Co., Ltd, Tokyo).

Results

The effects of HGF in NhHeps

NhHeps in number were increased under stimulation of HGF in various concentrations (data not shown). Above all, the concentration of HGF 5 ng/mL represented the largest cells proliferations inducer. Therefore, we decided to use 5 ng/mL concentration of HGF in this study, and this HGF's hepatocyte growth stimulation was regarded as the liver regeneration in early phase after living-donor liver transplantation.

HGF increased the expression of PKR

To evaluate if HGF influences the expression of IFN-induced antiviral proteins, NhHeps were incubated in the absence or presence of IFN-α with or without HGF for 48 h and then were harvested for the Western blotting *(Fig. 1)*. Pretreated HGF demonstrated an enhancement of the effect on IFN-α-induced PKR in our experiments *(Fig. 2)*. Data were representative examples of three similar experiments.

Differential effects of immunosuppressant on IFN-induced antiviral protein expression

To investigate the influence of HGF and immunosuppression, NhHeps treated by IFN-α were incubated with or without immunosuppressive drugs (Tac, CyA and PLS) for 16 h, after pretreatment with HGF for 48 h or in a combination of both, and then analyzed by Western blotting *(Fig. 3)*. In the absence of HGF, the expression of PKR was specifically suppressed by immunosuppressive drugs, with the effect of Tac > CyA > PLS *(Fig. 4)*. Data were representative examples of three similar experiments. In cells pretreated with HGF, PKR expression was higher than that without nontreatment. Moreover, the expression of PKR was suppressed at levels similar to those observed in the nontreated group.

Discussion

HCV infection is a major concern after LTx due to the universal recurrence, more rapid progression of fibrosis,

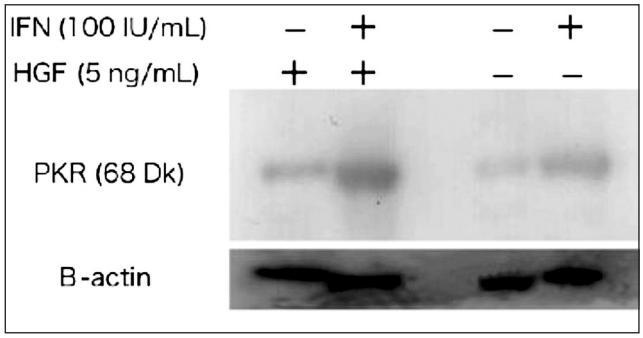

Fig. 1. The effects of HGF on IFN-induced PKR expression. NhHeps were incubated with or without of 5 ng/mL HGF for 48 h, and 100 IU/mL IFNα for 24 h or both. Thereafter, PKR expression was determined by Western blotting

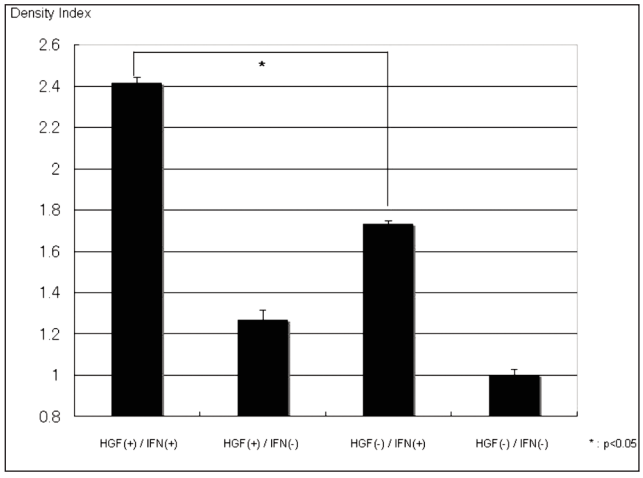

Fig. 2. The relative PKR expression in NhHeps treated with HGF and/or IFN. Density index represented the ratio to the control (no treatments of HGF and IFN). Data are expressed as the means ± SD and were representative examples of three similar experiments

and potential graft failure. The high rates of hepatic cirrhosis and the harmful impact of this complication on patients and graft survival, the development of new therapeutic strategies to reduce the severity of recurrent HCV are vitally important [8–11]. At present, the optimal timing for initiation of HCV therapy and the length of treatment in LTx has not been defined, and various authors have tried antiviral treatment during all phases of HCV infection.

Tac interferes with the translocation of STAT-1 more than CyA [12], which leads the inhibition of IFN-induced antiviral proteins by Tac. Therefore, Tac is used for induction of immunosuppression after LTx for patients with HCV infection, and subsequently, Tac is replaced with CyA to facilitate IFN therapy [13]. The current data revealed that the antiviral effect induced by IFN was greater with CyA than with Tac in the presence of HGF in order to increase the proliferation of hepatocytes.

The effect of a high concentration CyA was compared with a low concentration Tac, since rejection is usually controlled by serum trough values of Tac of 10 ng/mL and of CyA of 100 ng/mL during the period of stability after LTx [14].

Glucocorticoid inhibits the expression of STAT-1, as signal transduction factors for IFN, and diminished the signaling by IFN [15]. However, the effects $PSL_{[AU4]}$ and calcineurin inhibitors on IFN therapy have not yet been evaluated. This study revealed that PKR was slightly suppressed by PSL, while it was more strongly suppressed by Tac and CyA.

PEGylated IFN plus RBV, has emerged as the therapy of choice, but the optimum antiviral doses and treatment duration have not been established. Dose reductions and treatment discontinuation rates remain high, and limit the response rates. The need for safer and more effective antiviral therapy in this population of HCV-infected patients is obvious [16]. The current report showed that HGF had upregulated PKR expression. HGF might be a

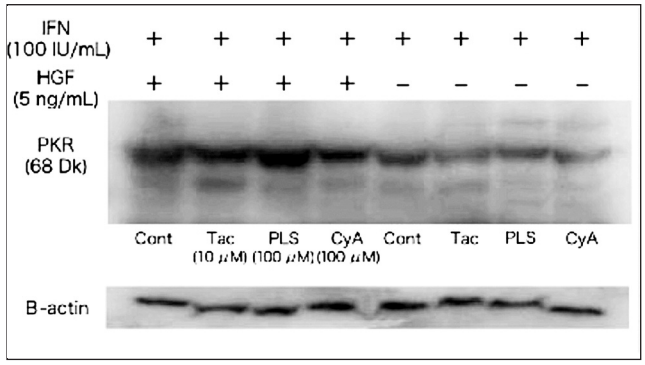

Fig. 3. Effects of calcineurin inhibitors, methylprednisolone and IFNα on the IFN-induced expression of PKR. NhHeps were incubated with or without 5 ng/mL HGF for 48 h, and the effect of immunosuppressive drugs was investigated with each concentration or both. The expression of PKR was determined by Western blotting

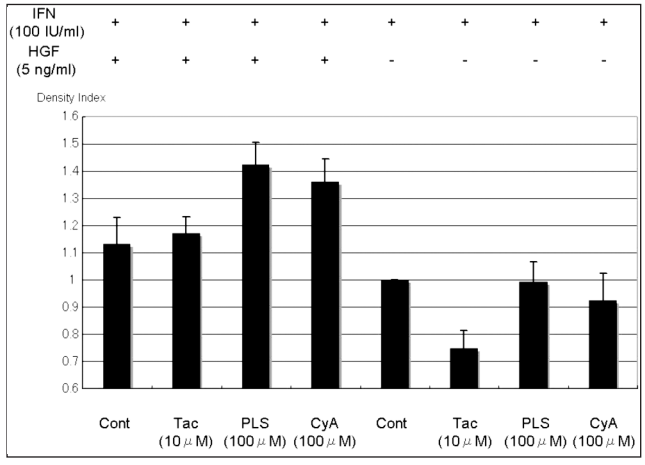

Fig. 4. The relative PKR expression in NhHeps treated with HGF and/or IFNα and immunosuppressive drugs. The density index represents the ratio to the control (no IFN or immunosuppression). The data are expressed as the means ± SD and are representative examples of three similar experiments

new option for this therapy to improve the rates of SVR, and to reduce the rates of treatment discontinuation.

HGF stimulated IFN-induced PKR expression in a cell culture system, and that mechanism does not appear to act via the standard IFN pathways. HGF interacts with the mitogen-activated protein kinase (MAPK)-signaling cascade and phosphoinositide 3-kinase (PI3K)-Akt pathway [17]. This study, did not investigate the interaction between IFN and MAPK-signaling and the PI3K-Akt pathway. This study demonstrated that liver regeneration induced by HGF, did not interfere with the signaling by IFN.

In conclusion, these experiments demonstrated that HGF serves as potent modulator of IFN-α-induced antiviral protein expression in NhHeps, although many questions remain unanswered. Understanding the interactive effects of HGF on IFN-signaling pathways may lead to the development of more effective therapeutic approaches to the control of viral clearance and liver inflammation in patients with HCV. Hepatocyte proliferation induced by HGF did not interfere with IFN signaling. However, the presence of immunosuppressive drugs negatively affects the IFN signaling.

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