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Hepatocyte Growth Factor Dampens Liver Immune-Mediated Pathology in Acute Viral Hepatitis without Compromising Antiviral Activity

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Abstract

Background and aim—Hepatocyte growth factor (HGF) is a pleiotropic cytokine related with cell proliferation and survival; however, its role in viral hepatitis is not elucidated. In this study we studied HGF immune role in viral hepatitis.

Methods—Mice received hydrodynamically-delivered HGF plasmid or control plasmid, and then infected with adenovirus (Ad); and parameters of immune-mediated liver damage were evaluated. We studied dendritic cell (DC) activation in the presence of HGF. T cells collected from infected mice were re-stimulated with virally-infected DC to measure cytokine production *in vitro*.

Results—HGF ameliorated the liver inflammation during viral hepatitis, as alanine transferase (ALT), intrahepatic lymphocytes (IHL) and splenocyte counts were diminished by HGF. Lower histological scores of liver pathology were observed in the HGF group. DC from the HGF group expressed reduced CD40. The hepatic expression and serum concentration of IL-12p40 were diminished in HGF-transfected mice. *In vitro* experiments with DC confirmed that HGF diminished CD40 expression and IL-12p40 production. The expression and serum levels of IFN- γ , IL-6 and CXCL9 were significantly decreased in the HGF group. HGF over-expression diminished the expression and concentration of IL-10 and TGF- β . The frequency of PD-1⁺Tim-3⁺ in CD8 T cells was decreased by HGF over-expression. Moreover, T cells in the HGF group at day 14 secreted more IFN- γ and TNF- α than those in the control group, when re-stimulated with virally-infected DC.

Conclusion—HGF modulated DC activation and T cell priming, thereby limiting the immune-mediated damage in the liver. However, viral clearance was not compromised by HGF.

Keywords

Hepatocyte growth factor; viral hepatitis; dendritic cells; T cell inhibition

Introduction

Fulminant hepatitis (FH) is a fatal disease characterized by massive hepatocyte death that leads to consumptive coagulopathy and encephalopathy. The only effective treatment for FH

is liver transplantation¹ and thus new therapies are needed. As viral hepatitis accounts as an important cause of FH², the pathogenic mechanisms are being examined. For example, adenovirus (Ad), a promising tool in gene and cancer therapy and vaccine vector³, has been found to elicit strong and pathogenic immune activation leading to FH.^{4,5}

HGF is a pleiotropic molecule with pro-mitogenic functions.⁶ HGF is a potential therapeutic agent for treatment of fatal liver disease⁷ and peripheral arterial disease,⁸ and is produced in injured tissues by stromal-derived cells, monocytes/macrophages⁹ and neutrophils.¹⁰ HGF has anti-inflammatory features by inhibiting NF- κ B activation.¹¹ HGF also diminishes TGF- β biological functions¹², DC migration is c-Met (HGF receptor) dependent¹³ and HGF promotes T cell reconstitution.¹⁴ Thus, HGF may affect the immune system through different mechanisms.

Despite the widely recognized role of HGF in chemically-induced fulminant hepatitis¹⁵, cirrhosis secondary to bile duct ligation¹⁸ and liver regeneration^{7, 16}, the immune effects of HGF in acute viral hepatitis are not elucidated. In this report, we use a model Ad-induced hepatitis and *in vitro* cell culture systems to examine the immune effects of HGF in acute viral hepatitis. Here we showed that HGF diminished the expression of pro-inflammatory cytokines but, in contrast to previous studies, in which HGF was shown to be highly immunosuppressive^{17, 18}, we found that HGF enhanced antiviral immune responses by inhibiting TGF- β and IL-10 production. Our findings that HGF ameliorates liver damage in viral hepatitis may provide insight into the treatment for FH having a viral etiology.

Materials and Methods

Mouse experiments

C57BL/6 mice (6–8 weeks, Jackson Laboratories, Bar Harbor, ME) were maintained in germ-free conditions in the animal research facility at the University of Texas Medical Branch. All animal experiments were done in accordance with an institutional review approved protocol. Mice received hydrodynamically-delivered, 20 μ g of naked DNA plasmid encoding the human HGF gene in a pCDNA3.1 plasmid¹⁹, 20 μ g of empty plasmid, or a mixture of both (10 μ g each). Three days after gene delivery, the groups received 3×10^9 pfu of AdLacZ virus (Vector Development Laboratory, Houston, TX). Mice were sacrificed at day 1, 6 or 14 post-infection. Blood, liver and spleen samples were collected for further analyses.

Intrahepatic lymphocytes and splenocyte collection and *ex vivo* T cell stimulation

Livers were mechanically disrupted and digested in a solution of 0.05% of collagenase D (Roche, Mannheim, Germany) in RPMI media (Corning, Mannassas, VA). Mononuclear cells were separated in a Percoll (Sigma Life Sciences, St. Louis, MO) discontinuous gradient. Intrahepatic lymphocytes (IHL) or splenocytes collected from the experimental animals were plated in 24-well plates (2×10^6 cells/well) and stimulated with phosphor 12-myristate 13-acetate (10 ng/mL) and ionomycin (500 ng/mL) in the presence of 1 μ g/mL of GolgiPlug (BD Biosciences, San Diego, CA) in complete RPMI media (10% heat inactivated FBS, Penicillin, ampicillin, pyruvate and 2-ME) incubated for 5 h at 37°C and 5% CO₂. Additional experiments we used T cells from Ad-infected mice stimulating them with plate-bound anti-CD3/CD28 antibodies for 72 h in the presence or absence of HGF (40 ng/mL). Intrahepatic T cells were re-stimulated *ex vivo* with virally-infected dendritic cells in 96-well plates (1:5 ratio) for 48 h in complete RPMI media. GolgiPlug was added 4 h prior to cell collection. Cells were stained for intracellular cytokines, and supernatants were used for measuring IFN- γ and TNF- α by ELISA.

Evaluation of ALT and cytokines in serum

ALT activity in serum was measured in the Department of Clinical Chemistry (UTMB, Hospital University Clinics). HGF, TGF- β and CXCL9 concentrations were evaluated with specific ELISA (R&D Systems, Minneapolis, MN). The levels of IL-2, IL-12 (p40), IL-7, IL-10 and IL-17 were measured by using a multiplex magnetic beads kit (Millipore, Billerica, MA) and detected in a Bioplex 200 system (Bio-Rad, Hercules, CA).

Histological grading

A nominal grading scale was defined by taking into consideration the following parameters: lymphocyte infiltration into acinar zones (1 point if they were present in zone 1, 2 points if they were present in zone 2, and 3 points if they were present in zone 3), inflammatory affection to the central vein (3 points), number of Councilman bodies per view (0 to 1 bodies = 0 points, 2 to 3 = 1 point, 3 to 4 bodies = 2, and more than 5 bodies = 3). We graded the observations as follows: 0 for preparations with less than 1 point, I for the preparations with 2 to 4 points total, II for preparations with 5 to 6 points, and III for those adding more than 6 points. Slides were double-blinded and evaluated by 3 independent individuals.

Flow cytometry analysis

IHL and splenocytes isolated were stained with monoclonal antibodies after being treated with FC blocking antibody (BD, Franklin Lakes, NJ). Antibodies against mouse IFN- γ , CD4, CD8, CD3, c-Met, MHC-II, CD40, CD86, CD44, CD62L, PD-1 and Tim-3 were purchased from eBioscience (San Diego, CA). Data were acquired with the LSR II Fortessa system (BD Biosciences) and analyzed with FlowJo 8.5 software (TreeStar, Ashland, OR).

Gene expression analysis and viral quantification

Freshly isolated tissues were preserved in RNA-later solution (Ambion, Austin, TX). RNA was isolated by using an RNAeasy kit (Qiagen, Maryland, USA), and cDNA was synthesized with a commercial kit (Bio-Rad). Our real-time PCR protocol was as follows: denaturing 10 min at 95°C, 40 cycles of 15 s at 95°C, and 60 s at 60°C. Melt-curve analysis was also used to check the specificity of the amplification reaction. The relative quantity of mRNA expression was calculated by using the $2^{-\Delta\Delta CT}$ method. Virus titers were calculated by using a standard curve of AdLacZ DNA.

Bone marrow-derived dendritic cell (BMDC) cultures

BMDC were differentiated in IMDM media (Gibco, Grand Island, NY) supplemented with 20 ng/mL of recombinant GM-CSF (PeproTech, Rocky Hill, NY). Fresh media was added every 3 days. Non-adherent cells were harvested at day 7 and plated in 24-well plates at 1×10^6 cells/well. BMDC were stimulated with 100 ng of LPS (Sigma, St. Louis, MO), Poly IC, CL096, (Invivogen, San Diego, CA), or AdLacZ virus (MOI 1:200) for 24 hours in the presence or absence of 40 pg/mL of recombinant murine HGF (PeproTech). Additionally, BMDC were treated with 1 μ M of c-Met inhibitor SU11274 (Calbiochem, Darmstadt, Germany), in the presence of HGF as described previously.²⁰

Statistical analyses

Data were analyzed with Graph Pad Prism 4 software. For normally distributed and non-parametric data, a two-tailed Student T test was done. For non-normally distributed data, a Mann-Whitney and X^2 test were performed. A p value of less than 0.05 was considered significant.

Results

The HGF/c-Met axis is up-regulated in Ad-induced hepatitis

HGF is secreted in response to partial hepatectomy or chemical damage to the liver.⁶ Hence, we evaluated the kinetics of the HGF/c-met axis during Ad-induced hepatitis. We found that HGF serum concentrations increased considerably during the first 12 h and were followed by a second peak at day 6 (Fig. 1A). The hepatic expression of the *hgf* gene started at 12 h of infection and peaked at day 3 prior to the second peak in the serum (Fig. 1B). We speculated that the biphasic HGF peaks resulted from protease-mediated activation and release of pro-HGF into serum (peak 1), and *de novo* gene expression (peak at day 6).⁶ The *c-met* expression in liver was increased within the first 24 h and fell on day 6 (Fig. 1C). Since c-Met prevents apoptosis through Bcl-2²¹, we evaluated its expression which rose considerably during the first 12 h followed by a second increase at day 6 (Fig. 1D). This pattern was reminiscent of that of serum HGF, suggesting that the HGF/c-Met axis played a critical role in Ad-induced hepatitis.

HGF ameliorates immune-mediated pathological changes in the liver

It is known that HGF protects against chemically induced injury of the liver²², kidneys²³ and heart.²⁴ To explore the biological role of HGF in viral hepatitis, we administered a plasmid with human HGF, control plasmid, or a mixture of both.¹⁹ This strategy was chosen over antibody neutralization because we wanted to examine HGF's local effects in the liver and hydrodynamically-delivered vectors have a predominant hepatic expression²⁵. Secondly, HGF is involved in lymphopoiesis,¹⁴ and thus antibody neutralization could potentially affect this process and obscure data interpretation. We found that hepatic expression of exogenous HGF was at 24 hours in the HGF group as in previous publications¹⁹ (Fig. 2A). Three days later, both groups were infected with Ad. At day 6, the serum ALT levels were significantly decreased in the HGF group and the group with the plasmid mixture (Fig. 2B). IHL and splenocyte counts were also significantly reduced in the groups that received the HGF plasmid (Fig. 2C and D). In H&E studies the HGF group had lower histopathological scores compared to the control group (Fig. 2E and F) findings which correlated with the ALT levels (Spearman's rho = 0.92). Altogether, these data indicate that HGF exerts cyto-protection via immune-modulation in viral hepatitis.

HGF reduces DC activation during Ad-induced hepatitis

We evaluated the expression of c-Met in hepatic DC and Kupffer cells (KC) at different time points during Ad infection and found a steady increase in these cell subsets (Fig. 3A and not shown). However, the expression of CD40 in KC was not affected by HGF over-expression (not shown). Previous studies showed that HGF inhibits DC activation in experimental autoimmune encephalitis (EAE)⁶ and airway hypersensitivity.¹⁷ However, the effect of HGF on virally infected DC has not been evaluated. Therefore, we asked whether HGF mediates its protective effects by inhibiting CD40. There was a significant decrease in the expression of CD40 and MHC-II in the DC collected from the HGF group after 24 h (Fig. 3B, C and not shown). Importantly, CD40 is up-regulated during the course of Ad infection and correlates with liver damage.²⁶ Consistently, there was a reduced serum concentration of IL-12p40 in the HGF group at day 6 (Fig. 3D). Splenic DC from both groups were not different (data not shown) due perhaps to the hepato-specificity of Ad and hydrodynamic injection of HGF plasmid.^{25, 27}

To determine whether HGF exerts a direct effect on DC, we performed *in vitro* studies in which BMDC were stimulated with 100 ng/mL of different toll like receptors (TLRs) agonists or with AdLacZ (MOI 1:200). We found that BMDC displayed c-Met upon stimulation of TLRs and virus (Fig. 4A). BMDC received different stimuli for 24 h in the

presence or absence of recombinant HGF (40 ng/mL). BMDC stimulated in the presence of recombinant HGF did not up-regulate the expression of CD40 (Fig. 4B and C) and CD86 (not shown). Furthermore, IL-12p40 production was significantly decreased by HGF in BMDC stimulated with TLR3, TLR7/8 and Ad but not with TLR4 agonist (Fig. 4D). HGF's effects were counteracted when c-Met inhibitor SU11274 was used during BMDC activation (Fig. 4E), indicating that c-Met mediates HGF biological effects in BMDC. Our *in vivo* and *in vitro* studies indicated that HGF directly modulates DC maturation and activation upon viral infection, which may modulate T cell activation and cytokine production.

HGF diminishes IFN- γ and acute phase protein production

We examined IFN- γ expression, an important effector molecule secreted in viral hepatitis²⁸. In the HGF group, there was a significant decrease in the serum concentration and liver expression of IFN- γ (Fig. 5A and B). Furthermore, the percentage of IFN- γ -producing T cells was significantly decreased in the HGF group when compared to those in control group (Fig. 5C and D).

CXCL9 and IL-6 are produced by DC and are critical for T cell recruitment to the liver following stimulation with TLR agonist and IFN- γ .^{29, 30} Based on the diminished T cell recruitment (Fig. 2B) and diminished DC activation in the presence of HGF (Fig. 4B), we asked whether HGF affects CXCL9 and IL-6. We found that hepatic expression and serum concentrations were significantly decreased in the HGF group (Fig. 6 A–D). Importantly, type I interferon was also decreased in the HGF group (not shown). These data indicate that the inhibitory effect of HGF on T cell recruitment is in part attributable to a lower production of IL-6 and CXCL9.

HGF decreases IL-10 and TGF- β production and modulates T cell activation

Having demonstrated that IFN- γ , IL-6 and CXCL9 were down-regulated by HGF (Fig. 5 and 6), we asked whether HGF could affect T cell activation. We collected IHL from Ad-infected mice and stimulated them with anti-CD3/CD28 antibodies in the presence of HGF, and found no effect on IFN- γ and TNF- α expression (not shown). We then asked whether HGF protection resulted from an augmented concentration in anti-inflammatory cytokines as previous reports showed contradictory effects of HGF on TGF- β and IL-10.⁶ We then observed a significant reduction in the expression and production of IL-10 and TGF- β in the HGF group (Fig. 7A, B and not shown). Since TGF- β and IL-10 are known to be related to the expression of PD-1 and Tim-3 T cell inhibitory markers,^{31, 32} and are also associated with low antiviral activity in CD8⁺ T cells,³³ we measured whether HGF affects their expression. HGF significantly diminished the frequency of PD-1⁺ Tim-3⁺ CD8 T cells in the liver (45 $\pm$ 3.6% vs. 25.25 $\pm$ 1.8%, Fig. 7C).

Furthermore, when T cells from HGF groups were re-stimulated with virally-infected DC (at a 1:5 ratio), they secreted higher quantities of IFN- γ and TNF- α compared to T cells from the control group (Fig. 7D and E). Consistent with better T cell recall, viral clearance was more efficient in the HGF group at day 14 (Fig. 7F). Altogether, these data indicate that exogenous HGF decreases pro-inflammatory cytokine production in the liver microenvironment, but at the same time reduces T cell inhibition with the subsequent enhancement of viral clearance.

Discussion

Fulminant hepatitis caused by viruses is a fatal disease.^{2, 4, 34} Fortunately, HGF is a potential therapeutic agent for treatment of patients with this disease.⁷ Clinical observations showed that serum HGF levels were elevated in patients infected with HCV.^{35, 36} However,

there is a paucity of mechanistic studies on the role of HGF in viral hepatitis. In this study, we found that HGF was drastically elevated during the first several hours of Ad infection (Fig. 1). When HGF was over-expressed in the mouse liver, it reduced the total pathologic changes (Fig. 2). We concluded that HGF has immunomodulatory effects on DC and T cell functions in a multi-layered fashion to protect the liver from immune pathology mediated by Ad infection.

HGF did not affect KC virally-mediated activation at 24 h, however we cannot discard the possibility that HGF may modulate them at later time points of infection. In contrast, we found that HGF down-regulated DC activation marker CD40 as well as IL-12p40 production (Fig. 3 and 4). This effect on DC has a particular relevance in FH because the CD40 is involved in the exacerbation of liver damage in Ad-induced hepatitis.^{26, 37} Previous studies have shown that HGF inhibits DC function after exposure to antigens and adjuvants^{17, 18} and that sustained expression of HGF induces tolerogenic DC.⁶ These results are in agreement with our observations in that HGF suppressed DC activation through TLRs during Ad infection (Fig. 3 and 4). However, further characterization of the immune function indicates that antiviral response was not abrogated by HGF (Fig. 7). Therefore, HGF plays a complex immunomodulatory role, depending in part on the dose of HGF given and the organ in question.

In addition, HGF acts directly on fully differentiated T cells or other immune cells,^{38, 39} and has been found to induce tyrosine phosphorylation on immune cells via mannose receptor.³⁹ In our *in vitro* studies with a c-Met inhibitor we found that HGF mediates its modulatory effects in a c-Met-dependent manner in BMDC (Fig. 4). Additionally, *ex vivo* stimulation of T cells collected from Ad-infected mice in the presence of HGF did not change the cytokine expression (not shown). Thus, we concluded that HGF modulates the micro-environment of T cells suppressing TGF- β and IL-10 production^{12, 16, 40, 41} and also CXCL9 and IL-6 expression (Fig. 5 and 6).

TGF- β and IL-10 directly promotes PD-1 expression.⁴² In our study, HGF over-expression decreased TGF- β in Ad-induced hepatitis (Fig. 7). This effect may have relevant translational implications, because suppression of TGF- β signaling, but not antibody neutralization^{43, 44}, can result in decreased T inhibition and enhanced viral clearance.⁴² In an EAE model, the constitutive expression of HGF led to increased production of IL-10.⁶ In contrast, hydrodynamic injection of HGF plasmid inhibited IL-10 production in viral hepatitis (Fig. 7). Possibly, sustained HGF expression causes up-regulation of IL-10, whereas transient HGF expression leads to its down-regulation. IL-10 and TGF- β are associated with viral persistence.³¹ In our study, hepatic CD8⁺ T cells in the HGF-transfected mice had a lower percentage of PD-1⁺Tim-3⁺ population, two markers of T cell anergy.^{45–48} When these T cells were re-stimulated with virally-infected DC, cells from the HGF group secreted more IFN- γ and TNF- α in comparison to cells from control animals. Consistently, viral clearance was higher in the HGF group (Fig. 7). The anti-inflammatory HGF effect and an increase in CD8 T function are apparently paradoxical. However, it is in line with the notion that an overwhelming production of effector molecules causes T cell inhibition in the liver²⁸ and that their decrease leads to a better viral clearance^{49, 50}.

In conclusion, we found that HGF was elevated during Ad infection. HGF over-expression modulated DC activation, and shaped liver microenvironment reducing the exacerbated production of pro-inflammatory cytokines, and anti-inflammatory cytokines. As such, there is an efficient T cell infiltration that leads to better viral clearance. Thus, HGF should be further explored in clinical studies as therapy for FH of viral etiology.

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Figure 1. The HGF axis is activated in the course of viral hepatitis

C57BL/6 mice received 3×10^9 pfu of AdLacZ i.v. and were sacrificed at specific time points. Serum and liver were collected and we measured the serum concentration of HGF with an ELISA (A). (B) Liver expression of the *hgf* gene was normalized to the GAPDH. (C and D) Liver expression of *c-met* (C) and *bcl-2* were evaluated with RT PCR and normalized to GAPDH. Data are representative of three independent experiments with three mice at each time-point (* $p < 0.05$, Standard error of the mean).

Figure 2. HGF over-expression ameliorated Ad-induced immune-pathology

Mice were i.v. injected with 20 μ g of an empty plasmid (white bars), a combination of 10 μ g of control plasmid and 10 μ g of the HGF plasmid (gray bars), or 20 μ g of HGF plasmid (black bars). Three days later mice were i.v. infected with 3×10^9 pfu of AdLacZ. Mice were sacrificed at 6 days post-infection. (A) Hepatic expression of the HGF plasmid was presented as fold increase normalized to GAPDH gene expression. (B) ALT activity measured in serum. (C and D) Cell counts of intra-hepatic lymphocytes (IHL) and splenocytes recovered from each mouse. (E) H&E stains of liver from experimental animals. (F) Distribution of the histological scores from animals of two independent experiments. Data are representative of five independent experiments with four mice per group. * $p < 0.05$.

Figure 3. HGF diminishes DC activation *in vivo*

Intra-hepatic lymphocytes were isolated from Ad-infected mice and stained for CD11c and c-Met expression (A). (B) The percentage of CD40⁺ DC (C) and expression of CD40 in DC collected at day 1 of infection. (D) Concentration of IL-12 (p40) in mice receiving the HGF or control plasmids at day 6 post-infection. Data are representative of three independent experiments with three mice per group. Error bars represent the standard error of the mean, * $p < 0.05$.

Figure 4. HGF modulates bone marrow-derived DC

DCs were generated from bone marrow culture in the presence of recombinant GM-CSF. DCs were stimulated for 24 h with LPS (TLR4), Poly I:C (TLR3), CL097 (TLR7/8), and AdLacZ (MOI 1:200), respectively. (A) Expression of c-Met in activated DC. Expression of CD40 in DC, histogram (B) and the pooled data from five technical replicates (C). (D) Concentration of IL-12p40 in the supernatant of the stimulated DC. CD40 expression in DC without any treatment (white bars) or treated with HGF in the presence of c-Met inhibitor (black bars) (E). Data are representative of three independent experiments with five technical replicates per treatment. * $p < 0.05$. Open bars represent control groups and solid bars the HGF groups.

Figure 5. HGF abrogates the production of IFN- γ during Ad-induced hepatitis

Mice were treated and sacrificed at day 6 of infection as described in figure 2. (A and B) Serum concentration and liver expression of IFN- γ at day 6 post-infection. (C and D) Percentage of IFN- γ production in T cells. Results are representative of three independent experiments with four mice per group. Standard error represented in the error bars. * $p < 0.05$.

Figure 6. CXCL9 and IL-6 production is decreased by HGF

Mice were treated with HGF plasmid or control, infected and sacrificed as described in figure 2. (A) Liver expression of CXCL9 normalized with GAPDH gene and serum concentration evaluated with ELISA (B). Expression of IL-6 normalized with GAPDH (C), Serum concentration of IL-6. Data are representative of three independent experiments, four mice per group. * $p < 0.05$.

Figure 7. HGF modulates T cells by decreasing the expression of TGF-β and IL-10

Mice were treated as previously specified in figure 2 and sacrificed at day 6 of infection. (A) Liver expression of IL-10 normalized to GAPDH expression (left) and IL-10 serum concentration (right). (B) Hepatic expression of TGF-β normalized to GAPDH expression (left) and serum concentration of TGF-β (right). (C) Representative FACS plot of PD-1 and Tim-3 expression on CD8⁺ T cells collected from the liver at day 14 post-infection (left). Percentage of PD-1⁺Tim-3⁺ CD8⁺ T cells collected from liver (right). Intra-hepatic T cells isolated were re-stimulated with virally-infected BMDC (M.O.I. 1:200) for 48 h. (D) IFN-γ concentration in the supernatants from the stimulated T cells (left) and representative FACS dot plots for IFN-γ. (E) TNF-α concentration in the supernatants of stimulated cells (left) and representative FACS dot plots for intracellular TNF-α expression. (F) Viral copy numbers of AdLacZ normalized with DNA concentration of the sample at different days of infection. Data is representative of three independent experiments with four mice per group. Error bars are standard error of mean. * $p < 0.05$.