Suppression of Acute and Chronic Rejection by Hepatocyte Growth Factor in a Murine Model of Cardiac Transplantation

Induction of Tolerance and Prevention of Cardiac Allograft Vasculopathy

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Background—Although treatment with immunosuppressive agents has contributed to overcoming acute rejection and improving the midterm survival of transplanted hearts, cardiac allograft vasculopathy (CAV) has remained the main cause of primary graft failure. Recent approaches have shown that hepatocyte growth factor (HGF) exhibits cardioprotective functions. We therefore addressed whether HGF would regulate acute and chronic rejection in cardiac transplantation.

Methods and Results—We used a murine heterotopic cardiac transplantation model between fully incompatible strains and administered 500 µg·kg⁻¹·d⁻¹ HGF during the initial 14 days after transplantation. The HGF-treated allografts showed significantly prolonged survival (42.3±4.1 days, P<0.001) compared with the controls (11.1±0.6 days), with tolerance induction in 47.4%. Histopathologically, the number of infiltrating cells was significantly decreased and myocardial necrosis was less prominent with a reduction of apoptosis in the allografts by HGF treatment during acute rejection. In the long-term surviving allografts, HGF significantly inhibited the development of CAV and interstitial fibrosis. With respect to intragraft cytokine mRNA expression, HGF treatment reduced the early expression of interferon-γ and enhanced the expression of transforming growth factor-β1 during the acute phase and of interleukin-10 continuously through the acute phase to the chronic phase.

Conclusions—Our findings demonstrate that HGF can prolong the survival of allografts by its cardioprotective and immunomodulative potencies. Thus, HGF administration may constitute a new therapeutic approach to preventing cardiac graft failure that has not been overcome by conventional immunosuppressive agents. (Circulation. 2004;110:1650-1657.)

Key Words: pathology ■ growth substances ■ immune system ■ rejection ■ transplantation
We used adult male C3H/He (H2 b), BALB/c (H2 d), and C57BL/10 (H2 k) mice between 6 and 8 weeks of age (Japan Charles River Laboratories, Tokyo). The investigation conformed to the guidelines for the handling of animals from the Research Committees of Shinshu University.

**Heterotopic Cardiac Transplantation**

Donor hearts were heterotopically transplanted into recipient mice. 13 The mice were anesthetized by a single intraperitoneal injection of ketamine/xylazine (100:10 mg/kg). BALB/c hearts were transplanted into C3H/He recipients as allografts, and C3H/He hearts were transplanted into the same strain as isografts. Graft function was measured daily by palpation, and rejection of the cardiac graft was defined as the cessation of pulse.

**Admnistration of Human Recombinant HGF**

Human recombinant HGF was purified as described previously. 4 The recipients received 100 (n=7) or 250 (n=19) μg/kg HGF every 12 hours (total, 200 or 500 μg · kg⁻¹ · d⁻¹ for 14 days after transplantation. Control mice were treated with the same volume of phosphate-buffered saline (PBS; n=14). To investigate the effects of HGF on long-surviving grafts, we administered 0.1 mg · kg⁻¹ · d⁻¹ tacrolimus (Fujisawa Pharmaceutical Co) for 60 days to recipient mice with (n=4) or without (n=8) administration of 500 μg · kg⁻¹ · d⁻¹ HGF for the initial 14 days after transplantation.

**Methods**

**Animals**

We used adult male C3H/He (H2 b), BALB/c (H2 d), and C57BL/10 (H2 k) mice between 6 and 8 weeks of age (Japan Charles River Laboratories, Tokyo). The investigation conformed to the guidelines for the handling of animals from the Research Committees of Shinshu University.

**Figure 1.** Effects of HGF on graft survival. Recipient mice were treated with PBS, 200 μg · kg⁻¹ · d⁻¹ HGF (HGF 200), 500 μg · kg⁻¹ · d⁻¹ HGF (HGF 500), 0.1 mg · kg⁻¹ · d⁻¹ tacrolimus (Tac), or 0.1 mg · kg⁻¹ · d⁻¹ tacrolimus with 500 μg · kg⁻¹ · d⁻¹ HGF for initial 14 days after transplantation. Abbreviations are as defined in text.

**Figure 2.** Skin graft assay for recipient mice that accepted cardiac allografts over 60 days. A, Representative photos of mice after skin transplantation. Left, both shaved skin grafts were viable at 1 week. Right, C57BL/10 skin (black hair) was completely rejected (arrow), whereas BALB/c skin (white hair) was accepted at 4 weeks. * indicates C57BL/10 skin; **, BALB/c skin. B, Survival curves of skin grafts. Abbreviations are as defined in text.

**Skin Transplantation**

Recipient mice were sensitized by 1-cm² full-thickness skin grafts from the BALB/c and C57BL/10 mice. The grafts were considered rejected when >80% of the graft was necrotic.

**Histopathologic and Immunohistochemical Study**

The specimens were stained with hematoxylin-eosin, elastica–van Gieson’s, and Masson’s trichrome (MT) stains. For evaluation of cell infiltration, 5 fields (1.6×10⁻³ mm² for 1 field) were randomly selected from 1 section, and the number of nuclei in each field was counted. The difference in number between the naive hearts and grafts was considered to be the representative number of infiltrating cells. To evaluate CAV, the area encompassed by the lumen and internal elastic lamina of each coronary artery was traced, and the luminal occlusion rate was calculated by the following formula: luminal occlusion rate=(internal elastic lamina area–luminal area)/ internal elastic lamina area. To evaluate the proportion of interstitial fibrosis, we measured the whole area of the coronal section of the upper ventricle and the area of fibrosis (green area with MT staining) of the same section in each specimen, and then the fibrosis rate was calculated. Anti–c-Met antibody (SP260G, Santa Cruz Biotechnology Inc), anti-α-smooth muscle actin (α-SMA) antibody (E2464, Spring Bioscience), and anti-nonmuscle myosin heavy-chain B (MHC-B) antibody (PRB-445P, Covance Research Products) were used for immunohistochemical staining.

**Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick-End-Labeling (TUNEL) Staining**

Apoptotic cells were detected with the ApopTag in situ apoptosis detection kit (Serologicals Corp). Cardiomyocytes were subsequently detected with anti-desmin antibody (H-76, Santa Cruz Biotechnology Inc). Quantitative evaluation was performed with the same method that was used in the histological study.

**Real-Time RT-PCR Assay for mRNA Quantification**

Relative levels of mRNAs for the genes of interest were assessed by real-time reverse transcription–polymerase chain reaction (RT-PCR) with use of the ABI Prism 5700 system (PE Applied Biosystems, Inc); the primers and probes have been described previously. 8,14 The expression levels of each target gene were normalized by subtracting the corresponding glyceraldehyde 3-phosphate dehydrogenase threshold cycle (CT) values by using the ∆∆CT comparative method. 14

**ELISA for Endogenous HGF**

The levels of endogenous HGF in serum were measured with a rat-HGF ELISA kit (Institute of Immunology), which specifically detects rat and mouse HGF but not human HGF.
Statistics
Kaplan-Meier curves were used to estimate graft survival time, and the log-rank test was used for statistical analysis. Other results were analyzed by 1-way or 2-way ANOVA followed by Bonferroni correction. A value of $P < 0.05$ was considered statistically significant. Data were expressed as mean±SEM.

Results
Effect of HGF on Cardiac Allograft Survival
The survival of allografts treated with HGF was significantly prolonged, whereas all allografts treated with PBS were
rejected within 14 days (PBS-treated, 11.1±0.6 days; 200 μg · kg⁻¹ · d⁻¹ HGF, 28.8±8.3 days [P<0.05]; 500 μg · kg⁻¹ · d⁻¹ HGF, 42.3±4.1 days [P<0.001]). Nine of 19 allografts treated with 500 μg · kg⁻¹ · d⁻¹ HGF and 2 of 7 allografts treated with 200 μg · kg⁻¹ · d⁻¹ HGF showed prolonged survival of >60 days. All allograft hearts that received tacrolimus daily with HGF kept beating throughout the observation period (Figure 1).

**Development of Stable Transplantation Tolerance in the Recipients**

To determine whether HGF induced tolerance, we challenged the secondary-skin transplantation in the recipients with long-term surviving allografts in the group treated with 500 μg · kg⁻¹ · d⁻¹ HGF. We tested 4 of 9 recipients with long-term surviving BALB/c hearts >60 days; all of these mice accepted the BALB/c skin grafts. However, these mice rejected the third-party skin grafts within 2 weeks (Figure 2).

**Effects of HGF in the Course of Acute Rejection**

First, we investigated the effect of HGF on acute rejection between the PBS-treated group and the group treated with 500 μg · kg⁻¹ · d⁻¹ HGF. Histopathologically, extensive interstitial infiltration of mononuclear cells and focal myocardial necrosis were observed in PBS-treated allografts from day 4. Subsequently, the myocardial necrosis in PBS-treated allografts increased in a time-dependent manner and reached a plateau by day 7 (Figure 3A). However, myocardial necrosis was less prominent in the HGF-treated allografts (Figure 3B). In addition, the number of infiltrating cells in the HGF-treated allografts was significantly decreased compared with that in the PBS-treated allografts after day 7 (Figure 3D). By TUNEL staining, both the number of total apoptotic cells and of apoptotic cardiomyocytes was significantly reduced by HGF at day 12; moreover, inhibition of apoptosis was more remarkable in the cardiomyocytes (Figure 3E through 3H).

To evaluate whether c-Met expression was regulated after transplantation, the levels of c-Met mRNA in the allografts were measured by real-time RT-PCR, and the localization of c-Met was evaluated by immunohistochemistry. In the PBS-treated allografts, c-Met mRNA levels increased steadily over time and reached a plateau on day 7 (Figure 4A). Consistent with the low-level expression of c-Met mRNA in naive hearts, we were unable to immunohistochemically detect c-Met expression in the naive hearts (Figure 4D). Seven days after transplantation, clearly positive staining for c-Met was observed in the cardiomyocytes in both control and HGF-treated allografts.
To assess the effects of exogenous HGF on endogenous serum HGF levels in cardiac transplantation, we measured endogenous HGF by ELISA. In PBS-treated mice, the levels of endogenous HGF increased until day 7 and then decreased to normal levels on day 12. Significantly higher levels of endogenous HGF were observed in the HGF-treated mice on days 12 and 21 (Figure 4E). Although the source of endogenous HGF induced by exogenous HGF remains to be elucidated in our model, the upregulation of endogenous HGF by administration of exogenous HGF was consistent with the previous reports of in vivo experimental models.9,10

Because previous studies have provided evidence of the involvement of interferon (IFN)-γ, interleukin (IL)-10, and transforming growth factor (TGF)-β in immunomodulation in recipients after transplantation,15–21 we focused on whether exogenous HGF could modulate the expression of these genes in cardiac allografts. The expression of IFN-γ mRNA in control allografts was maximal on day 4 and then decreased by ≈30% by day 7. On the one hand, HGF treatment significantly reduced the level of IFN-γ mRNA in the allografts by day 4 (Figure 5A). IL-10 mRNA expression reached a maximal level by day 4 but declined thereafter in control allografts while continuing to increase in the HGF-treated allografts (Figure 5B). In control allografts, the levels of TGF-β mRNA increased biphasically; in contrast, levels of TGF-β mRNA in the HGF-treated allografts rose to a higher level than those in control allografts on day 4 and remained at a high level until day 12 (Figure 5C).

Effects of HGF on Allografts in the Chronic Phase

Next, to determine the effects of HGF on the allografts during the chronic phase, we investigated the difference among the following 4 groups on day 60: recipients treated with 500 μg · kg⁻¹ · d⁻¹ HGF for the initial 14 days with a long-term surviving allograft, recipients receiving daily tacrolimus with HGF for the initial 14 days, recipients receiving daily tacrolimus alone, and isografts. In allografts receiving tacrolimus alone, many of the intramyocardial and epicardial arteries were severely occluded by thickened intima (Figure 6A and 6P). In contrast, in HGF-treated allografts, the arterial intima was almost intact (Figure 6C and 6P), and only a few arteries were mildly occluded in the allografts that received tacrolimus with HGF (Figure 6B and 6P). By immunohistochemistry, α-SMA was expressed in the thickened intima and media in allografts that received tacrolimus alone, and MHC-B, which represents a phenotypic change of vascular smooth muscle cells,22 was also expressed in the thickened intima and media of the allografts that received tacrolimus alone (Figure 6D and 6G). In contrast, although α-SMA was expressed in the media of HGF-treated allografts regardless of tacrolimus administration, the intima was almost negative for α-SMA (Figure 6E and 6F). Moreover, MHC-B expression was almost negative in both the intima and media of HGF-treated allografts (Figure 6H and 6I). To evaluate another aspect of the changes during the chronic phase, cardiac remodeling of the allografts was assessed by interstitial fibrosis. In allografts that received tacrolimus alone, 2 of 7 allografts showed severe fibrosis in both the epicardium and intramyocardium, without a reduction in contractility, and the others showed mild fibrosis (Figure 6J, 6M, and 6Q). In the long-surviving allografts treated with HGF alone, interstitial fibrosis was significantly milder than that in the allografts treated with tacrolimus (Figure 6L, 6O, and 6Q).

To evaluate the involvement of the c-Met/HGF receptor-ligand system during the chronic phase, intragraft levels of c-Met mRNA and serum levels of endogenous HGF were measured. The levels of c-Met mRNA in HGF-treated allografts and in those that received tacrolimus together with HGF tended to be higher than those in both the tacrolimus-treated allografts and isografts (Figure 7A). In the recipients treated with HGF alone and in recipients that received tacrolimus together with HGF, the levels of endogenous HGF

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(Figure 5). Effect of HGF on cytokine mRNA expression in cardiac allografts during acute phase. Relative levels of IFN-γ (A), IL-10 (B), and TGF-β (C) mRNAs in cardiac allografts were determined by real-time RT-PCR. *P<0.05. Abbreviations are as defined in text.
were significantly higher than those of transplanted mice that received tacrolimus alone and isografts (Figure 7B).

To determine the intragraft levels of cytokines during the chronic phase, we measured the mRNA levels. Expression of IFN-γ mRNA was significantly higher in the allografts than in the isografts, regardless of the presence or absence of HGF treatment (Figure 8A). The expression of IL-10 mRNA was significantly higher in both the HGF-treated allografts and in allografts that received tacrolimus together with HGF than in the isografts, whereas the allografts that received tacrolimus alone showed lower expression levels than did the HGF-treated groups (Figure 8B). In contrast, similar levels of TGF-β1 mRNA expression were observed in all groups (Figure 8C). However, the levels of IFN-γ and TGF-β1 mRNA in the HGF-treated allografts were remarkably reduced during the chronic phase compared with those in the acute phase ($P<0.05$, except day 1).

**Discussion**

In the present study, we have demonstrated that the administration of HGF alone significantly prolonged survival in cardiac allografts in the murine model and that HGF could induce tolerance of the cardiac allografts. Furthermore, HGF prevented the progression of CAV and interstitial fibrosis.
We have demonstrated the upregulation of c-Met expression after transplantation in the myocardium of cardiac allografts, which was enhanced by the administration of HGF. Moreover, we have demonstrated that the administration of HGF resulted in a significant reduction of apoptosis in cardiomyocytes. Taken together, our findings indicate that HGF can produce cardioprotective effects on the transplanted heart through the inhibition of apoptosis in cardiomyocytes in the acute phase after transplantation.

Previous studies of renal and liver transplantation models had demonstrated that HGF administration prolonged graft survival. However, it still remained uncertain whether tolerance could be induced by HGF in each model. In our study, administration of HGF could induce tolerance in the recipient, which was an unexpected but favorable effect on cardiac allografts. This result indicates that HGF not only may provide cardioprotective properties but also may play an immunomodulative role for the recipient. It is generally understood that IFN-γ plays a central role in acute rejection. In our study, administration of HGF resulted in a significant reduction of IFN-γ expression in the allografts in the acute phase, suggesting that the prolongation of graft survival observed in HGF-treated recipients may be mainly attributable to the HGF-induced modulation of IFN-γ, IFN-γ-inducible factors, and a modification of the alternative pathways to IFN-γ by HGF in the acute phase. On the other hand, regulatory T (T_{reg}) cells play a role in the immunologic responses involved in allograft rejection and tolerance, and IL-10 and TGF-β, modulate allograft rejection by regulating T_{reg} cell function. Although the role of TGF-β, in the function of T_{reg} cells remains somewhat controversial, the expression of TGF-β, facilitates T_{reg} cell–mediated immunosuppression, and the administration of TGF-β, delays allograft rejection in cardiac transplantation models. We found that HGF increased the cardiac expression of IL-10 in the acute and chronic stages. Likewise, HGF increased the expression of TGF-β, in the acute stage after cardiac allograft transplantation. Therefore, T_{reg} cell–mediated immunosuppression may be responsible for the beneficial effects of HGF at least in part, and the increased expression of IL-10 and TGF-β, by HGF is likely to regulate T_{reg} cell function toward immunologic suppression and allograft tolerance.

The immune reaction after transplantation has been successfully suppressed by the continuous administration of calcineurin inhibitors. However, these agents cannot inhibit the development of vasculopathy and remodeling in the allografts. Although the exact pathogenesis of CAV remains to be established, previous studies suggest that CAV is primarily an immune system–mediated disease and have demonstrated the pivotal role of IFN-γ in triggering the pathological changes associated with CAV. Therefore, suppression of the cardiac expression of IFN-γ by HGF seems to be a predominant mechanism responsible for the preventive effect of HGF on the development of CAV. In
addition, it is noteworthy that HGF facilitated reendothelialization and the inhibition of neointimal formation in models of balloon injury. Together with the increased expressions of HGF and c-Met in the chronic phase of cardiac transplantation in HGF-treated mice, the effects of HGF on vascular endothelial cells and the subsequent inhibition of neointimal formation by HGF may decrease susceptibility to the development of CAV. In addition, HGF strongly suppressed interstitial fibrosis. Although expression of TGF-β was upregulated in the early phase of cardiac transplantation in HGF-treated mice, it declined to levels equivalent to those of isografts in the chronic phase. Consequently, it is possible that the antifibrogenic effect of continuously high levels of HGF surpassed the fibrogenic effect of TGF-β. However, it should be noted that tacrolimus allowed the development of both CAV and cardiac remodeling, even though it prevented acute rejection, whereas early treatment with HGF remarkably inhibited them.

In conclusion, our results provide strong evidence that HGF plays important roles in preventing acute and chronic rejection by its cardioprotective and immunomodulatory effects in the murine cardiac transplantation model. Although further investigations are needed to fully clarify the precise molecular and cellular mechanisms involved in its regulation of the immune system, the administration of HGF may provide an alternative to conventional immunosuppressive therapy for clinical cardiac transplantation.

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References
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