Thrombosis Increases Circulatory Hepatocyte Growth Factor by Degranulation of Mast Cells

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Background—Plasma concentrations of hepatocyte growth factor (HGF), a powerful angiogenic growth factor inducible by heparin, increase in thrombus-associated disorders such as myocardial infarction and unstable angina. The mechanism of this thrombus-associated HGF release, however, is unknown.

Methods and Results—Wistar rats received through the tail vein (1) normal saline (NS), (2) 50 μg of the mast cell–degranulating agent CP48/80, or (3) 1000 U/kg heparin. Blood samples were collected at 10 minutes or 30 minutes after the injections, or from untreated rats, for measurements of HGF. The same experiments were performed in mast cell–deficient white spotting (Ws) rats. Ws rats have a small deletion of the c-kit gene and are deficient in mast cells. Intravenous heparin immediately increased plasma HGF in both Wistar (38.02±2.08 ng/mL versus 1.11±0.70 ng/mL in untreated rats, P<0.0001) and Ws rats (36.39±4.15 ng/mL versus 0.66±0.18 ng/mL in NS-treated rats, P<0.0001). Injection of CP48/80 also increased plasma HGF in Wistar rats (9.12±1.11 ng/mL versus 0.65±0.24 ng/mL in NS group, P<0.004) but not in Ws rats (0.67±0.27 ng/mL versus 0.66±0.18 ng/mL in NS group, P=0.997). In a rat carotid artery microthrombus model, intra-arterial thrombus formation increased circulating HGF in Wistar rats (2.12±0.70 ng/mL versus sham 0.61±0.15 ng/mL in sham-operated Ws rats, P=0.0064) but not in Ws rats (0.76±0.33 ng/mL versus 0.21±0.04 ng/mL in sham-operated Ws rats, P=0.29). In addition, in vitro stimulation of rat peritoneal mast cells with thrombin rapidly induced degranulation in a dose-dependent manner.

Conclusions—These observations indicate that mast cell degranulation stimulated by thrombin is necessary for the rapid induction of plasma HGF in intravascular thrombus-associated disorders. (Circulation. 2002;106:3133-3138.)

Key Words: growth substances ■ heparin ■ thrombus ■ cells ■ thrombosis

Progress in interventional cardiology has improved the quality of life of patients with atherosclerotic cardiovascular disorders, though many are not candidates for percutaneous interventions or surgery. In the last two decades, the discovery of several angiogenic compounds and progress in molecular biology have focused the efforts on gene therapy as an alternate treatment of these severe forms of vascular disease. Heparin, an anticoagulant familiar not only to cardiologists but also to general practitioners, is endowed with potent angiogenic activity. In addition, HGF, also known as scatter factor, is a powerful angiogenic factor, along with other growth factors, such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and others. These factors, also called heparin-binding growth factors, are known to be inducible by heparin administration in vivo. In a recent study, however, we demonstrated that serum HGF is more prominently induced by heparin administration than the other heparin-binding growth factors in patients with coronary artery disease. The same study also showed that serum obtained from patients treated with heparin promoted vascular tube formation in vitro, a process inhibited by a neutralizing antibody against HGF but not by antibodies against FGF or VEGF. In addition, it has been reported that serum HGF is increased in the acute phase of thrombus-associated clinical disorders such as acute myocardial infarction and unstable angina or in rats with experimentally induced myocardial ischemia-reperfusion, and experimentally induced arterial carotid artery thrombosis. However, the precise mechanism of HGF elevation in these disorders has not been clarified. On the other hand, mast cells are the only cells capable of producing heparin in living animals, have been related to angiogenesis,11,12 and have been observed to accumulate around newly formed vessels in tumors and healing wounds. This study was performed to examine the role of mast cells in the increase in HGF, which occurs after arterial thrombus formation.

Methods

Biochemicals and Miscellaneous Supplies

Mast cell–degranulating agent compound 48/80 (CP48/80), rat thrombin, and xylazine were purchased from Sigma Chemical and heparin from Takeda Chemical Industries Ltd. Argatroban was
obtained from Daiichi Pharmaceutical Co Ltd. Fogarty (2F) balloon catheters were obtained from Baxter Healthcare Corp (model 12 to 060 to 2F, CV-1035). An ELISA kit for HGF was provided by the Institute of Immunology (Tokyo, Japan). Ketamine was obtained from Sankyo Co Ltd. Histamine measurements were made by Otsuka Pharmaceutical Industries Ltd.

Animals
Male Wistar rats and mast cell-deficient white spotting (Ws) rats were purchased from Shizuoka Agricultural Cooperation Association (Shizuoka, Japan) and housed in a special pathogen-free animal facility of the Kyoto University Hospital. Ws rats have a small deletion of the c-kit gene and are deficient in mast cells. All experiments were performed in accordance with the Guidelines for Animal Experiments of Kyoto University.

Plasma HGF Measurement After Arterial Injection in Wistar and Ws Rats
Seven- to 10-week-old male Wistar rats (weight, 200 to 260 g) were anesthetized intraperitoneally with ketamine (100 mg/kg) and xylazine (5 mg/kg). Normal saline (NS, 0.5 mL), 50 μg CP48/80 diluted in 0.5 mL NS, or 1000 U/kg heparin diluted in NS were injected intravenously through the tail vein. Five-milliliter blood samples were collected from the inferior vena cava in tubes containing 5 mg Na2 EDTA at 10 minutes or 30 minutes after injection or from untreated rats (0 minutes control). After centrifugation at 3000 rpm for 10 minutes at 4°C, the plasma was collected and HGF measured by ELISA. The same experiments were performed in 8- to 10-week-old mast cell–deficient Ws rats weighing 200 to 220 g. However, blood was sampled at 10 minutes only, since elevation of plasma HGF after CP48/80 injection was confirmed at this time point in the Wistar rats. To confirm the essential role of thrombus formation in HGF release, thrombin (500 U/kg) was injected in Wistar rats only. Plasma HGF concentrations were measured 10 minutes after the injection.

Plasma HGF Measurements After Arterial Thrombus Formation in Wistar and Ws Rats
A well-established intra-arterial thrombus formation and injury-stenosis–reperfusion rat model was used. Briefly, 12- to 18-week-old male Wistar rats weighing 300 to 480 g and Ws rats weighing 220 to 380 g were exposed left common carotid artery from the left common iliac artery exposed 0.35-mm stainless steel wire, were then left common carotid artery from the left common iliac artery exposed. Histamine measurements were made by Otsuka Pharmaceutical Industries Ltd. as previously reported, the administration of 1000 U/kg of heparin to Wistar rats rapidly increased the mean plasma concentration of HGF to 38.02 ± 2.08 ng/mL at 10 minutes (n = 4) and 39.72 ± 0.77 ng/mL at 30 minutes (n = 2) compared with 1.11 ± 0.70 ng/mL in the untreated group (n = 3, P < 0.0001, Figure 1A). The injection of 50 μg of the mast cell–degranulating agent CP48/80 also raised the mean plasma HGF to 9.12 ± 1.11 ng/mL at 10 minutes (n = 5, P = 0.004 versus NS) and to 8.70 ± 1.11 ng/mL (n = 4, P = 0.020 versus NS) at 30 minutes, whereas after the injection of NS, mean HGF was 0.65 ± 0.24 ng/mL at 10 minutes (n = 4) and 1.19 ± 0.39 ng/mL at 30 minutes (n = 3). However, in mast cell–deficient Ws rats, at 10 minutes after the administration of 50 μg of CP48/80, the mean plasma HGF was 0.67 ± 0.27 ng/mL (n = 5), comparable to that measured after the injection of NS (0.66 ± 0.18 ng/mL, n = 4, P = 0.997). In contrast, the administration of heparin was followed by the same increase in HGF as that measured in Wistar rats (36.39 ± 4.15 ng/mL, n = 4, P < 0.0001 compared with NS, Figure 1B). Plasma concentrations of HGF were increased 10 minutes after the injection of heparin in Wistar rats (3.41 ± 0.05 ng/mL, n = 4), compared with NS (1.11 ± 0.70 ng/mL, n = 3, P < 0.05, Figure 1C).

Results

Mast Cell Stimulation by Thrombin In Vitro
Rat peritoneal mast cells were harvested from 3 Wistar rats weighing 400 to 500 g by lavaging the peritoneal cavity with 100 mL of mast cell medium (MCM) composed of 150 mmol/L NaCl, 3.7 mmol/L KCl, 3.0 mmol/L Na2HPO4, 3.5 mmol/L KH2PO4, 5.6 mmol/L D-glucose, 0.1% BSA, and 0.1% gelatin, at pH 6.8. The peritoneal lavage was centrifuged at 350 rpm for 7 minutes at 4°C. The pellets were resuspended in MCM, stratified over 38% BSA for 20 minutes, and centrifuged at 1500 rpm for 7 minutes at 4°C. The upper aqueous phase was removed, and the lower phase was resuspended in 10 mL MCM and centrifuged at 350 rpm for 10 minutes at 4°C. The pellets were suspended in 1 mL MCM, and the number of mast cells was counted by staining with toluidine blue and adjusted to 1.8 × 10⁶ per 450 μL MCM. This mast cell suspension was incubated at 37°C with 50 μL of preheated MCM for 0 minutes, by immediately adding cold MCM (presaturation control) or for 10 minutes with, respectively, 50 μL of preheated MCM (unstimulated control), 50 μL of thrombin at a concentration of 1 U/mL, or 50 μL of thrombin at a concentration of 10 U/mL. Fifteen hundred microliters of cold MCM was added to stop the reaction, centrifuged at 1000 rpm for 10 minutes at 4°C, and the histamine content of the supernatant was measured by radioimmunoassay. HGF concentrations of the supernatants were also measured by ELISA. Measurements were made in triplicate.

Plasma Histamine Measurement After Thrombin Injection in Wistar Rats
To confirm the degranulation of mast cells after thrombus formation, 500 U/kg thrombin or NS was injected in Wistar rats only. Plasma histamine concentrations were measured 10 minutes after the injection.

Statistical Analysis
All comparisons were made by 1-way ANOVA, followed by the Fisher’s protected least significant difference test. A value of P < 0.05 was considered significant. Data are expressed as mean ± SEM.
Sham-operated animals (Figure 2C and D). As we have observed in the smooth surface of the intact endothelium observed in the injury-stenosis-reperfusion model of both the endothelium-denuded internal surface of the left common carotid artery in the Wistar rats, compared with the sham-operated rats (0.61±0.15 ng/mL, n=6, P=0.0064). In contrast, the mean plasma HGF measured in Wistar rats (0.76±0.33 ng/mL, n=4) was not significantly different from that measured in the sham-operated rats (0.21±0.04 ng/mL, n=7, P=0.29, Figure 2E).

Furthermore, the induction of HGF in Wistar rats was significantly attenuated by argatroban (0.45±0.25 ng/mL, n=7) compared with NS (1.79±0.62 ng/mL, n=4, P<0.05, Figure 2F).

### Mast Cell Degranulation Stimulated by Thrombin In Vivo

The histamine contents in the supernatant of mast cells stimulated with thrombin at 10 minutes increased significantly in a dose-dependent manner compared with the pre-stimulation at 0 minutes and in the unstimulated controls at 10 minutes (Figure 3). However, the concentrations of HGF in the supernatants were not significantly changed by the stimulation of mast cells with thrombin (data not shown).

### Mast Cell Degranulation After Thrombus Formation In Vivo

Plasma concentrations of histamine were increased 10 minutes after the injection of thrombin in Wistar rats (251.4±65.43 nmol/l, n=7) compared with NS (98.43±30.28 nmol/l, n=4, P<0.05, Figure 4).

### Discussion

This study showed that HGF, a potent angiogenic growth factor, is induced by mast cell degranulation in a rat model of arterial thrombus formation. HGF was initially discovered as a mitogen for hepatocytes but was later found to participate in several biological processes, including embryogenesis, organ regeneration, wound healing, and, in particular, angiogenesis. Its angiogenic property is now being considered in the treatment of chronic limb ischemia. It is stored in the extracellular matrix, binding heparan-sulfate proteoglycan as well as the other heparin-binding growth factors. Heparin induces angiogenesis in vivo and is under clinical investigation for the treatment of chronic myocardial ischemia. Its angiogenic effects are mainly attributable to the liberation of angiogenic peptides from the extracellular matrix and their recruitment into the target lesions. In a recent study, we demonstrated in patients with coronary artery disease that serum HGF was more prominently induced by heparin than the other heparin-binding growth factors such as FGF and VEGF. In addition, the in vitro formation of vascular tubes induced by the serum obtained from patients treated with heparin was inhibited by neutralization of the antibody against HGF and not by neutralization of the antibodies against FGF or VEGF. These observations highlight the importance of HGF in heparin-induced angiogenesis and are behind our choice of this heparin-binding growth factor for these experiments. In this and in an earlier study from our laboratory, plasma HGF increased to the same extent immediately after the administration of heparin in both

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**Figure 1.** Plasma HGF concentration at 10 minutes and 30 minutes after intravenous injection of normal saline (hatched bars, n=3 at 10 minutes, n=4 at 30 minutes), mast cell–degranulating agent CP 48/80 (solid bars, n=4 at 10 minutes, n=5 at 30 minutes), and 1000 U/kg heparin (lattice bars, n=4 at 10 minutes, n=2 at 30 minutes) in Wistar rats (A). Plasma HGF at 0 minutes indicates concentration in untreated rats (open bar, n=3). *P<0.05, #P<0.0001, and NS (difference not significant) vs 0 minutes. Plasma HGF concentration at 10 minutes after intravenous injection of normal saline (hatched bar, n=4), mast cell–degranulating agent CP 48/80 (solid bar, n=5), and 1000 U/kg heparin (lattice bar, n=4) in mast cell–deficient Ws rats (B). *P<0.0001 and NS (difference not significant) vs normal saline. Plasma HGF concentration at 10 minutes after intravenous injection of normal saline (open bar, n=4) and 500 U/kg thrombin (hatched bar, n=4) in Wistar rats (C). *P<0.05 vs normal saline.

**Arterial Thrombus–Induced Plasma HGF Elevation by Mast Cell Degranulation In Vivo**

Numerous mural microthrombi were observed on the endothelium-denuded internal surface of the left common carotid artery in the injury-stenosis-reperfusion model of both the Wistar (Figure 2A) and Ws (Figure 2B) rats, as opposed to the smooth surface of the intact endothelium observed in the sham-operated animals (Figure 2, C and D). As we have previously reported, plasma HGF increased significantly to 2.12±0.70 ng/mL (n=5) immediately after thrombus formation in the injured carotid artery of the Wistar rats, compared with the sham-operated rats (0.61±0.15 ng/mL, n=6, P=0.0064). In contrast, the mean plasma HGF measured in Wistar rats (0.76±0.33 ng/mL, n=4) was not significantly different from that measured in the sham-operated Ws rats (0.21±0.04 ng/mL, n=7, P=0.29, Figure 2E).

In a recent study, we demonstrated in patients with coronary artery disease that serum HGF was more prominently induced by heparin than the other heparin-binding growth factors such as FGF and VEGF. In addition, the in vitro formation of vascular tubes induced by the serum obtained from patients treated with heparin was inhibited by neutralization of the antibody against HGF and not by neutralization of the antibodies against FGF or VEGF. These observations highlight the importance of HGF in heparin-induced angiogenesis and are behind our choice of this heparin-binding growth factor for these experiments. In this and in an earlier study from our laboratory, plasma HGF increased to the same extent immediately after the administration of heparin in both
Wistar and mast cell–deficient Ws rats. These observations indicate that mast cell–deficient Ws rats retain the ability to store and produce HGF as well as the Wistar rats. Mast cells have been associated with angiogenesis in wound healing, tumors, rheumatoid synovium, and tissue ischemia, accumulating around the small vessels in these lesions. Morphometric observations in rats injected with CP 48/80 have shown that mast cell degranulation has stimulatory effects on the growth and expansion of the vascular network in the mesentery. Mast cells contain several chemical mediators, among which histamine, tumor necrosis factor-α, tryptase, and especially heparin, have angiogenic activity. Mast cells are the only cells known to produce heparin in living animals. The immediate increase in plasma HGF in response to CP 48/80 in Wistar rats, in contrast to the absence of response in Ws rats, confirms that mast cell degranulation causes a rapid increase in plasma HGF.
An increase in plasma HGF has been observed in diseases associated with intravascular thrombosis, including myocardial infarction,30 unstable angina,6,7 cerebral infarction,31 diabetic retinopathy,32 and represents a new diagnostic marker in the early phase of these thrombotic disorders. In investigations of tumoral processes, thrombin is also known to be a potent stimulator of angiogenesis, as shown in the chick chorioallantoic membrane system and the mouse Matrigel system.33 In this study, we have also shown that the formation of thrombus by thrombin increased the plasma concentrations of HGF in Wistar rats. The precise mechanism of increase in plasma HGF and its relation with intravascular thrombosis, however, have not been clarified. To confirm the obligatory participation of mast cell degranulation in this phenomenon, mast cell–deficient Ws rats were used in our experimental arterial thrombus model. The rapid increase in plasma HGF in Wistar rats was confirmed in this and our previous study.9 The induction of HGF also was attenuated by the specific thrombin inhibitor argatroban. These observations confirm that thrombin contributes to HGF release in this model. In contrast, the absence of increase in mast cell–deficient Ws rats indicates that mast cells are an essential participant in this rapid increase in plasma HGF associated with intravascular thrombosis.

Mast cells have a thrombin receptor,34 and their degranulation by thrombin stimulation has been observed in vitro34 and in vivo.35 Our study also ascertained the rapid, dose–dependent release of histamine (as a maker of degranulation) from mast cells by thrombin stimulation in vitro, whereas the levels of HGF remained unchanged with or without thrombin stimulation. In this study, we have also demonstrated that the formation of thrombus by thrombin increased the plasma concentrations of histamine in Wistar rats. These experiments provide an explanation for the rapid increase in plasma HGF in intravascular thrombosis-associated diseases. Thrombin does not directly release HGF from mast cells. The thrombi formed within the vessels stimulate the degranulation of mast cells, and the content of the granules, most likely heparin, immediately releases HGF trapped in the extracellular matrix into the blood stream, to be used for angiogenesis at appropriate sites.

This study contributes information of clinical importance, indicating that living organisms are prepared to adapt to life-threatening crises such as myocardial infarction by releasing heparin from mast cells in order to liberate several heparin-binding growth factors. Therefore, it appears justified to use heparin in the treatment of disorders benefiting from angiogenesis such as ischemic heart disease or limb ischemia.

The separate importance of mast cells, heparin22 and heparin-binding growth factors,24 and thrombosis33 in angiogenesis was recognized early, though the relation among these factors was unclear. This study is the first to show that mast cell degranulation stimulated by thrombin is necessary for the rapid induction of plasma HGF, a potent angiogenic heparin-binding growth factor, in disorders associated with intravascular thrombus formation.

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References

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