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Induction of Hepatocyte Growth by Intraportal Infusion of HGF into Beagle Dogs

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Hepatocyte growth factor (HGF), originally identified as a potent mitogen for mature parenchymal hepatocytes, is a hepatotrophic factor involved in liver regeneration and is even essential for development of the liver. We report here that human recombinant HGF at a very low concentration and given intraportaly stimulated liver regeneration in dogs. *In vitro,* HGF dose-dependently stimulated DNA synthesis of primary cultured hepatocytes isolated from a dog. The maximal activity was twofold higher than that of epidermal growth factor, and insulin potentiated the mitogenic activity of HGF. When human recombinant HGF was infused through the portal vein into 30% partially hepatectomized dogs at 0.25 μg/kg body weight in order to directly target the liver, HGF stimulated DNA synthesis of hepatocytes and liver weight at 72 h after the operation; labeling indices in saline- and HGF-injected groups were 0.75 and 1.82%, respectively, and the liver weights in saline- and HGF-injected groups were 302 and 374 g, respectively. Since HGF exerts potent antihepatitis activity as well as mitogenic activity, these results indicate that intraportal administration of HGF may be particularly important to enhance liver regeneration and prevent the severe hepatic insufficiency after hepatic surgery.

Hepatocyte Growth Factor (HGF) was originally isolated as a potent mitogen for mature hepatocytes (1–4), but it is now known to have multiple actions for a wide variety of epithelial cells, endothelial cells, and some mesenchymal cells (reviewed in refs. 5–8). In addition to mitogenic activity, HGF has an unique morphogenic activity such as an induction of epithelial tubulogenesis including in cells derived from the kidney, liver, and mammary gland (9–11). Characterization of scatter factor, which enhances epithelial cell motility, revealed that it is the same molecule as HGF (12–14). HGF is a heterodimeric glycoprotein composed of a 69kDa α-chain and a 34kDa β-chain, and has four homologous kringle domains in the α-chain (15, 16). The receptor capable of signal transduction of HGF is c-met protooncogene product of heterodimeric tyrosine kinase (17, 18). Extensive studies on physiological functions of HGF have established that this growth factor functions as a potent hepatotrophic factor for liver regeneration (5–8). Expression of HGF is rapidly induced in organs after various types of liver injuries (5, 6, 19, 20), and elevated plasma HGF levels were noted in patients with hepatic diseases (21, 22). HGF is produced in non-parenchymal liver cells (20, 23), thus a paracrine-related mechanism of HGF is functioning in the liver.

We previously showed that intravenously injected recombinant HGF markedly enhanced replication of hepatocytes following liver injuries in experimental animals (24), and it suppressed hepatic dysfunction in the model that induces experimental cholestasis (24). Subsequent studies also noted a potent mitogenic action of HGF for hepatocytes following acute liver injury (25–28). More importantly, we recently found that HGF remarkably suppressed the onset of hepatic fibrosis/cirrhosis and abrogated lethal hepatic dysfunction caused by chronic hepatic injury (29). However, since plasma HGF is rapidly cleared (30–32), and plasma HGF levels rapidly fall down after the intravenous injection of HGF, relatively high doses are required for the maximal activity of HGF in experimental animals.

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Abbreviations: HGF, hepatocyte growth factor; hrHGF, human recombinant HGF.

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We here show that human recombinant HGF at very low concentrations stimulated hepatic regeneration in dogs when HGF was infused locally through the portal vein. Directly targeted therapeutic strategies for clinical application can be considered.

MATERIALS AND METHODS

Materials. Human recombinant HGF (hrHGF) was purified from conditioned medium of CHO cells transfected with an expression vector containing full size human HGF cDNA as described previously (15, 33). Recombinant epidermal growth factor (EGF) and insulin were purchased from Sigma Co. (St. Louis). [^{3}H]Thymidine for the measurement of DNA synthesis was purchased from Amersham Co. (Tokyo).

Primary culture of beagle dog hepatocytes. Hepatocytes were isolated from the median lobe of beagle dog liver, as described previously (34). Viability of the hepatocytes exceeded 85%. The yield from the perfused liver was 3×10^7 cells/g liver. Cells (2.5×10^5) were placed in collagen-coated 24-well culture plates and cultured in Williams medium E supplemented with 5% calf serum, 1 nM insulin and 10 nM dexamethasone under 5% CO_2 and 30% O_2 in air at 37°C. After 4 h, the medium was changed to serum-free William's medium E supplemented with 0.1 μg/ml of aprotinin (bovine pancreatic trypsin inhibitor), as described elsewhere (35).

Assay of DNA synthesis. hrHGF (10 ng/ml), insulin (0.1 μM) and EGF (10 ng/ml) were added to the cultures 20 h after plating. Twelve hours later, [^{3}H] thymidine (2.5 μCi/ml, 0.27 Ci/mmol) was added and the culture was continued for the following 24 h. Incorporation of [^{3}H]thymidine into DNA was measured as described previously (36).

Animal treatment. Male beagle dogs aged 11 to 12 months (9–12 kg, Kyushu Animal Laboratories) were used following a 12 h deprivation of food are water. After giving an intramuscular injection of ketamine (50 mg/body) and an intravenous injection of thiopental sodium (25–30 mg/kg), a cuffed endotracheal tube was inserted into the trachea and connected to a respirator. Five percent glucose solution (500 ml) was infused into the external jugular vein during the treatment. After an abdominal midline incision, the superior mesenteric vein was cannulated with a catheter, and a 30% hepatectomy (excision of left lateral lobe) was carried out. Our preliminary experiment using five animals indicated that weight of the excised left lateral lobe accounted for 30% of the total liver weight. hrHGF (0.25 μg/kg) in saline or saline alone was injected into the superior mesenteric vein immediately after and 12, 24 and 48 h after partial hepatectomy. Four or five animals were included in each experimental group.

Assessment of liver regeneration. The dogs were killed 24 or 72 h after hepatectomy, and the livers were removed and weighed. Fifty mg/kg of bromodeoxyuridine (BrdU) was injected 90 min before they were killed. After fixation, the liver was embedded in paraffin and cut into 4 μm sections. After deparaffinization, the sections were treated with 2 M HCl to denature DNA. Immunohistochemical detection of BrdU was done using the routine avidin-biotin complex method with anti-BrdU monoclonal antibody as the first antibody. Neighboring sections were stained with hematoxylin-eosin. To determine the labeling index, 1,000 cells were counted randomly in several fields and the BrdU positive nuclei were recorded in each field, under light microscopic examination. The labeling index was expressed as a percent of labeled cells among total cells. Values were expressed mean ± SD. Statistical analysis was carried out through appropriate use of the student's t-test. p values less than 0.05 were considered significant.

Liver regeneration ratio. Liver regeneration ratio was calculated by the conventional formula:

Liver regeneration ratio = Remnant liver weight (g) / Total liver weight (g) Total liver weight was calculated from weight of the excised left lateral lobe, based on calculation that the weight of the excised lobe was 30% of a total liver weight.

RESULTS

Mitogenic activity of hrHGF for canine hepatocytes in primary culture. We first examined effects of hrHGF on DNA synthesis of canine hepatocytes in primary culture. Mature hepatocytes were isolated from the liver using collagenase perfusion. Addition of hrHGF alone stimulated DNA synthesis of canine hepatocytes in a dose-dependent manner (Fig. 1). hrHGF was effective at 2 ng/ml and the maximal mitogenic activity was seen with 6 ng/ml. EGF (10 ng/ml) also stimulated the DNA synthesis, but the activity was half of that of HGF. Although insulin (0.1 μM) alone had only weak mitogenic activity for canine hepatocytes, the simultaneous addition of insulin and HGF resulted in a remarkable enhancement of DNA synthesis; in the presence of HGF and insulin, there was a two-fold higher enhancement compared with that seen with HGF alone. The result indicates that insulin potentiate the mitogenic action of HGF for canine hepatocytes.

Mitogenic action of HGF for liver cells after partial hepatectomy. On the basis of the potent mitogenic activity of HGF for primary cultured canine hepatocytes, we measured the efficacy of hrHGF on DNA synthesis of liver cells in dogs subjected to 30% partial hepatectomy. In the present

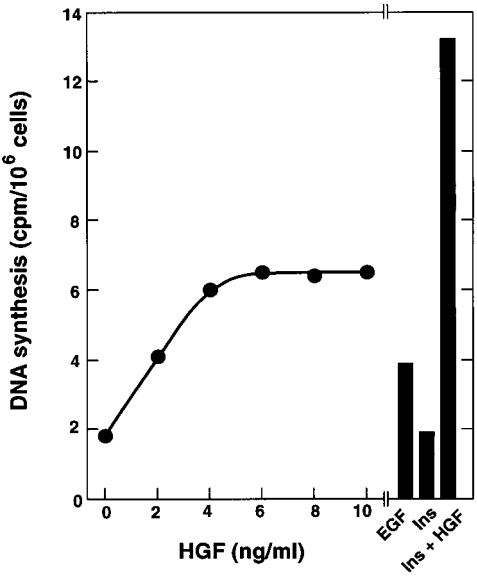

FIG. 1. Effects of human recombinant HGF (hrHGF) on DNA synthesis of canine hepatocytes in primary culture. Hepatocytes isolated from a beagle dog were cultured. DNA synthesis was measured by [^{3}H]thymidine incorporation. Human recombinant EGF (EGF) and insulin (Ins) were added at 10 ng/ml and 0.1 μM, respectively. Each value represents the mean of triplicate measurements. Standard deviations were lower than 10% of each values.

study, a low dose of HGF (0.25 μg/kg body weight) was injected through the superior mesenteric vein immediately after and 12, 24, and 48 h after partial hepatectomy.

In the normal liver, the labeling index (cells undergoing DNA synthesis in %) of hepatocytes is lower than 0.1% (not shown), whereas it increased to 0.4 ± 0.14% at 24 h after 30% partial hepatectomy, indicating that hepatocytes do respond to endogenous mitogenic stimulus. hrHGF-injection only slightly increased the labeling index to 0.5 ± 0.3% at 24 h after the surgery. However, at 72 h after the operation, the labeling index of hepatocytes was increased from 0.75 ± 0.44% to 1.82 ± 0.76% following hrHGF-injection (Fig. 2). The labeling index of hrHGF-injected group was significantly higher than that of the control saline-injected group at 72 h after the operation. Hepatocytes undergoing DNA synthesis after hrHGF-injection were predominantly distributed around portal zones (not shown).

Liver regeneration enhanced by hrHGF. When weight of the remnant liver was measured, there was no significant difference between the control saline-injected group (270 ± 37.4 g) and the hrHGF-injected group (266 ± 39 g) at 24 h after the operation (Fig. 3A). At 72 h after the operation, however, the increase in remnant liver weight was enhanced from 302 ± 32 g (control saline-injected group) to 374 ± 48.6 g (hrHGF-injected group) (Fig. 3A). When the liver regeneration ratio was calculated based on these data, the regeneration ratio in the control saline-injected group was 95 ± 12%, whereas it increased to 117 ± 14% in case of hrHGF-injection (Fig. 3B).

DISCUSSION

hrHGF-injection stimulates hepatic DNA synthesis and regeneration of the liver in experimental animals with induced liver injuries, including partial hepatectomy (24–28). In all these reports on the efficacy of HGF *in vivo*, relatively high doses of HGF were used (higher than 50μg/kg/day and up to 4.8 mg/kg/day), since intravenous (tail vein or jugular vein) or intraperitoneal administration was used. Plasma hrHGF levels rapidly decreased following the intravenous administration of hrHGF (30–32). In the present study, we found that a very low dose of hrHGF (0.5 μg hrHGF/kg/day) is effective in stimulating replication of hepatocytes *in vivo*, when intra-portally injected

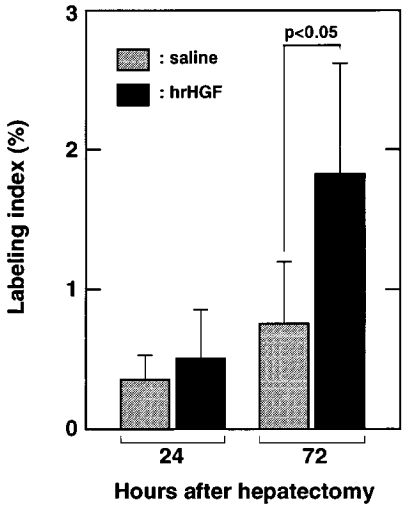

FIG. 2. Labeling index in hepatocytes of the liver in dogs with or without hrHGF-infusion after 30% partial hepatectomy. Labeling index was measured by BrdU incorporation and following immunohistochemical detection using monoclonal anti-BrdU antibody. Four animals were used in both saline-injected and HGF-injected groups at 24 h and five animals were used in both saline-injected and HGF-injected group at 72 h, respectively.

into dog after partial hepatectomy. The doses of HGF used in the previous experiments were over 100-fold higher than those given in the present study. Since the liver is the main organ contributing the clearance of circulating plasma HGF (30–32), hrHGF infused through the portal vein may be effectively trapped by the liver without dilution during blood circulation, thus the dose can be remarkably reduced. An additional benefit of this system is that the insulin concentration may be highest in the portal vein and insulin markedly potentiates mitogenic activity of HGF.

Hepatic lobectomy or extended lobectomy often leads to a high operative mortality due to hepatic insufficiency (37, 38). Since postoperative liver failure is predominantly related to a reduction in functioning liver mass (37, 39), the promotion of remnant liver regeneration would be expected to reduce postoperative mortality. In our initial trial, a 70% partial hepatectomy in beagles was done in view of postoperative hepatic insufficiency, but the animals failed to survive due to anatomical difficulty. In case of 30% partial hepatectomy, there was no significant hepatic insufficiency, as determined by serum bilirubin, serum enzyme activities (alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase), prothrombin time, and C-reactive protein. These values were not significantly changed compared with data on intact dogs (not shown). Nevertheless, previous studies *in vivo* as well as *in vitro* clearly indicated that HGF prevented the onset of severe hepatic insufficiency in experimental animals with acute or chronic liver injury (24, 27, 29). The protective effect of HGF is likely to be a direct one on hepatocytes. HGF stimulates protein synthesis, including albumin, and prevents cytosolic enzyme leakage induced by CCl_4 in primary cultured hepatocytes (40, 41). A 30% partial hepatectomy led to only a weak hepatic dysfunction, but liver cells in the remnant liver are expected to respond to mitogenic stimulus, because most mature hepatocytes in the normal liver are in the resting G_0 state and will not respond sensitively to mitogenic factors (24, 42).

The intra-portal infusion of HGF proved useful against postoperative hepatic failure following major hepatic resection in dogs, because of its potent mitogenic action, as well as its cytoprotective effect on hepatic functions. In conclusion, because of the targeted administration of HGF to the liver through an intraportal delivery method, HGF, even at a very low dose will induce hepatocyte mitogenesis and enhance liver regeneration.

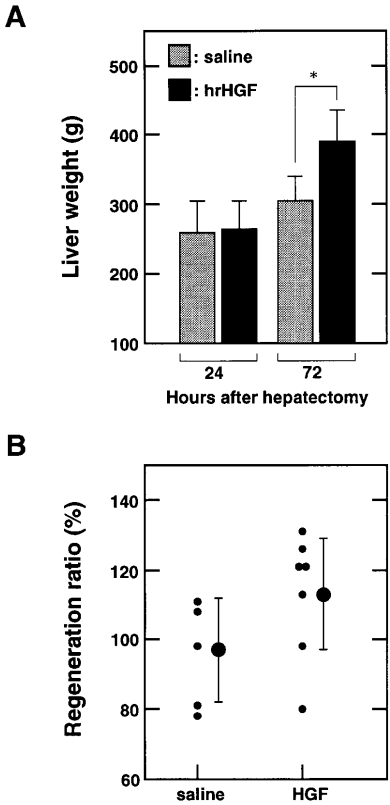

FIG. 3. Change in the remnant liver weight and regeneration ratio of the liver in dogs with or without hrHGF-infusion after 30% partial hepatectomy. (A) Change in the remnant liver weight at 24 h and 72 h after the operation. (B) Liver regeneration ratio at 72 h after the operation. Four animals were used in both saline-injected and HGF-injected groups at 24 h and five animals were used in both saline -injected and HGF-injected groups at 72 h, respectively. *$p < 0.05$.

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REFERENCES

1. Nakamura, T., Nawa, K., and Ichihara, A. (1984) *Biochem. Biophys. Res. Commun.* **122,** 1450–1459.
2. Nakamura, T., Teramoto, H., and Ichihara, A. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83,** 6489–6493.
3. Ghoda, E., Tsubouchi, H., Nakamura, H., Hirono, S., Sakiyama, O., Takehashi, K., Miyazaki, H., Hashimoto, S., and Daikuhara, Y. (1988) *J. Clini. Invest.* **81,** 414–419.
4. Zarnegar, R., and Michalopoulos, G. K. (1989) *Cancer Res.* **49,** 3314–3320.
5. Nakamura, T. (1991) *Prog. Growth Factor Res.* **3,** 67–85.
6. Jiang, W. G., Hallett, M. B., and Puntis, M. C. A. (1993) *Br. J. Surg.* **80,** 1368–1373.
7. Zarnegar, , and Michalopoulos, G. K. (1995) *J. Cell Biol.* **129,** 1177–1180.
8. Boros, P., and Miller, C. M. (1995) *Lancet* **345,** 293–295.
9. Montesano, R., Matsumoto, K., Nakamura, T., and Orci, L. (1991) *Cell* **67,** 901–908.
10. Johnson, M., Koukoulis, G., Matsumoto, K., Nakamura, T., and Iyer, A. (1993) *Hepatology* **17,** 1052–1061.
11. Soriano, J. V., Pepper, M. S., Nakamura, T., Orci, L., and Montesano, R. (1995) *J. Cell Sci.* **108,** 413–430.
12. Weidner, K. M., Arakaki, N., Hartmann, G., Vanderkerkhove, J., Weingart, S., Rieder, H., Fonatsch, C., Tsubouchi, H., Hishida, T., Daikuhara, Y., and Birchmeier, W. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88,** 7001–7005.
13. Furlong, D. L., Takehara, T., Taylor, W. G., Nakamura, T., and Rubin, J. S. (1991) *J. Cell Sci.* **100,** 173–177.
14. Naldini, L., Weidner, K. M., Zarnegar, R., Michalopoulos, G. K., Birchmeier, W., and Comoglio, P. M. (1991) *EMBO J.* **10,** 2867–2878.

15. Nakamura, T., Nishizawa, T., Hagiya, M., Sugimura, A., Tashiro, K., and Shimizu, S. (1989) *Nature* **342,** 440–443.
16. Tashiro, K., Hagiya, M., Nishizawa, T., Seki, T., Shimonishi, M., Shimizu, S., and Nakamura, T. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87,** 3200–3204.
17. Bottaro, D., Rubin, J. S., Faletto, D. L., Chan, A. M. I., Kmiecik, T. E., Vande Woude, G. F., and Aaronson, S. A. (1991) *Science* **251,** 802–804.
18. Naldini, L., Vigna, E., Narcimham, R. P., Gaudino, G., Zarnegar, R., Michalopoulos, G. K., and Comoglio, P. M. (1991) *Oncogene* **6,** 501–504.
19. Kinoshita, T., Tashiro, K., and Nakamura, T. (1989) *Biochem. Biophys. Res. Commun.* **165,** 1229–1234.
20. Noji, S., Tashiro, K., Koyama, E., Nohno, T., Ohyama, K., Taniguchi, S., and Nakamura, T. (1990) *Biochem. Biophys. Res. Commun.* **173,** 42–47.
21. Tsubouchi, H., Hirono, S., Gohda, E., Nakayama, H., Takahashi, K., Sakiyama, O., Miyazaki, H., Sugihara, J., Tomita, E., Muto, Y., Daikuhara, Y., and Hashimoto, S. (1989) *Hepatology* **6,** 875–881.
22. Shiota, G., Okano, J. I., Kawasaki, H., Kawamoto, T., and Nakamura, T. (1995) *Hepatology* **21,** 106–112.
23. Ramadori, G., Neubauer, K., Odenthal, M., Nakamura, T., Knittel, T., Schwogler, K.-H., and Meyer zum Buschenfelde, (1992) *Biochem. Biophys. Res. Commun.* **183,** 739–742.
24. Ishiki, Y., Ohnishi, H., Muto, Y., and Nakamura, T. (1992) *Hepatology* **16,** 1227–1235.
25. Fujiwara, K., Nagoshi, S., Ohno, A., Hirata, K., Ohta, Y., Mochida, S., Tomiya, T., Higashio, K., and Kurokawa, K. (1993) *Hepatology* **18,** 1443–1449.
26. Ishii, T., Sato, M., Suzuki, K., Nakai, H., Hishida, T., Niwa, T., Umezu, K., and Yuasa, S. (1995) *J. Biochem.* **117,** 1105–1112.
27. Roos, F., Terrell, T. G., Godowski, P. J., Chamow, S. W., and Schwall, R. H. (1992) *Endocrinology* **131,** 2540–2544.
28. Roos, F., Ryan, A. M., Chamow, S. M., Bennett, G. L., and Schwall, T. (1995) *Am. J. Physiol.* **268,** 380–386.
29. Matsuda, Y., Matsumoto, K., Ichida, T., and Nakamura, T. (1995) *J. Biochem.* **118,** 643–649.
30. Liu, K-X, Kato, Y., Narukawa, M., Kim, D. C., Hanano, M., Higuchi, O., Nakamura, T., and Sugiyama, Y. (1992) *Am. J. Physiol.* **263,** 642–649.
31. Liu, K., Kato, Y., Yamasaki, M., Higuchi, O., Nakamura, T., and Sugiyama, Y. (1993) *Hepatology* **17,** 651–660.
32. Ziochech, T. F., Richardson, L., Deguzman, G. G., Modi, N. B., Hansen, S. E., and Godowski, P. J. (1994) *Endocrinology* **134,** 1879–1887.
33. Seki, T., Ihara, I., Sugimura, A., Shimonishi, M., Nishizawa, T., Asami, D., Hagiya, M., Nakamura, T., and Shimizu, T. (1990) *Biochem. Biophys. Res. Commun.* **172,** 321–327.
34. Tanaka, K., Sato, M., Tomita, Y., and Ichihara, A. (1978) *J. Biochem.* **84,** 937–946.
35. Asami, O., Nakamura, T., Mura, T., and Ichihara, A. (1984) *J. Biochem.* **95,** 299–309.
36. Nakamura, T., Tomita, Y., and Ichihara, A. (1983) *J. Biochem.* **94,** 1029–1035.
37. Didolkar, M. S., Fitzpatrick, J. L., Elias, E. G., Whitley, N., Keramati, B., Suter, C. M., and Brown, S. (1989) *Surg. Gynecol. Obstet.* **169,** 17–26.
38. Tsuzuki, T., Sugioka, A., Ueda, M., Iida, S., Kanai, T., Yoshii, H., and Nakayasu, K. (1990) *Surgery* **107,** 511–520.
39. Makuuchi, M., Thai, B. L., Takayasu, K., Kosuge, T., Gunver, P., Yamazaki, S., Hasegawa, H., and Ozaki, H. (1990) *Surgery* **107,** 521–527.
40. Takehara, T., Matsumoto, K., and Nakamura, T. (1992) *J. Biochem.* **112,** 330–334.
41. Takehara, T., and Nakamura, T. (1991) *Biomed. Res.* **12,** 335–338.
42. Mizuno, K., Higuchi, O., Tajima, H., Yonemasu, T., and Nakamura, T. (1993) *J. Biochem.* **114,** 96–102.