ductal carcinoma after curative resection (51). Our findings suggest that HDGF expression, as evaluated by immunohistochemistry, can be used as a new prognostic factor for pancreatic cancer.

HDGF in cholangiocarcinoma

The expression levels of HDGF and VEGF were examined in patients with human hilar cholangiocarcinoma (52). HDGF and VEGF had a positive correlation, and patients with positive HDGF expression had a significantly poorer overall survival rate than those with negative HDGF expression. A multivariate analysis showed that HDGF expression was an independent prognostic factor. These findings suggested that a high expression of HDGF plays an important role in the development and progression of human hilar cholangiocarcinoma and that HDGF expression can be a valuable prognostic factor for this cancer.

HDGF in esophageal cancer

As described in the "HDGF in apoptosis" section, a previous study suggested that there is a relationship between the expression of HDGF and the radiosensitivity of esophageal cancer cells. HDGF is highly expressed in radiosensitive esophageal cancer cells, and radiotherapy is more effective in patients with a high expression of HDGF than in those with low expression (35). In addition, the induction of apoptosis in cancer cells is thought to be one of the important mechanisms involved in the anti-tumor effects of radiotherapy, and HDGF expression is suggested to be involved in the resistance to apoptotic signals (38-44). Therefore, the mechanism(s) underlying how HDGF expression is associated with the sensitivity to radiation still remains to be clarified.

Although an increased expression of HDGF leads to a higher effect of radiotherapy, HDGF expression is associated with the recurrence and prognosis of cancer in patients with esophageal cancer (53). Patients with a higher expression of HDGF had a poorer disease-free and overall survival compared with those with a lower expression. Further studies are therefore necessary to determine the functional role of HDGF in esophageal cancer.

HDGF in gastric cancer

HDGF is expressed in gastric cancer cells (15, 32). Transfection of HDGF stimulated cellular proliferation via the activation of Erk1/2 signaling, and promoted the anchorage-independent growth of gastric cancer cells (15). Furthermore, a reduction of HDGF in gastric cancer cells induced Bad-mediated apoptotic signaling, and decreased the invasive activity of the cells (32). Interestingly, HGF (hepatocyte growth factor) induced HDGF in a dose-dependent manner, and HDGF induced VEGF expression, thus suggesting that HDGF may be involved in tumor growth by means of its cooperation with these growth factors. In

patients with gastric cancer, higher expression levels of HDGF are significantly associated with higher rates of infiltrative tumor growth, as well as vascular and lymphatic invasion (54). Thus, HDGF is suggested to have a significant role in the development and progression of gastric cancer.

HDGF in colorectal cancer

HDGF has been reported to be involved in the development and progression of colorectal cancer cells (42, 43). HDGF expression is remarkably high in human colorectal cancers, especially in tumors proficient in DNA mismatch repair (25). We have documented that recombinant HDGF stimulated the proliferation of colonic HT-29 cells, whereas a polyclonal antibody against recombinant HDGF significantly suppressed their proliferation (55). Knockdown of HDGF induced apoptosis through the activation of the mitochondrial pathway (42), whereas the overexpression of HDGF resulted in the resistance of colorectal cancer cells to drug-induced apoptosis (43). These findings suggest that HDGF plays an important role in gut epithelial cell proliferation, including that of colorectal cancer cells.

HDGF in gastrointestinal stromal tumors (GIST)

The HDGF protein was detected in GIST tissues (56, 57). An immunohistochemical evaluation suggested that there is a significant relationship between HDGF expression and tumor growth. HDGF expression correlates with tumor mitosis and tumor size. A high expression of HDGF in patients with GIST was related to an early recurrence and poor prognosis, and HDGF expression was reported to be an independent prognostic factor for the disease-free and overall survival of patients after surgical resection (56). Furthermore, with regard to the surgically resected colorectal stromal tumors, it was reported that patients with high HDGF expression had shorter disease-free survival than patients with low HDGF levels, and that HDGF was an independent prognostic factor for patients with colorectal stromal tumors (57).

HDGF in malignant diseases of non-gastroenterological tissues

In addition to the gastroenterological cancers, HDGF is thought to be associated with the development and progression of various malignant diseases in non-gastroenterological tissues. HDGF has been shown to function as a mitogenic factor for lung epithelial cells both *in vitro* and *in vivo*, and previous studies have shown the high HDGF expression correlates with the aggressive biological behavior and poor clinical outcomes reported for non-small cell lung cancer (NSCLC) (5, 16, 58-60). Patients with a high expression level of HDGF show a poorer overall and disease-free survival than those with a low HDGF expression level, thus indicating that HDGF

is a significant independent prognostic factor in NSCLC (58, 59). Furthermore, HDGF has been also reported to be involved in the unfavorable clinical features of many other malignant diseases, including malignant melanoma, nasopharyngeal carcinoma, breast cancer and prostate cancer (61-63). Although HDGF was originally identified as a growth factor for hepatoma cells, HDGF also appears to have significant roles in many kinds of malignant disease, including non-gastroenterological cancers.

Conclusion

HDGF is a novel growth factor belonging to a new gene family. It is a unique molecule with multiple malignant characteristics, such as its ability to act as a growth stimulating factor, an angiogenic factor and a possible anti-apoptotic factor. Numerous studies have demonstrated that the overexpression of HDGF correlates with poor outcomes in various types of malignant diseases. HDGF is considered to play significant roles in the proliferation and survival of cancer cells and also in the induction of angiogenesis. HDGF is therefore suggested to be a potential therapeutic target for many malignant diseases.

Acknowledgements

This study was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1 Fernández M, Semela D, Bruix J, Colle I, Pinzani M, Bosch J. J Hepatol. 2009; 50:604-620.

2. Evan GI, Vousden KH. Nature. 2001; 411(6835):342-348.

3. Nakamura H, Kambe H, Egawa T, Kimura Y, Ito H, Hayashi E, Yamamoto H, Sato J, Kishimoto S. Clin Chim Acta. 1989; 183:273-284.

4. Nakamura H, Izumoto Y, Kambe H, Kuroda T, Mori T, Kawamura K, Yamamoto H, Kishimoto T. J Biol Chem. 1994; 269:25143-25149.

5. Nakamura H, Weidong L, Enomoto H. Trends in Cancer Research. 2009; 5:1-11.

6. Enomoto H, Nakamura H, Nishiguchi S. Current Research in Gastroenterology & Hepatology. 2010; 4:79-88.

7. Izumoto Y, Kuroda T, Harada H, Kishimoto T, Nakamura H. Biochem Biophys Res Commun. 1997; 238:26-32.

8. Ikegame K, Yamamoto M, Kishima Y, Enomoto H, Yoshida K, Suemura M, Kishimoto T, Nakamura H. Biochem Biophys Res Commun. 2000; 266:81-87.

9. Dietz F, Franken S, Yoshida K,

Nakamura H, Kappler J, Gieselmann V. Biochem J. 2002; 366(Pt 2):491-500.

10. Ge H, Si Y, Roeder RG. EMBO J. 1998; 17: 6723-6729.

11. Thakar K, Kröcher T, Savant S, Gollnast D, Kelm S, Dietz F. Biol Chem. 2010; 391:1401-1410.

12. Everett AD, Lobe DR, Matsumura ME, Nakamura H, McNamara CA. J Clin Invest 2000; 105: 567-575.

13. Enomoto H, Yoshida K, Kishima Y, Kinoshita T, Yamamoto M, Everett AD, Miyajima A, Nakamura H. Hepatology. 2002; 36:1519-1527.

14. Enomoto H, Yoshida K, Kishima Y, Okuda Y, Nakamura H. J Gastroenterol. 2002; 37 Suppl 14:158-161.

15. Mao J, Xu Z, Fang Y, Wang H, Xu J, Ye J, Zheng S, Zhu Y. Cancer Sci. 2008; 99: 2120-2127.

16. Everett AD, Narron JV, Stoops T, Nakamura H, Tucker A. Am J Physiol Lung Cell Mol Physiol. 2004; 286:L1194-1201.

17. Abouzied MM, El-Tahir HM, Prenner L, Häberlein H, Gieselmann V, Franken S. J Biol Chem. 2005; 280:10945-10954.

18. Kishima Y, Yamamoto H, Izumoto Y, Yoshida K, Enomoto H, Yamamoto M, Kuroda T, Ito H, Yoshizaki K, Nakamura H. J Biol Chem. 2002; 277:10315-10322.

19. Everett AD, Stoops T, McNamara CA. J Biol Chem. 2001; 276:37564-37568.

20. Stec I, Nagl SB, van Ommen GJ, den Dunnen JT. FEBS Lett. 2000; 473:1-5.

21. Qiu C, Sawada K, Zhang X, Cheng X. Nat Struct Biol. 2002; 9:217-224.

22. Nameki N, Tochio N, Koshiba S, Inoue M, Yabuki T, Aoki M, Seki E, Matsuda T, Fujikura Y, Saito M, Ikari M, Watanabe M, Terada T, Shirouzu M, Yoshida M, Hirota H, Tanaka A, Hayashizaki Y, Güntert P, Kigawa T, Yokoyama S. Protein Sci. 2005; 14:756-764.

23. Yang J, Everett AD. BMC Mol Biol. 2007; 8:101.

24. Oliver JA, Al-Awqati Q. J Clin Inves 1998; 102:1208-19.

25. Lepourcelet M, Tou L, Cai L, Sawada J, Lazar AJ, Glickman JN, Williamson JA, Everett AD, Redston M, Fox EA, Nakatani Y, Shivdasani RA. Development. 2005; 132:415-427.

26. Michalopoulos GK. Am J Pathol.

2010; 176:2-13.

27. Fausto N, Campbell JS, Riehle KJ. Hepatology. 2006; 43(2 Suppl 1):S45-53.

28. Enomoto H, Nakamura H, Liu W, Yoshida K, Okuda Y, Imanishi H, Saito M, Shimomura S, Hada T, Nishiguchi S. Hepatol Res. 2009; 39: 988-997.

29. Cilley RE, Zgleszewski SE, Chinoy MR. J Pediatr Surg. 2000; 35:113-118.

30. Mori M, Morishita H, Nakamura H, Matsuoka H, Yoshida K, Kishima Y, Zhou Z, Kida H, Funakoshi T, Goya S, Yoshida M, Kumagai T, Tachibana I, Yamamoto Y, Kawase I, Hayashi S. Am J Respir Cell Mol Biol. 2004; 30:459-469.

31. Okuda, Nakamura H., Yoshida K., Enomoto H., Uyama H., Hirotani T., Funamoto M., Ito H., Everett A.D., Hada T., Kawase I. Cancer Sci. 2004; 94:1034-1041.

32. Lee KH, Choi EY, Kim MK, Lee SH, Jang BI, Kim TN, Kim SW, Kim SW, Song SK, Kim JR, Jung BC., Oncol Res. 2010; 19:67-76.

33. Liu W, Nakamura H, Deng H, Enomoto H., Yamamoto T, Iwata Y, Koh N, Saito M, Imanishi H, Shimomura S, Nishiguchi S, Trends in Cancer Research. 2009; 5: 29-36.

34. Thompson CB. Science. 1995; 267:1456-1462.

35. Matsuyama A, Inoue H, Shibuta K, Tanaka Y, Barnard GF, Sugimachi K, Mori M. Cancer Res. 2001; 61:5714-5717.

36. Clermont F, Gonzalez NS, Communi D, Franken S, Dumont JE, Robaye B. J Cell Biochem. 2008; 104:1161-1171.

37. Machuy N, Thiede B, Rajalingam K, Dimmler C, Thieck O, Meyer TF, Rudel T. Mol Cell Proteomics. 2005; 4:44-55.

38. Zhou Z, Yamamoto Y, Sugai F, Yoshida K, Kishima Y, Sumi H, Nakamura H, Sakoda S. J Biol Chem. 2004 5; 279:27320-6.

39. Marubuchi S, Okuda T, Tagawa K, Enokido Y, Horiuchi D, Shimokawa R, Tamura T, Qi ML, Eishi Y, Watabe K, Shibata M, Nakagawa M, Okazawa H. J Neurochem. 2006 ; 99:70-83.

40. Borders AS, Hersh MA, Getchell ML, van Rooijen N, Cohen DA, Stromberg AJ, Getchell TV. Physiol Genomics. 2007; 31:531-543.

41. Tsang TY, Tang WY, Tsang WP, Co

NN, Kong SK, Kwok TT. Apoptosis. 2008; 13:1135-1147.

42. Liao F, Dong W, Fan L. Med Oncol. 2010; 27:1219-1226.

43. Liao F, Liu M, Lv L, Dong W. Eur J Pharmacol. 2010; 645:55-62.

44. Tsang TY, Tang WY, Tsang WP, Co NN, Kong SK, Kwok TT. Cell Physiol Biochem. 2009; 24: 253-262.

45. Kishima Y, Yoshida K, Enomoto H, Yamamoto M, Kuroda T, Okuda Y, Uyama H, Nakamura H. Hepatogastroenterology. 2002; 49:1639-1644.

46. Soga M, Kishimoto Y, Kawamura Y, Inagaki S, Makino S, Saibara T. Cancer Lett. 2003; 196:43-48.

47. Yoshida K, Nakamura H, Okuda Y, Enomoto H, Kishima Y, Uyama H, Ito H, Hirasawa T, Inagaki S, Kawase I. J Gastroenterol Hepatol. 2003; 18:1293-1301.

48. Yoshida K, Tomita Y, Okuda Y, Yamamoto S, Enomoto H, Uyama H, Ito H, Hoshida Y, Aozasa K, Nagano H, Sakon M, Kawase I, Monden M, Nakamura H. Ann Surg Oncol. 2006; 13:159-167.

49. Hu TH, Huang CC, Liu LF, Lin PR, Liu SY, Chang HW, Changchien CS, Lee CM, Chuang JH, Tai MH. Cancer. 2003; 98:1444-1456.

50. Zhou Y, Zhou N, Fang W, Huo J. Diagn Pathol. 2010; 5: 58

51. Uyama H, Tomita Y, Nakamura H, Nakamori S, Zhang B, Hoshida Y, Enomoto H, Okuda Y, Sakon M, Aozasa K, Kawase I, Hayashi N, Monden M. Clin Cancer Res. 2006; 12(20 Pt 1):6043-6048.

52. Liu YF, Zhao R, Guo S, Wang XQ, Lian PL, Chen YG, Xu KS. Ann Surg Oncol. 2011; 18:872-879.

53. Yamamoto S, Tomita Y, Hoshida Y, Morii E, Yasuda T, Doki Y, Aozasa K, Uyama H, Nakamura H, Monden M. Ann Surg Oncol. 2007; 14:2141-2149.

54. Yamamoto S, Tomita Y, Hoshida Y, Takiguchi S, Fujiwara Y, Yasuda T, Doki Y, Yoshida K, Aozasa K, Nakamura H, Monden M. Clin Cancer Res. 2006; 12:117-122.

55. Nakamura H, Yoshida K, Ikegame K, Kishima Y, Uyama H, Enomoto H. J Gastroenterol. 2002; 37 Suppl 14:8-14.

56. Chang KC, Tai MH, Lin JW, Wang

CC, Huang CC, Hung CH, Chen CH, Lu SN, Lee CM, Changchien CS, Hu TH. Int J Cancer. 2007; 121:1059-1065.

57. Hu TH, Lin JW, Chen HH, Liu LF, Chuah SK, Tai MH. Dis Colon Rectum. 2009; 52:319-326.

58. Ren H, Tang X, Lee JJ, Feng L, Everett AD, Hong WK, Khuri FR, Mao L. J Clin Oncol. 2004; 15:3230-3237.

59. Iwasaki T, Nakagawa K, Nakamura H, Takada Y, Matsui K, Kawahara K. Oncol Rep. 2005; 13:1075-1080.

60. Zhang J, Ren H, Yuan P, Lang W, Zhang L, Mao L. Cancer Res. 2006; 66:18-23.

61. Bernard K, Litman E, Fitzpatrick JL, Shellman YG, Argast G, Polvinen K, Everett AD, Fukasawa K, Norris DA, Ahn NG, Resing KA. Cancer Res. 2003; 63:6716-6725.

62. Wang S, Fang W. Histopathology. 2011; 58:217-224.

63. Guo Z, He Y, Wang S, Zhang A, Zhao P, Gao C, Cao B. Oncol Rep. 2011; 26:511-517.

Curr. Res. in Gastroenterology & Hepatology 4, 2010 (79-88)

Role of Hepatoma-derived Growth Factor (HDGF) in hepatocyte proliferation and differentiation

Hirayuki Enomoto, Hideji Nakamura and Shuhei Nishiguchi

Division of Hepatobiliary and Pancreatic Medicine, Department of Internal Medicine, Hyogo College of Medicine, Mukogawa-cho 1-1, Nishinomiya, Hyogo 663-8501, Japan.

Abstract

Hepatoma-derived growth factor (HDGF) is a novel growth factor, which was identified from the conditioned medium of Huh-7 hepatoma cells. HDGF is a unique growth factor which is both a secreted protein and a nuclear protein. We have investigated the roles of HDGF in hepatocyte growth and differentiation. HDGF is highly expressed in the fetal liver and stimulates the growth of immature hepatocytes, suggesting that HDGF participates in the proliferation of hepatocyte during liver development. In rodent models of liver regeneration, HDGF expression was induced in parenchymal hepatocytes, showing the possible involvement of HDGF in the proliferation of mature hepatocytes. HDGF stimulates the

Correspondence: : Hirayuki Enomoto, Division of Hepatobiliary and Pancreatic Medicine, Department of Internal Medicine, Hyogo College of Medicine, Mukogawa-cho 1-1, Nishinomiya, Hyogo 663-8501, Japan; E-mail:enomoto@hyo-med.ac.jp

proliferation of various hepatoma cells, and HDGF expression in HCC (hepatocellular carcinoma) is closely related to the disease-free and overall survival of patients, indicating that HDGF expression could be a prognostic factor for the disease. Transgenic expression of HDGF in the liver of mice resulted in the partial maturational disturbance of hepatocytes during the post-natal stage, thus suggesting the inhibitory role of HDGF in the hepatocyte differentiation. Our findings have shown that HDGF promotes the proliferation of both normal and malignant hepatocytes In addition, HDGF has a suppressive role in the differentiation of hepatocytes. We conclude that HDGF is a unique growth factor, which has important roles in both hepatocyte proliferation and differentiation.

Introduction

In the normal adult liver, most hepatocytes stay in a quiescent state, and the cell turnover is extremely low. However, hepatocytes actively proliferate under certain situations such as liver development, regeneration and carcinogenesis (1,2). In the developing liver, immature hepatocytes decrease their proliferative activity, acquiring the highly specialized functions of mature hepatocytes. In contrast, malignant hepatic cells acquired unregulated growth capacity, losing the characteristics of mature hepatocytes. Therefore, the proliferative activity of hepatocytes depends on the cellular conditions, and it is usually in opposition to the degree of the cellular differentiation. Clarifying the molecular mechanisms that regulate hepatic cell proliferation and differentiation would provide us with important knowledge for the understanding of liver development, regeneration and carcinogenesis.

We have identified a novel factor, hepatoma-derived growth factor (HDGF) from the human hepatoma-derived cell line Huh-7, which autonomously proliferates in serum-free chemically defined medium (3, 4). We have shown that HDGF participates in liver development as an important growth stimulating factor for fetal hepatocytes (5-7). In addition, we found that the expression of HDGF was induced during liver regeneration, thus suggesting a functional role of HDGF in the proliferation of mature hepatocytes (8). Furthermore, we have shown that HDGF is a growth factor for hepatoma cells, and that HDGF expression is closely associated with the prognosis of patients with HCC (hepatocellular carcinoma) (9). We recently generated a HDGF transgenic mouse and reported that HDGF inhibited hepatocyte differentiation in these mice (10). In this article, we describe the characteristics of this novel growth factor and its possible roles in hepatocyte proliferation and differentiation.

HDGF and its dual characteristics as a secreted protein and a nuclear protein

HDGF is a 26kDa heparin-binding acidic glycoprotein, which we identified from the conditioned medium of the human hepatoma-derived cell line, Huh-7 (3, 4). In addition, several groups including ours have found 4 additional novel genes, HDGF-related proteins (HRPs) (11-13). These proteins (named HRP1- HRP4) share a highly homologous N-terminal amino region consisting of about 100 amino acids, which we call the "HATH (homologous to the amino terminus of HDGF) region". Besides HRP proteins, Lens epithelium-derived growth factor (LEDGF), which was reported to be a survival factor for lens epithelium, contains a HATH region and is regarded as a member of the HDGF family (14). HDGF was originally purified from the conditioned medium of hepatoma cells, however, HDGF mRNA is ubiquitously expressed in adult non-cancerous tissues, and the exogenous administration of HDGF stimulates the proliferation of various types of cells, including fibroblast, vascular smooth muscle cells, as well as several hepatoma cell lines (3-5, 15). These findings suggest that HDGF therefore plays some functional roles in these normal tissues as well as the proliferation of cancer cells.

Although HDGF was originally identified from the conditioned media of cultured cells, the primary sequence of the HDGF protein was lacking the hydrophobic sequence which functioned as a signal peptide. However, previous studies have reported that HDGF can be detected in the conditioned media of various types of cells (3, 4, 15, 16), thus suggesting that HDGF is likely secreted via a process different from the classical secreting pathway of the Golgi system. Recently, amino acid residues 81-100 in the HATH region have been reported as a possible receptor-binding site (17), and we have found the putative receptor of HDGF (Liu et al, in submission). Furthermore, exogenously administration of HDGF protein activated the Erk/MAPK signaling pathway in both in hepatoma cells and endothelial cells (15, 18). These recent findings indicate that HDGF functions via a receptor-mediated signal transduction pathway to act as a growth stimulating factor.

While suggestive receptor-mediated signaling of HDGF has been reported, we noted that the HDGF protein contains 2 putative nuclear localization signals (NLSs) (4, 19). The first NLS (NLS1) resides in the HATH region and the second NLS (NLS2) is in the gene-specific region, and previous studies have shown that HDGF is detected in the nucleus as well as in the cytoplasm, implying that HDGF could have some characteristics of a nuclear protein. We have shown that HDGF can be transported to the nucleus by means of the NLSs, and

that the nuclear translocation is important for the growth stimulating activity of HDGF (19). Regarding the function of HDGF in the nucleus, recent reports have suggested that the HATH region serves as a DNA binding domain. The HATH regions of HDGF family contain a PWWP motif, which was first reported in a candidate gene WHSC1 for Wolf-Hirschhorn syndrome (20, 21). HDGF and HRPs form one of the gene families with PWWP motif, and an NMR analysis has revealed that the PWWP domain of HDGF has a characteristic of hydrophobic cavity, suggesting that HDGF binds to some component of chromatin through this cavity (22). Furthermore, HDGF has been proved to function as a transcriptional repressive factor through the binding to a conserved DNA sequence in the promoter regions of target genes, and the putative DNA binding site is considered to reside in the PWWP domain (23). These findings suggest that the PWWP motif of the HDGF protein acts as a DNA binding domain.

Based on these findings, we consider HDGF to be a unique growth factor that has dual characteristics; thereby acting as a secreted protein and as a nuclear protein.

HDGF in Hepatocyte Proliferation

HDGF and fetal hepatocyte proliferation

Unlike mature hepatocytes in the adult liver, immature hepatocytes in the fetal stage can autonomously proliferate *in vitro*, even in the absence of any growth factors (24, 25). However, the growth regulation of fetal hepatocytes has been poorly understood. During liver development, HDGF was highly expressed in immature fetal hepatocytes, especially in the mid-gestation stage, and its expression was dramatically decreased near birth (6). Using an *in vitro* model of hepatocyte maturation, we demonstrated that HDGF expression in hapatocytes decreased with cellular differentiation, suggesting that HDGF was related to the hepatocyte proliferation. Indeed, exogenous administration of recombinant HDGF stimulated the growth of primary cultured fetal hepatocytes. Furthermore, the reduction of HDGF by an antisense adenovirus suppressed the proliferation of fetal hepatocytes. The growth inhibitory effect of the HDGF antisense virus was reversed by the exogenous administration of recombinant HDGF (6). These findings strongly suggest that HDGF is associated with the proliferation of hepatocytes during liver development.

Several growth factors such as EGF (Epidermal growth factor), HB-EGF (heparin-binding EGF-like growth factor), TGF-α (transforming growth factor-α) and HGF (hepatocyte growth factor) have been shown to play significant roles in hepatocyte proliferation (1, 2). The expression levels of

these factors and their receptor in the fetal stage were lower than those observed in the post-natal stage, thus suggesting that these growth factors and their signals could have more significant roles during the post-natal stage compared with the fetal stage. In contrast, HDGF expression in the developing liver was highly detected during the mid-gestation stage and was markedly decreased with hepatocyte maturation. Despite the normal development of HDGF-null mice, possibly as a result of the functional redundancy that has been considered to exist among HDGF and its related genes (26), HDGF is presumed to participate in liver development as a unique growth stimulating factor for fetal hepatocytes.

HDGF and adult hepatocyte proliferation

Although adult hepatocytes are cells which rarely replicate in their normal state, their proliferative capacity appears in the regenerating liver such as after hepatectomy or drug-induced hepatic injury. Many growth factors have been reported to be involved in the liver regeneration (1, 2). For example, EGF is a paracrine or endocrine growth factor that has growth stimulating effects on primary cultured hepatocyets. TGF-α is a growth factor which is induced in hepatocytes after partial hepatectomy and promotes their growth in an autocrine manner. HB-EGF is a paracrine growth factor which stimulates DNA synthesis of primary cultured rat adult hepatocytes and is induced in non-parenchymal cells during liver regeneration. HGF is the most potent growth stimulating factor for mature hepatocytes and HGF promotes hepatocyte replication by a paracrine or endocrine mechanism. The concentrations of plasma HGF increase within 1hour after partial hepatctomy, and the phosphorylation of the HGF receptor (c-Met) is observed before the DNA synthesis, suggesting an important role of HGF/c-Met system in liver regeneration (27). In addition, other growth factors such as aFGF (28), bFGF (29) and VEGF (30) have been suggested to be involved in liver regeneration, but their roles are still unclear.

Although the expression level of HDGF in non-proliferative hepatocytes of the adult liver is lower than fetal hepatocytes, we considered that HDGF expression could be induced in the proliferative hepatocytes of the regenerating liver. Therefore, we examined the expression patterns of HDGF in two different liver regeneration models (8). In the CCl_4-treated liver, HDGF expression was up-regulated, and a single peak was observed prior to the DNA synthesis peak. HDGF expression was also induced in the hepatectomized liver, and its peak induction was also detected before the peak of DNA synthesis. The HDGF expression in the regenerating liver was

predominantly induced in hepatocytes, but barely increased in non-parenchymal cells. Our findings showed that the HDGF expression to increase in parenchymal hepatocytes before DNA synthesis in the regenerating liver, suggesting that HDGF acts as an autocrine factor promoting liver regeneration. These findings suggest that HDGF plays a major role in liver regeneration, especially in the hepatocyte proliferation.

HDGF and hepatoma cell proliferation

As described above, we have purified HDGF from the conditioned media of Huh-7 hepatoma cells and demonstrated that HDGF participates in the proliferation of both fetal and adult non-transformed hepatocytes. However, malignant transformation is an important event which induces active proliferation of hepatocytes, and our original purpose of finding HDGF was to identify a novel growth factor involved in the proliferation of hepatoma cells. Therefore, we investigated the role of HDGF in the proliferation of hepatic cancer cells.

As expected, HDGF is expressed in various hepatoma cell lines such as Huh-7, HLF and HepG2, and HDGF stimulates the proliferation of these cells. In addition, endogenous overexpression of HDGF significantly increases the cell number and DNA synthesis of hepatoma cells (19), whereas antisense treatment targeting HDGF reduced the cellular proliferation (31). These findings strongly suggested that HDGF has a pivotal role in the proliferation of hepatoma cells. Therefore, we further examined the relationship of HDGF expression to the prognosis of the patients with hepatocellular carcinoma (HCC).

The expression level of HDGF was strongly associated with the prognosis of heptocellular carcinoma and higher expression of HDGF resulted in more unfavorable prognosis (9, 32). In our study, high expression of HDGF was more frequently observed in well-differentiated carcinomas than in poorly-differentiated carcinomas (9). However, another group reported that HDGF was expressed more highly in poorly-differentiated HCC than in well-differentiated HCC (32). Although we cannot fully explain this discrepancy, it may depend on the differences between the anti-HDGF antibodies used in these studies. Despite the inconsistency, both studies reported that the HCC patients with a higher HDGF expression showed an earlier recurrence and a poorer overall survival rate than those with a lower level of expression. Furthermore, both univariate and multivariate analyses revealed a significantly poorer disease-free and overall survival in patients with higher HDGF expression after a resection of HCC (9, 32). These findings suggest that HDGF could be a prognostic

factor for the disease-free and overall survival of patients with HCC.

HDGF in Hepatocyte Differentiation

In the previous study, we used an *in vitro* culture system, which recapitulated the maturational process of hepatocytes ranging from mid-gestation to the newborn stage. Using the culture system, we have demonstrated that HDGF expression is high in immature hepatocytes and markedly decreased with cellular differentiation (6). However, we could not clarify the functional role of HDGF in the hepatocyte differentiation using the *in vitro* model. On the other hand, Lepourcelet et al (33) have reported that overexpression of HDGF showed inhibitory effects on epithelial cellular maturation, suggesting a suppressive role of HDGF in cellular differentiation. Therefore, we generated transgenic mice which overexpressed HDGF in hepatocytes under the transcriptional control of the mouse albumin promoter/enhancer, and investigated the effects on hepatocyte differentiation *in vivo* (10). The HDGF transgenic mice did not have any apparent morphological abnormalities in the liver. However, their gene expression patterns showed the possibility that the maturational process of hepatocytes during the post-natal stage was partially disturbed. In light of these findings, the HDGF expression may therefore be important for sustaining the characteristics of immature cells, and it may also be associated with the high growth activity of cancer cells.

Recently, Lee et al (34) reported that patients with HCC who had a gene expression pattern similar to fetal hepatoblasts showed a poor prognosis. The gene expression pattern was characterized by expression of markers of oval cells (hepato-cholangio progenitor cells), thus suggesting that the HCC of this subtype may originate from immature hepatic progenitor/stem cells. As described above, two groups including our group have shown a high expression of HDGF to be closely associated with a poor prognosis for HCC patients. Although we believe that such a poor prognosis depends on the growth stimulating effects of HDGF on HCC cells, HDGF may promote the proliferation of hepatic progenitor/stem-derived cells and thereby cause a poor prognosis, because HDGF can function as a growth factor for immature fetal hepatocytes. Therefore, clarifying the role of HDGF in hepato-cholangio progenitor cells (like oval cells) should lead to the development of a new therapeutic strategy for HCC.

Conclusion

HDGF is a novel nuclear/growth factor belonging to a new gene family. HDGF not only promotes hepatocyte proliferation

but it also suppresses the differentiation of hepatocytes, thus indicating that HDGF participates in both hepatocyte growth and differentiation. Clarifying the functional role of HDGF will provide further insights into the molecular mechanisms of liver development, regeneration and carcinogenesis.

Acknowledgements

This study was supported in part by grants from the Hyogo Prefecture Health Promotion Association and from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1. Michalopoulos GK, Khan Z. Gastroenterology. 2005; 128:503-6.

2. Fausto N, Campbell JS, Riehle KJ. Hepatology. 2006; 43(2 Suppl 1):S45-53.

3. Nakamura H, Kambe H, Egawa T, Kimura Y, Ito H, Hayashi E, Yamamoto H, Sato J, Kishimoto S. Clin Chim Acta. 1989;183: 273-84.

4. Nakamura H, Izumoto Y, Kambe H, Kuroda T, Mori T, Kawamura K, Yamamoto H, Kishimoto T. J Biol Chem. 1994;269:25143-49.

5. Enomoto H., Nakamura H., Nishiguchi S. Current Research in Hepatology, 2009; 3:27-35.

6. Enomoto H, Yoshida K, Kishima Y, Kinoshita T, Yamamoto M, Everett AD, Miyajima A, Nakamura H. Hepatology. 2002; 36:1519-27.

7. Enomoto H, Yoshida K, Kishima Y, Okuda Y, Nakamura H. J Gastroenterol. 2002; 37 Suppl 14:158-61.

8. Enomoto H, Nakamura H, Liu W, Yoshida K, Okuda Y, Imanishi H, Saito M, Shimomura S, Hada T, Nishiguchi S. Hepatol Res. 2009; 39: 988-97.

9. Yoshida K, Tomita Y, Okuda Y, Yamamoto S, Enomoto H, Uyama H, Ito H, Hoshida Y, Aozasa K, Nagano H, Sakon M, Kawase I, Monden M, Nakamura H. Ann Surg Oncol. 2006; 13: 159-67.

10. Enomoto H, Nakamura H, Komatsu-Kanatani N, Liu Y, Yoshida K, Okuda Y, Yamamoto T, Liu W, Nishiguchi S. World J Hepatol. 2009; 1: 98-102.

11. Izumoto Y, Kuroda T, Harada H, Kishimoto T, Nakamura H. Biochem Biophys Res Commun. 1997; 238:26-32.

12. Ikegame K, Yamamoto M, Kishima Y,

Enomoto H, Yoshida K, Suemura M, Kishimoto T, Nakamura H. Biochem Biophys Res Commun. 2000; 266:81-7.

13. Dietz F, Franken S, Yoshida K, Nakamura H, Kappler J, Gieselmann V. Biochem J. 2002; 366(Pt 2):491-500.

14. Ge H, Si Y, Roeder RG. EMBO J. 1998; 17: 6723-9.

15. Everett AD, Narron JV, Stoops T, Nakamura H, Tucker A. Am J Physiol Lung Cell Mol Physiol. 2004; 286:L1194-1201.

16. Zhou Z, Yamamoto Y, Sugai F, Yoshida K, Kishima Y, Sumi H, Nakamura H, Sakoda S. J Biol Chem. 2004;279:27320-6.

17. Abouzied MM, El-Tahir HM, Prenner L, Häberlein H, Gieselmann V, Franken S.J Biol Chem. 2005; 280:10945-54.

18. Mao J, Xu Z, Fang Y, Wang H, Xu J, Ye J, Zheng S, Zhu Y. Cancer Sci. 2008; 99: 2120-7.

19. Kishima Y, Yamamoto H, Izumoto Y, Yoshida K, Enomoto H, Yamamoto M, Kuroda T, Ito H, Yoshizaki K, Nakamura H. J Biol Chem. 2002; 277:10315-22.

20. Stec I, Nagl SB, van Ommen GJ, den Dunnen JT. FEBS Lett. 2000; 473:1-5.

21. Qiu C, Sawada K, Zhang X, Cheng X. Nat Struct Biol. 2002; 9:217-24

22. Nameki N, Tochio N, Koshiba S, Inoue M, Yabuki T, Aoki M, Seki E, Matsuda T, Fujikura Y, Saito M, Ikari M, Watanabe M, Terada T, Shirouzu M, Yoshida M, Hirota H, Tanaka A, Hayashizaki Y, Güntert P, Kigawa T, Yokoyama S. Protein Sci. 2005; 14:756-64.

23. Yang J, Everett AD. BMC Mol Biol. 2007; 8:101.

24. Curran TR, Bahner RI, Oh W, Gruppuso PA. Exp Cell Res. 1993; 209: 53-7.

25. Nakamura T, Fujii T, Ichihara A. J Biochem. 1988; 103: 700-6.

26. Gallitzendoerfer R, Abouzied MM, Hartmann D, Dobrowolski R, Gieselmann V, Franken S. Dev Dyn. 2008; 237: 1875-85.

27. Stolz DB, Mars WM, Petersen BE,

Kim TH, Michalopoulos GK. Cancer Res. 1999; 59: 3954-60.

28. Marsden ER, Hu Z, Fujio K, Nakatsukasa H, Thorgeirsson SS, Evarts RP. Lab Invest. 1992; 67: 427-33.

29. Baruch Y, Shoshany G, Neufeld G, Enat R. J Hepatol. 1995; 23: 328332.

30. Mochida S, Ishikawa K, Inao M, Shibuya M, Fujiwara K. Biochem Biophys Res Commun. 1996; 226:176-9.

31. Kishima Y, Yoshida K, Enomoto H, Yamamoto M, Kuroda T, Okuda Y, Uyama H, Nakamura H. Hepatogastroenterology. 2002; 49: 1639-44.

32. Hu TH, Huang CC, Liu LF, Lin PR, Liu SY, Chang HW, Changchien CS, Lee CM, Chuang JH, Tai MH. Cancer. 2003; 98: 1444-56.

33. Lepourcelet M, Tou L, Cai L, Sawada J, Lazar AJ, Glickman JN, Williamson JA, Everett AD, Redston M, Fox EA, Nakatani Y, Shivdasani RA. Development. 2005; 132: 415-27.

34. Lee JS, Heo J, Libbrecht L, Chu IS, Kaposi-Novak P, Calvisi DF, Mikaelyan A, Roberts LR, Demetris AJ, Sun Z, Nevens F, Roskams T, Thorgeirsson SS. Nat Med. 2006; 12: 410-6.

J Gastroenterol 2009; 44:228–235
DOI 10.1007/s00535-008-2304-4

Journal of Gastroenterology

Involvement of hepatoma-derived growth factor in the growth inhibition of hepatocellular carcinoma cells by vitamin K_2

Teruhisa Yamamoto, Hideji Nakamura, Weidong Liu, Ke Cao, Shohei Yoshikawa, Hirayuki Enomoto, Yoshinori Iwata, Noritoshi Koh, Masaki Saito, Hiroyasu Imanishi, Soji Shimomura, Hiroko Iijima, Toshikazu Hada, and Shuhei Nishiguchi

Division of Hepatobiliary and Pancreatic Medicine, Department of Internal Medicine, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan

Background. Vitamin K_2 has been reported to suppress the growth of human hepatocellular carcinoma (HCC) in vitro and hepatocarcinogenesis in hepatitis C virus (HCV)-related cirrhosis in vivo. Hepatoma-derived growth factor (HDGF) is a unique nuclear targeting growth factor that is highly expressed in HCC cells and is a possible prognostic factor for patients with HCC. We investigated the regulation of HDGF expression by vitamin K_2. ***Methods.*** Three HCC-derived cell lines, HepG2, HuH-7, and SK-Hep-1, were used. Cell number was determined with the MTT assay. The expression levels of HDGF mRNA and protein were measured by the real-time reverse transcriptase-polymerase chain reaction (PCR) method and ELISA and Western blot analysis, respectively. The HDGF promoter activity was measured by a dual luciferase-reporter assay. ***Results.*** Vitamin K_2 suppressed the growth of the three HCC cell lines in a dose-dependent manner. Vitamin K_2 significantly suppressed the expression of the HDGF protein and mRNA in three cell lines. By a luciferase assay, vitamin K_2 significantly suppressed the promoter activity of the HDGF protein. Based on some luciferase-reporter plasmids containing truncated promoter regions, the possible responsive site of vitamin K_2 seems to reside in the region –1 to –150 bp of the HDGF gene. ***Conclusions.*** These findings suggested that regulation of the HDGF gene expression is one of the crucial mechanisms of vitamin K_2-induced cell growth suppression for HCC.

Key words: HDGF, HCC, vitamin K_2, luciferase assay

Introduction

Hepatoma-derived growth factor (HDGF) is a unique nuclear targeting growth factor with heparin affinity that was purified and cloned from a human hepatocellular carcinoma (HCC) cell line.[1–4] HDGF has both oncogenic and angiogenic activity.[5,6] HDGF stimulates the proliferation of HCC cells, in addition to fibroblasts, endothelial cells, vascular smooth muscle cells, and fetal hepatocytes, after translocation to the nucleus by use of the bipartite nuclear localization signals.[1–9] HDGF is highly expressed in several cancers including HCC and is closely related to the aggressive biological potential of cancer cells.[10–16] A downregulation of HDGF by antisense oligonucleotides or siRNA treatment suppresses the proliferation of cancer cells that express HDGF endogenously.[14,17] Recently, a significant correlation has been shown between HDGF expression and the prognosis for the recurrence-free and overall survival in patients with HCC.[18,19] HDGF is considered to play an important role in both hepatocarcinogenesis and cancer progression. If HDGF expression is suppressed by drugs or chemical agents, then the growth of HCC cells should be regulated efficiently. However, the regulation mechanism of HDGF expression has not yet been clarified.

Vitamin K, an essential hydrophobic vitamin, and its derivatives have been shown to inhibit the proliferation of cancer cells including HCC.[20–23] However, the precise mechanism of their growth inhibitory action has not yet been clarified. Vitamin K consists of different forms, vitamin K_1–K_5. Vitamin K_2 (menaquinone) is produced by the intestinal flora and is used as an oral medication for patients with osteoporosis. The in vivo preventive effect of vitamin K_2 on the development of HCC, or the recurrence after treatment of HCC, in patients with HCV-related cirrhosis has been reported.[24,25] Recent in vitro studies have advocated some molecular mechanisms for the growth inhibition by vitamin K_2: a pathway

Received: February 21, 2008 / Accepted: September 27, 2008
Reprint requests to: H. Nakamura

via protein kinase A activation, induction of the cell cycle-regulating proteins including p21, and reduced expression of the cyclin-dependent kinases.[26–29] However, these mechanisms cannot explain the entire suppressive effects of vitamin K_2 on the proliferation of HCC cells. Other unknown mechanisms have been suggested for the cell proliferation inhibitory effects of vitamin K. The transcriptional regulation of the growth factor genes or growth factor receptor genes by vitamin K_2 has not yet been reported.

In the present study, we investigated the regulation of HDGF expression by vitamin K_2 in HCC cells.

Materials and methods

Materials

Vitamin K_2 (menatetrenone, MK-4) was supplied from Eisai Co. (Tokyo, Japan). The human HCC cell lines HepG2, HuH-7, and SK-Hep-1 were purchased from American Type Culture Collection (ATCC). These cell lines were cultured in Dulbecco's modified Eagle's essential medium (DMEM; Gibco BRL, Grand Island, NY, USA) with 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 µg/ml) at 37°C in a humidified incubator with 5% CO_2.

Cell proliferation assays

The cells were seeded onto 96-well plates at a density of 2.5×10^3 cells. After a 24-h culture, 100 µl fresh medium containing different concentrations of vitamin K_2 (10, 30, and 100 µM) was added in each well. Vitamin K_2 was dissolved in 99% ethanol at the concentration of 10 mM and then diluted with DMEM to the appropriate concentrations for the experiments. Forty-eight hours later, the culture medium was replaced with fresh medium containing different concentrations of vitamin K_2. The control cells were cultured in DMEM containing the corresponding concentration of ethanol to each dose of the vitamin K_2. After a 4-day culture with vitamin K_2 treatment, the number of viable cells in each well was determined with the 3-(4,5-dimethyl-2-thiazolyl-2,5-diophenyl-2*H*-tetrazolium bromide) (MTT) assay (Roche, Nutley, NJ, USA) according to the manufacturer's instructions.

All experiments were carried out in four wells concurrently, and then were repeated three times.

Western blotting

After a 96-h culture with vitamin K_2, the cells were washed twice with ice-cold phosphate-buffered saline (PBS), lysed, and sonicated in RIPA buffer [1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 100 µg/ml phenylmethylsulfonyl fluoride, 45 µg/ml aprotinin, 100 mM sodium orthovanadate]. The supernatant of the homogenate was used for protein determination with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA) and electrophoresis. The samples with 5 µg total protein were electrophoresed on a 12.5% SDS-polyacrylamide gel under reducing conditions and blotted to a polyvinylidene difluoride (PVDF) membrane by electroblotting. The membranes were blotted with the anti-C terminus of the HDGF polyclonal antibody at a dilution of 1:10000, which was generated by the New Zealand White rabbit.[3] The signals were developed with an ABC kit (Vector, Burlingame, CA, USA) and diaminobenzidine.

HDGF-overexpressing HepG2 cells

We constructed *myc*-tagged human HDGF in pEF-BOS plasmids and selected and cloned stable transfectants after transfection to HepG2 as described previously.[3]

HDGF-knock-down SK-Hep-1 cells by shRNA

SuperSilencing shRNA plasmid for human HDGF was purchased from SuperArray Bioscience Corporation (catalog number: KH10419N). SK-Hep-1 was seeded at 1×10^5 cells in 6-well plates with 2 ml 10% FBS-DMEM medium. Next day, 2 µg HDGF-shRNA and the negative control shRNA plasmids were transfected into the cells by 5.0 µl Lipofectamine 2000, according to the protocol from SuperArray. The transfected medium was changed by 10% FBS-DMEM with Geneticin (G418), 1200 mg/l, after 24 h. The G418 media were changed every 3 days. The knock-down expression of HDGF protein was confirmed by Western blot in SK-Hep-1 cells selected by G418 media.

A quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of HDGF mRNA levels

HDGF mRNA expression was measured by a quantitative real-time PCR according to the method previously reported.[18] In brief, the total RNA was extracted with the AGPC method using Isogen (Nippongene, Tokyo, Japan); 5 µg deoxyribonuclease I-treated total RNA was used for the reverse transcriptase reaction. An aliquot representing 100 ng input RNA was amplified by using a TaqMan PCR Reagent Kit (Applied Biosystems, Foster City, CA, USA) with the ABI PRISM 7700 sequence Detection System (Applied Biosystems) as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s, and 60°C for 1 min. The forward primer 5′-AAGTTTGGCAAGCCCAACA-3′, reverse primer 5′-GGCTCTTCCACACAGCTCTTT-3′, and probe

5′-FAM-AACCCTACTGTCAAGGCTTCCGGCT-TAMRA-3′ were used for HDGF. As an internal control, beta-actin mRNA was used. The RNA extracted from HuH-7 cells was used as a standard. After reverse transcription (RT), standard complementary DNA (cDNA) was serially diluted to obtain five standard solutions for use in the PCR reaction to generate the reference curve. The relative amount of cDNA in each sample was measured by the interpolation of the standard curve, and then the relative ratio of the HDGF/beta-actin expression was calculated for each sample.

Luciferase assay of HDGF promoter activity

Constructs of luciferase-reporter plasmids of the HDGF promoter region. The DNA from the HuH-7 cells was extracted by the Isogen method. Thereafter, the DNA was digested by Tth111 I, purified by phenol/chloroform, and precipitated by ethanol. The HDGF promoter DNA was acquired by a nested PCR. First, 0.5 μg digested and purified DNA was amplified by the forward primer HDGF P5F5 (5′-TACGACATCAGGAGTTCG AAACCA-3′) and the reverse primer HDGF P3R (5′-TGCGCGCTCGTCGAGTTGTTTGT-3′) using a LA TAKARA Taq Kit (RR002A) (Takara, Kyoto, Japan). This PCR product was used as the template of the second amplification. The second PCR was done by series primer pairs designed by DNASIS software using a TAKARA Taq Kit. The DNA amplification was performed in the condition of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, for 35 cycles.

Plasmid constructs

The PCR products were purified, polished, and inserted into the pGL3 Basic luciferase-reporter vector predigested by *Sma*I, and transfected into *Escherichia coli* by a PCR Cloning Kit (Stratagene, La Jolla, CA, USA), according to the manufacturer's instructions. The isolated plasmids containing the desired HDGF promoters were verified by *Kpn*I plus *Xho*I digestions and sequenced using the RV Primer 3 from the 5′-end and the GL Primer 2 from the 3′-end.

Luciferase assay of luciferase-reporter plasmids

The HepG2 cells (2×10^5 cells/well) were seeded in a 6-well culture dish (Iwaki, Funabashi, Chiba, Japan) in phenol red-free DMEM containing 5% charcoal-dextran-stripped fetal bovine serum (FBS-CCS). The cells were transfected with 2 μg luciferase-reporter vectors by using a Fugene 6 transfection reagent kit (Roche), according to the manufacturer's recommendations. Twenty-four hours later, the culture media were changed to the fresh media with several concentrations (0, 10, 30, and 100 μM) of vitamin K_2. After incubation for 24 h, the cells were harvested and lysed with luciferase lysis buffer (Promega, Madison, WI, USA). The proteins were measured by a BCA protein assay kit. The luciferase activity of each sample was measured by a luciferase assay kit (Promega). The level of induction was calculated by dividing the mean luciferase activity of the samples treated with vitamin K_2 by the mean activity of the untreated control samples. All experiments were carried out in triplicate and repeated at least three times.

Enzyme-linked immunosorbent assay (ELISA) of HDGF protein

The cells were lysed with the RIPA buffer, as described above. After centrifugation at 10000 rpm for 30 min, the supernatants of the cell lysate were used for the measurement of the HDGF protein by an ELISA. An ELISA for the HDGF was developed by the sandwich method using a monoclonal antibody and a polyclonal antibody against HDGF.

Statistical analysis

The results are expressed as the means ± SE. At least three separate experiments were performed for each data point. The statistical analyses were done using Student's unpaired *t* test (two tailed).

Results

Effect of vitamin K_2 on HCC cells

In patients whom vitamin K_2 are administered at clinically used doses, the serum concentration is calculated to reach to about 30 μM. Thus, we used three doses—10, 30, and 100 μM—of vitamin K_2 in the subsequent experiments. Vitamin K_2 suppressed the proliferation of three HCC cell lines in a dose-dependent manner. The inhibitory effects by vitamin K_2 after 96 h treatment at 30 μM and 100 μM are shown for the three HCC cell lines in Fig. 1.

HDGF was expressed in three cell lines (Fig. 2a). The intracellular HDGF amounts in these cell lines were measured by an ELISA (Fig. 2b). HCC cells with higher production of the HDGF protein seem to show higher inhibition of HCC cell proliferation by vitamin K_2, although not significantly. Some growth factors are involved in the proliferation of HCC cells. Next, we knocked down the HDGF expression and assessed its participating level on the proliferation of HCC cells. We obtained two stable HDGF-knock-down clones after transfection of HDGF-shRNA into SK-Hep-1 cells. In