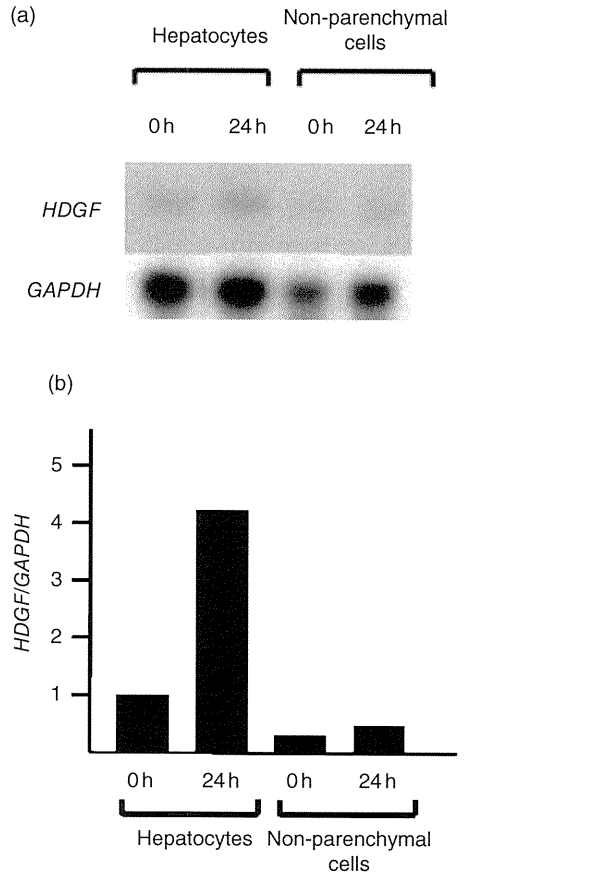

Figure 3 Hepatoma-derived growth factor (*HDGF*) was expressed in parenchymal cells in carbon tetrachloride (CCl_4)-treated liver. (a,b) *HDGF* expression in parenchymal and non-parenchymal cells. Parenchymal hepatocytes and non-parenchymal liver cells were isolated by *in situ* perfusion method. RNA was extracted from livers at 0 (no treatment) and 24 h after the CCl_4 treatment. (a) 7 μg of total RNA was loaded and hybridized with mouse cDNA of *HDGF* and hybridization with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. (b) *HDGF/GAPDH* ratio was shown. (c) Immunohistochemical analysis. Liver tissues were removed from mice at 0 h (no treatment, left) and 24 h (right) after the injection of CCl_4. Expression of HDGF protein was analyzed by immunohistochemical techniques. Bar, 200 μm.

CCl_4, expression of TGF-α showed two peaks at 12 h and 48 h,[29] and expression of HB-EGF peaks at 6 h and 36 h.[32] Although these expression patterns in previous studies were not completely the same as our data in Figure 4, the present results showed similar patterns as those observed in the rat models. Because the cell cycle period of mouse hepatocytes in regenerating livers is different from that of rat hepatocytes,[39] the difference of gene expression patterns could exist between mice and rats. Therefore, our experimental systems should not contradict the previous studies of rat models.

Although the immunohistochemical examinations of TGF-α and HB-EGF expression during drug-induced liver regeneration in mice have not been reported, several studies reported the expression patterns of TGF-α and HB-EGF in rat hepatectomized models.[40,41] According to these reports, the localization signals of TGF-α and EB-EGF shifted during the liver regeneration process after partial hepatectomy.

TGF-α was expressed in the cytoplasm of hepatocytes earlier in the study (at 6–12 h after hepatectomy) and the localization of TGF-α shifted to the nucleus of parenchymal cells 24–48 h after hepatectomy.[40] Because the liver regenerating process after CCl_4 treatment takes longer than partial hepatectomy,[39] our data in Figure 5 could be consistent with the data previously reported. HB-EGF expression in hepatectomized rat livers was induced mainly in non-parenchymal cells.[41] The expression pattern of HB-EGF protein in the present study was not completely equal to the previous report, because HB-EGF signals were detected in parenchymal hepatocytes as well as in non-parenchymal liver cells. Although we cannot fully elucidate the reason of the discrepancy, it could depend on the difference of animal models (partial hepatectomy of rats and CCl_4 treatment of mice) and/or the sensitivity and specificity of the antibodies used in different studies. In addition, we cannot completely deny the possibility that the HB-EGF antibody, which we commercially obtained and used in the present study, might be a little cross-reactive and detect the membrane-anchored precursor protein of HB-EGF (proHB-EGF).

As shown in Figures 3 and 5, the expression patterns of HDGF, TGF-α and HB-EGF after CCl_4 injection were markedly different from each other. We have shown that the HDGF sequence has two putative nuclear localization signals (NLS), and NLS sequences are essential for the growth promoting activity of HDGF, indicating that HDGF has a characteristic of the nuclear/growth factor. This point might be responsible for the unique expression pattern of HDGF, which is different from those of TGF-α and HB-EGF (Figs 1,4).

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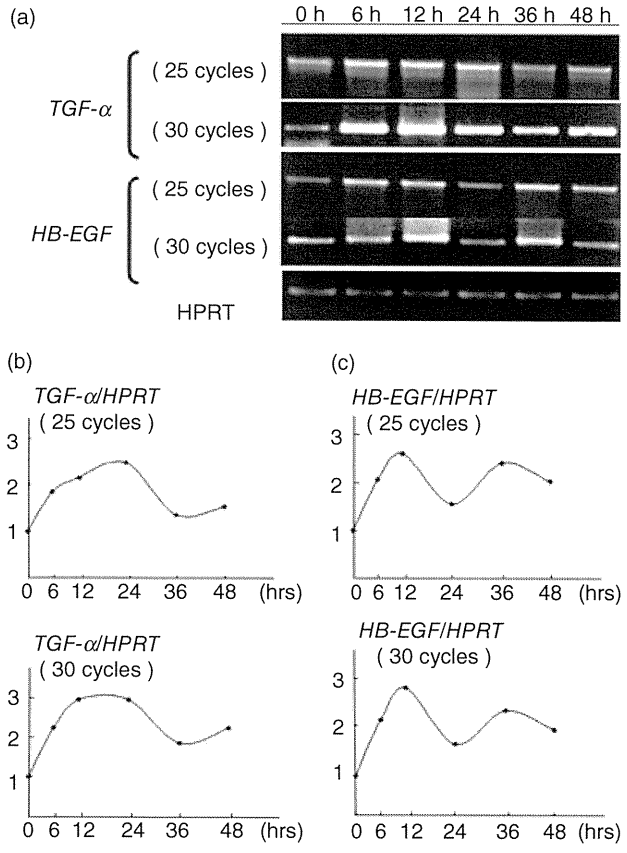

Figure 4 Expression of transforming growth factor-α (*TGF-α*) and heparin-binding epidermal growth factor-like growth factor (*HB-EGF*) mRNA during mouse liver regeneration induced by carbon tetrachloride (CCl_4). (a) Total RNA was isolated 0 (no treatment), 6, 12, 24, 36, 48 h after the injection. Expression patterns of *TGF-α* and *HB-EGF* were examined by RT–PCR as described in "Materials and Methods", and two different (25 and 30) cycles of PCR reactions were carried out. (b) The ratios of PCR band intensities (*TGF-α*/hypoxanthine guanine phosphoribosyl transferase [*HPRT*]) were measured, and the time courses of the ratio were graphically shown. (c) The ratios of PCR band intensities (*HB-EGF*/*HPRT*) were measured, and the time courses of the ratio were graphically shown.

The present study showed that HDGF was induced in two different models of mouse liver regeneration (after partial hepatectomy and CCl_4-induced liver damage). In both models, the peak HDGF expression was induced before the peak of DNA synthesis, and HDGF was detected dominantly in parenchymal cells. Genes, such as *HDGF* whose expression peak was 12–24 h earlier than the DNA synthesis peak, were reported to be involved in hepatocyte proliferation and to play important roles in liver regeneration.[24] Previously we showed that HDGF enhanced the proliferation of hepatoma-derived cell lines *in vitro*.[1,5] In addition, endogenous overexpression of HDGF in the hepatoblastoma cell line, HepG2, significantly stimulated their cellular growth.[27] HDGF also stimulated the proliferation of the hepatocytes isolated from newborn livers as well as fetal hepatocytes, although weakly.[21] Furthermore, HDGF has been shown to be expressed in proliferating cells characterized by the expression of PCNA or BrdU uptake by some authors, including the authors of the present study.[4,12] Although HDGF was expressed both in parenchymal and non-parenchymal liver cells, HDGF induction was observed mainly in the hepatocytes and its maximum expression occurred before the peak of DNA synthesis in the regenerating liver. The present findings suggest that HDGF should be actively induced in hepatocytes when liver volume is reduced, and then be involved in hepatocyte proliferation in liver regeneration as an autocrine factor. Thus, HDGF might play some significant roles in the proliferation of hepatocytes in liver regeneration as an autocrine factor.

Studies have shown that several molecules, which are involved in liver regeneration, also play important roles in liver development.[42–45] For example, the targeted disruption of HGF, which is one of the key molecules in liver regeneration, resulted in severe hypoplasia of the liver as a result of the growth disturbance of fetal hepatocytes.[42,43] One of the earliest response genes in liver regeneration, c-jun, was shown to be essential for hepatogenesis.[44] The NF-kB signal is one of the major pathways involved in liver regeneration and inactivation of NF-kB led to the hepatogenesis disorder.[45]

Previous studies have suggested that HDGF has important roles in organ development,[3,4,21,46] although HDGF-deficient mice normally developed, perhaps because of the redundancy among HDGF and its related genes.[47] HDGF is involved in glomerular capillary formation during renal development[3] and also in the development of lungs[46] and the cardiovascular system[4] as an angiogenic factor. We have reported that HDGF is highly expressed in fetal hepatocytes and promotes their proliferation, suggesting the important role of HDGF in liver growth.[21] Taken together, the early induction of HDGF in liver regeneration suggests that HDGF is a new member of molecules that are involved in both liver development and regeneration.

Besides growth factors, various kinds of cytokines take part in the process of liver regeneration. Among the cytokines, IL-6 is a key molecule in liver regeneration. IL-6 knockout mice show the severe retardation of liver

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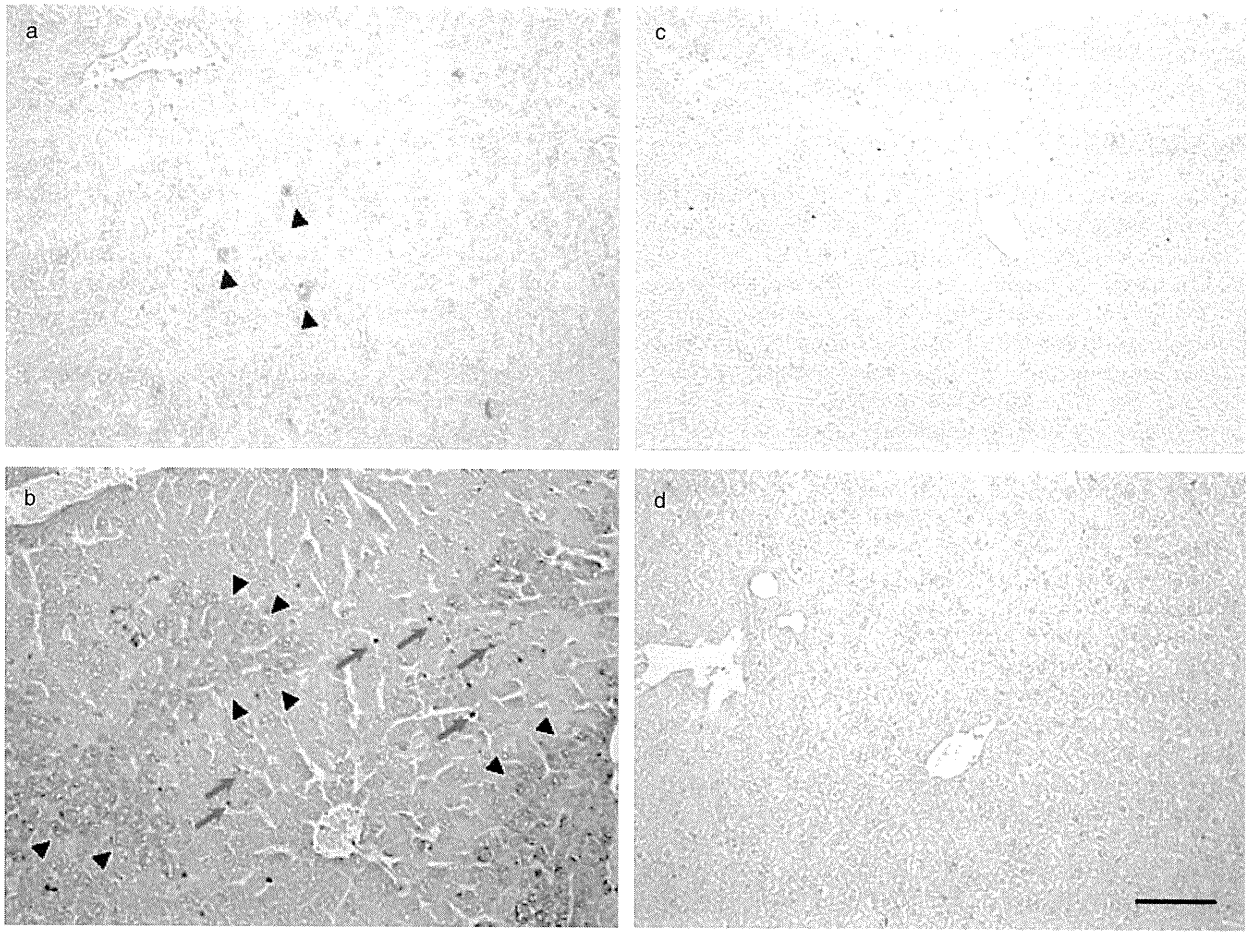

Figure 5 Immunohistochemistry of the transforming growth factor-α (TGF-α) and heparin-binding epidermal growth factor-like growth factor (HB-EGF) expression in carbon tetrachloride (CCl_4)-treated mouse liver. (a,c) Liver tissues were removed from mice at 0 h (no treatment; C) and 24 h after the injection of CCl_4 (a). Expression of TGF-α protein was analyzed using immunohistochemical techniques. TGF-α expression was detected in the cytoplasm of hepatocytes at 24 h after CCl_4 was given (a: arrowheads), whereas no signal was detected in the control liver (c). (b,d) Liver tissues removed from mice at 0 h (no treatment; d) and 24 h after the injection of CCl_4 (b) were also used for the detection of HB-EGF protein. HB-EGF expression was observed in peripheral hepatocytes (b: arrowheads) and non-parenchymal cells (b: arrows) in the liver tissues 24 h after CCl_4 was given. However, no signal was detected in the control liver (d). Bar, 200 μm.

regeneration, and IL-6 is regarded as a priming factor in liver regeneration.[48] Within the scope of the imagination, IL-6 might be related to the HDGF induction, because the IL-6 responsive DNA element resides in the promoter region of HDGF ([49] and data not shown). The regulatory mechanism(s) of HDGF induction is important and should be clarified.

In summary, we showed that HDGF was induced predominantly in the hepatocytes before the induction of DNA synthesis in the two models of liver regeneration. HDGF might be involved in liver regeneration as one of the autocrine factors. The present findings suggest that HDGF participates in human liver regeneration after drug-induced liver damage and surgical resection. HDGF might be a candidate for the protection and the enhancement of the recovery from liver damage as a result of surgical resection, viral infection or drug intoxicity.

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Involvement of Hepatoma-derived Growth Factor in the growth of hepatocytes during liver development and regeneration

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Abstract

Hepatoma-derived growth factor (HDGF) is a novel growth factor, which is purified from the conditioned media of Huh-7 hepatoma cells by the growth stimulating activity for Swiss 3T3 fibroblasts. HDGF is ubiquitously expressed in normal tissues, including the testes, kidney, heart, brain, and liver. HDGF promotes the proliferation of endothelial cells, vascular smooth muscle cells, some hepatoma cells as well as fibroblasts. Recent studies have suggested that HDGF plays an important role in fetal organ development and adult tissue repair. HDGF is highly expressed in fetal liver and stimulates the growth of immature hepatocytes. HDGF expression is induced in the regenerating liver both after partial hepatectomy and toxic liver damage. Furthermore, the expression patterns of HDGF in liver development and regeneration

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were different from those of other growth factors such as TGF- α (transforming growth factor-alpha), HB-EGF (heparin-binding EGF-like growth factor) and HGF (hepatocyte growth factor). Our findings suggest that HDGF is a unique growth factor, which plays a role in liver development and regeneration through the growth stimulation of fetal and adult hepatocytes.

Introduction

In order to resolve several clinical disorders in the liver, it is important to define the regulation of hepatocyte proliferation. Although most hepatocytes in the adult liver usually stay in the quiescent state and do not replicate, hepatic cells become proliferative during some situations such as liver development, regeneration and tumorigenesis (1-3).

From the fetus through to the newborn stage, immature hepatocytes differentiate throughout liver development and gain many functions specific to mature hepatocytes with decreased DNA synthesis and proliferative activity. During liver regeneration and carcinogenesis, mature hepatocytes often reduce their metabolic functions and restore their high growth activity, displaying the phenotypes similar to fetal hepatocytes (4). Therefore, an analysis of the fetal hepatocyte proliferation should help to gain new insights into the treatment and management of adult liver diseases, including acute/chronic hepatic dysfunction and hepatocellular carcinoma. Indeed, recent studies have demonstrated that several factors, which have been supposed to be important in liver regeneration and hepatocarcinogenesis, also play pivotal roles in hepatogenesis (5-8).

We have identified and cloned 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 (9). Previously, we showed that HDGF was involved in liver development through the growth stimulating effects of fetal hepatocytes (10). Recently, we found the expression of HDGF to be induced in liver regeneration, thus suggesting the functional role of HDGF in liver regeneration (11). In this article, we review the characteristics of this growth factor and its possible involvement in liver development and regeneration.

HDGF and HDGF related proteins

HDGF is a 26kDa heparin-binding acidic glycoprotein consisting of 240 amino acids. This growth factor was identified from the conditioned media of Huh-7 hepatoma cells. In addition to tumor cell lines, HDGF mRNA is ubiquitously expressed in various

adult normal tissues, including testis, kidney, liver, heart, brain, and skeletal muscle (9). These findings suggest that HDGF is not only related to the proliferation of cancer cells but also has some functional roles in these normal tissues and organs.

Although HDGF is originally purified from the conditioned media of cultured cells, the primary sequence of HDGF protein contains no N-terminal hydrophobic sequence functioning as a signal peptide. However, HDGF is detected in the conditioned media of several other types of cells, and HDGF is thought to be secreted by means of a different process from the classical secreting pathway of the Golgi system. On the other hand, we and others have reported that HDGF contains 2 putative nuclear localization signals (NLSs), and can be transported to the nucleus (12, 13). Immunohistochemical studies have revealed that HDGF is localized in the nucleus, suggesting that HDGF has the characteristics of a nuclear/growth factor.

Recently, other groups as well as ours have found 4 additional novel genes, HDGF- related proteins (HRPs) (14-16). These proteins shared a highly homologous amino terminal region consisting of about 100 amino acids, which we call HATH (homologous to the amino terminus of HDGF) region. Lens epithelium-derived growth factor (LEDGF), which was recently reported to function as a survival factor for lens epithelium, contains a HATH region in its N-terminal region and appears to be a member of HDGF family (17). It is thought that HDGF and HRPs form a new gene family. HDGF family proteins are characterized as follows; 1) contain HATH region in the N-terminal region, 2) have nuclear localization signals in the HATH region and remaining parts of the sequence other than HATH region, and 3) can be transported to the nucleus.

Dual mechanisms in the growth stimulating effects of HDGF

HDGF stimulates the growth of fibroblasts, endothelial cells, pulmonary epithelial cells, vascular smooth muscle cells, and hepatocytes as well as various cancer cells (9, 10, 18, 19). We have shown that the nuclear translocation is essential for the mitogenic activity of HDGF (12). As described above, HDGF has two NLS regions; the first NLS (NLS1) resided in the HATH region and the second NLS (NLS2) in the gene-specific regions. HDGF can be translocated to the nucleus using these NLSs, specially the NLS2 in the gene specific region.

Although it has not been fully elucidated how HDGF stimulates cellular growth after nuclear translocation, recent studies suggest the functional roles of the

HATH region. The HATH regions of HDGF family contain PWWP motif (20, 21), which has been first reported in a candidate gene WHSC1 for Wolf-Hirschhorn syndrome. An NMR analysis has shown that the PWWP domain of HDGF exhibits a characteristic hydrophobic cavity, suggesting that HDGF binds to some component of chromatin through this cavity (22). Furthermore, it has been reported that HDGF functions as a transcriptional repressive molecule 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 the functional role of the PWWP motif as a DNA binding domain of HDGF protein.

On the other hand, HDGF is first purified from the conditioned medium of hepatoma cells, and the exogenous administration of HDGF stimulates the proliferation of cells, including fibroblasts, endothelial cells, fetal hepatocytes and cancer cells (9, 10). Recently, amino acid residues 81-100 in the HATH region are reported as a possible receptor-binding site (24), and we have identified the putative receptor of HDGF (Liu et al, in submission). Moreover, exogenously supplied HDGF stimulates the phosphorylation of Erk/MAPK in endothelial cells and hepatoma cells (25). These findings indicate that the growth promoting effects of HDGF should depend on the receptor-mediated signal transduction pathway, at least partially.

Therefore, HDGF should have two different mechanisms of the growth stimulation: namely, one is a receptor-mediated pathway followed by the MAPK activation, and the other is a direct action through the DNA binding after nuclear translocation. This being the case, HDGF is a unique growth factor which functions by means of those dual pathways.

HDGF in organ development and tissue repair

HDGF is expressed at higher levels in fetal tissues than in adult tissues (10, 18, 26). Previous studies have suggested the important roles of HDGF in fetal organ development and adult tissue repair, although an HDGF-deficient mouse develops normally, possibly as a result of a redundant function which has been reported to be present among the HDGF family genes (27).

Oliver et al. purified an endothelial growth factor from the conditioned media of a rat metanephrogenic mesenchymal cell line, and this purified growth factor was identical to HDGF. Based on the expression pattern of HDGF in the developing kidney, they reported that HDGF should have an important role on glomerular capillary formation in nephron morphogenesis (26).

Everett et al. demonstrated that HDGF was strongly expressed in proliferating vascular smooth muscle cells (SMCs) and endothelial cells in fetus (18). They also indicated that HDGF expression was induced in vascular SMCs proximal to abdominal aorta constriction and in neointimal cells after endothelial injury. Exogenous HDGF and endogenous overexpression of HDGF stimulated the growth of vascular SMCs. These findings suggest that HDGF may regulate vascular SMC proliferation during cardiovascular development and neointimal formation in response to vascular injury.

The fetal airway ligation lung model in rodents suggested that HDGF expression was induced by airway pressure during pulmonary development. HDGF is highly expressed in the endothelial cells of developing blood vessels in the fetal lung (25, 28). In the bleomycin-induced lung damage model, HDGF expression is dominantly augmented in the bronchial and alveolar epithelial cells including type II alveolar cells (19). These reports suggest that HDGF is involved in various organ development and tissue repair processes.

HDGF is involved in liver development and regeneration

We previously investigated whether HDGF was associated with liver development (10). As a result, HDGF was found to be strongly expressed in immature fetal hepatocytes, especially in the mid-gestation stage, and its expression was dramatically reduced near birth. HDGF expression was reciprocal to the hepatocytes differentiation, suggesting that HDGF was significantly related to the hepatocytes proliferation. The exogenous administration of recombinant HDGF stimulated the growth of primary cultured fetal hepatocytes. Furthermore, adenoviral introduction of HDGF antisense cDNA into the fetal hepatocytes significantly reduced HDGF expression, resulting in the suppression of hepatocyte proliferation. The inhibitory effect of HDGF antisense virus was reversed by the exogenous administration of HDGF. These findings strongly suggest that HDGF is involved in the hepatocyte proliferation during liver development.

In addition, we examined whether HDGF was induced in the two different liver regeneration models, after partial hepatectomy and CCl4- induced liver damage (11). In the CCl4- treated liver, HDGF expression was induced and the single peak was detected prior to the DNA synthesis peak. HDGF expression was also increased in the hepatectomy model and its peak induction was also observed before the DNA synthesis peak as well. The induction of the HDGF gene expression in the regenerating liver was dominantly detected in parenchymal hepatocytes. Our

findings showed that HDGF expression was elevated in parenchymal hepatocytes prior to the DNA synthesis in the regenerating liver, suggesting the involvement of HDGF in liver regeneration as an autocrine factor. Based on these findings, we consider that HDGF plays significant roles in liver development and regeneration, especially in hepatocyte proliferation.

HDGF is a unique growth factor involved in hepatocyte proliferation

Several growth factors such as TGF-α (transforming growth factor-alpha, HB-EGF (heparin-binding EGF-like growth factor) and HGF (hepatocyte growth factor), have been reported to play important roles in hepatocyte proliferation (1-3). However, HDGF is a unique growth factor with characteristics different from these growth factors.

In liver development, the expression levels of TGF- α and HGF increase after birth and decrease after weaning, suggesting that these factors are implicated in the liver growth of post-natal stage (29-31). Although the expression pattern of HB-EGF during liver development has not been reported, the expression level of EGF receptor (the common receptor and signal transducer of TGF- α and HB-EGF), is lower at the fetal stage compared with the post-natal stage (32). Expression of the HGF receptor, c-met, is also up-regulated after birth (31). Therefore, these growth factors and their signals are presumed to participate in the liver development mainly in the post-natal stage rather than in the fetal stage. Conversely, HDGF is highly expressed in the immature hepatocytes and its expression was markedly reduced near birth, thus suggesting that HDGF may play a more important role in the fetal stage than in the post-natal stage during liver development (10). Despite the normal development of HDGF-null mice, possibly as a result of the redundancy that bas been reported to exist among HDGF and its related genes (27), HDGF is thought to play significant roles in the proliferation of fetal hepatocytes.

Regarding liver regeneration, previous studies have shown the possible involvement of numerous growth factors (1-3). Among these growth factors, TGF- α, HB-EGF, and HGF are strongly associated with liver regeneration. TGF- α is induced in replicating hepatocytes and stimulates the hepatocyte proliferation in an autocrine manner (29). The expression of HB-EGF mRNA is increased in non-parenchymal cells of a regenerating liver, suggesting a functional role of the growth factor in the hepatocyte proliferation as a paracrine factor (33). On the other hand, regarding HGF (a potent hepatocyte growth-stimulating factor), the extra-hepatic production of this growth factor has been suggested to play

more significant roles in the process of liver regeneration in comparison to the intra-hepatic production (34, 35). We consider that the growth factors induced in damaged tissues play an important role in the regeneration process of the organ repair, while the extra-hepatic production of growth factors should be also important.

In the liver regeneration animal model, TGF- α mRNA peaked at 12 to 24 hr after CCl4 treatment and its expression decreased at 36hr, and then again it increased at 48 hr time point. HB-EGF mRNA was also induced by CCl4 treatment, displaying the two peaks at 6 to 12 hr and 36 hr after the administration. Unlike these factors, the time course of HDGF mRNA expression showed a single peak at 24 hr after injection (11). In CCl4- treated liver of mice, the protein expression of TGF- α was observed in the cytoplasm of hepatocytes, but not in the non-parenchymal cells. HB-EGF protein was expressed in parenchymal cells, especially in the peripheral region of hepatic lobules, and was also expressed in non-parenchymal cells. HDGF protein was expressed in the whole hepatic lobules and was mainly detected in nucleus of hepatocytes, thereby indicating that the expression pattern of HDGF after CCl4 injection was markedly different from those of TGF- α and HB-EGF (11). These findings therefore suggest that HDGF plays a unique and important role in liver regeneration.

Conclusion

HDGF is a novel nuclear/growth factor belonging to a new gene family containing a HATH region in N-terminal region and nuclear translocation signals. These recently demonstrated findings suggest that HDGF should be a unique growth factor involved in both liver development and regeneration.

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BRIEF ARTICLE

Partial blockage of hepatocyte maturation in hepatoma-derived growth factor transgenic mice

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Abstract

AIM: To investigate the role of hepatoma-derived growth factor (HDGF) in liver development, especially in the hepatocyte differentiation.

METHODS: We generated transgenic mice which overexpressed HDGF in hepatocytes under the transcriptional control of mouse albumin promoter/enhancer. To examine the effects of HDGF overexpression on hepatocyte differentiation, we investigated the expression patterns of the differentiation marker genes.

RESULTS: The HDGF transgenic mice developed normally and showed no apparent abnormality in the liver. However, the gene expression patterns of the liver in adult transgenic mice were similar to those of the neonatal liver in control mice.

CONCLUSION: These findings suggest that HDGF-overexpression partially suppresses hepatocyte maturation.

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Key words: Hepatoma-derived growth factor; Hepatocyte; Maturation; Transgenic mice

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INTRODUCTION

The liver is the major hematopoietic organ during the fetal period, and immature hepatocytes function as stromal cells which support hematopoiesis. During liver development, immature hepatocytes differentiate and acquire many functions in preparation for the metabolic conditions after birth[1,2]. The expression patterns of differentiation marker genes can represent the maturational stage of hepatocytes. Alpha-fetoprotein (AFP) is one of the early marker genes of the immature hepatocytes and its expression remarkably decreases after birth[3]. The expression of albumin, the most abundant protein synthesized by hepatocytes, begins during the mid-gestational stage, and this expression increases with the progression of liver development, especially after birth[4]. In the late-gestational stage hepatocytes begin to produce metabolic enzymes including tyrosine amino transferase (TAT) and glucose-6-phosphatase (G-6-Pase)[5,6]. Subsequently, hepatocytes

gain a fully matured phenotype, characterized by the expression of tryptophan oxygenase (TO) within two weeks after birth[7]. The expression levels of TAT and G-6-Pase peak in the neonatal liver and decrease in the adult liver. In contrast, TO is barely expressed in the fetal and neonatal liver and is highly expressed in the adult liver. Although the gene expression patterns of hepatocytes continue to alter after birth, few studies have documented the growth and differentiation of post-natal hepatocytes.

Hepatoma- derived growth factor (HDGF) is a heparin-binding protein, which has been identified from the conditioned media of HuH-7 hepatoma cells[8,9]. HDGF stimulates the proliferation of fibroblasts, endothelial cells, vascular smooth muscle cells and hepatocytes[8-11]. Its primary sequence contains nuclear localization signals and the HDGF can be transported to the nucleus, thus indicating that HDGF is a unique nuclear/growth factor[12,13]. Recently, several novel genes have been identified for proteins which share a highly homologous amino terminal region consisting of about 100 amino acids; so-called HDGF- related proteins (HRPs)[14,15]. It is thought that HDGF and HRPs form a new gene family. Although HDGF was initially identified in human hepatoma-derived cells, HDGF mRNA is expressed in various normal adult tissues of mice and humans, thus suggesting that HDGF has some physiological functions in non-tumor cells[9].

Previous studies have suggested that HDGF participates in fetal organ development and adult tissue repair as an autocrine growth factor[10,11,16]. We have shown that HDGF is highly expressed in immature fetal hepatocytes and promotes their proliferation[16]. Furthermore, HDGF is induced in two animal models of liver regeneration[17], suggesting that HDGF plays an important role in the proliferation of both fetal and adult hepatocytes. Although the involvement of HDGF in cell differentiation has not been clarified, the suppressive effects of HDGF on gut cell maturation have been suggested[18]. We generated transgenic mice which overexpressed HDGF in hepatocytes under the control of the albumin promoter/enhancer, in order to examine the functional role of HDGF in liver development. The gene expression patterns of hepatocytes in adult transgenic mice resemble those of neonatal hepatocytes of wild-type mice, thus suggesting that HDGF-overexpression partially suppresses hepatocyte maturation.

MATERIALS AND METHODS

Mice

The DNA fragment covering the entire coding region of mouse HDGF was cloned into the Eco RI site of an expression vector which contains the promoter and enhancer of the mouse albumin gene[19]. A schematic representation of the constructed transgene (Alb-HDGF) is illustrated in Figure 1. Transgenic founders were generated by pronuclear injection according to standard techniques. Using a ^{32}P-labeled fragment of HDGF-specific cDNA as a probe, transgene integration and expression were identified by Southern and Northern blot analyses, respectively. C57BL/6CrSlc mice (Nihon SLC, Shizuoka, Japan) or non-transgenic mice were used as controls. All animal experiments were performed according to the guidelines of Osaka University Medical School.

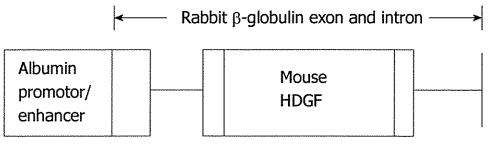

Figure 1 Schematic representation of the constructed transgene of HDGF. Schematic representation of the constructed fragments used in the generation of transgenic mice. A DNA fragment covering the entire cDNA of the mouse HDGF was inserted into the expression vector, which contains promoter and enhancer of mouse albumin gene.

Hybridization probes

The probes used for the Northern blot analysis were as follows: a 0.4 kb fragment of mouse HDGF cDNA[9], a 0.5 kb fragment of mouse G-6-Pase cDNA[20], and TO cDNA[20].

Southern blot and Northern blot analyses

Genomic DNA was isolated from individual mouse tails, and then was blotted onto nylon membranes according to standard protocols. Total RNA was extracted from liver tissues using ISOGEN (Nippon Gene, Tokyo, Japan), denatured with formamide and blotted onto nylon membranes. The mouse cDNAs described above were labeled with (α-^{32}P) dCTP using a Megaprime DNA labeling kit (Amersham Life Science, Tokyo, Japan) and then were used for hybridization[16,17].

RESULTS

An expression unit was constructed that contained the entire HDGF cDNA under the control of the mouse albumin promoter/enhancer (Alb-HDGF, Figure 1). Purified fragments were used for pronuclear injection and potential founders were analyzed for the genomic integration(s) of the transgene. Three founders containing the Alb-HDGF sequence were identified by Southern blot, and the transgenes were successfully transmitted in two lines (Figure 2A: Tg-48, and Tg-21). Northern blot analysis revealed that HDGF was highly expressed in the adult liver of Tg-48 mice, whereas HDGF expression in the liver of Tg-21 mice was almost equal to the wild-type mice (Figure 2B). We therefore used the Tg-48 mice to analyze the effects of HDGF overexpression in hepatocytes.

HDGF overexpressing mice (Tg-48) developed normally and did not show any abnormality in appearance. In addition, no obvious histological abnormality associated with the expression of HDGF was detected in these mice up to 12 mo of age (data not shown). The expression patterns of genes related to hepatocyte differentiation were investigated by Northern blotting to examine the effects of HDGF overexpression on liver development in detail. In normal mice, consistent with the previous studies, G-6-

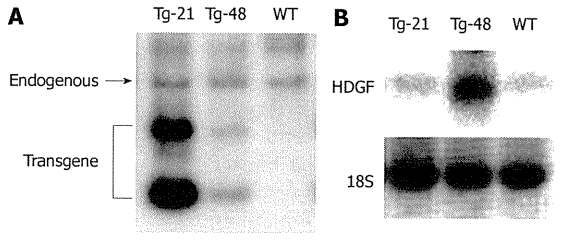

Figure 2 Genomic integration and mRNA expression of the HDGF-transgene. A: Southern blot analysis with HDGF cDNA probe. Genomic DNA was isolated from individual mouse tails and Southern blot analysis was performed according to standard methods. The bands representing endogenous HDGF and transgenes are shown. Genomic DNA of a transgenic mice (line number 21: Tg-21) contains high copy numbers of the transgene. Genomic DNA of the other transgenic mice (line number 48: Tg-48) contains low copy numbers of the transgene; B: Northern blot analysis with HDGF cDNA probe. Total RNA was isolated from liver tissues of both transgenic (Tg) and wild-type (WT) mice. Twenty micrograms of total RNA extracted was loaded and hybridized with mouse cDNA of the HDGF-specific sequence. HDGF expression was high in the liver of the Tg-48 mouse. The expression level of HDGF in the liver of Tg-21 was almost equal to the level expressed in the control liver. Ribosomal RNA of 18S is shown in the lower panel.

Pase expression was high in the neonatal liver and relatively low in adult (8 wk old) liver, whereas TO expression was higher in the adult liver in comparison to the neonatal liver (Figure 3A). Conversely, the expression of G-6-Pase was high, while the TO expression was low in the adult livers of transgenic mice (Figure 3B and D). These gene expression patterns observed in the livers of adult transgenic mice, were similar to the patterns observed in the neonatal stage of control livers. These findings have indicated that HDGF overexpressing hepatocytes in adult (8 wk old) transgenic mice appear to have the characteristics of hepatocytes in neonatal wild-type mice, suggesting the possible maturational retardation of hepatocytes in the transgenic mice. However, G-6-Pase expression in the transgenic liver in 24 wk-old mice decreased to the level of the normal liver, and the expression level of TO in the transgenic liver was increased almost to the level of the control liver, although small differences were still observed (Figure 3C and D). Therefore, HDGF overexpression did not completely block hepatocyte maturation and HDGF overexpressing hepatocytes could acquire almost fully differentiated phenotypes. These findings suggest that HDGF-overexpression partially suppresses the hepatocyte differentiation observed in the post-natal stage and thus causes the maturational delay of the hepatocytes.

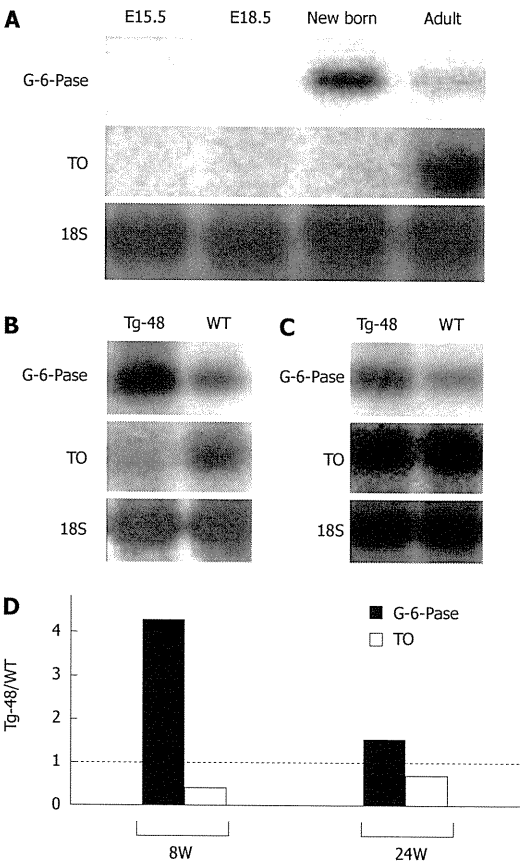

Figure 3 The expression of differentiation marker genes of hepatocytes. A: The expression of differentiation marker genes of hepatocytes in normal mice. RNA was extracted from fetal mice of E (embryonic day) 13.5 and 15.5, and postnatal mice of zero weeks (new born) and 8 wk after birth. Twenty micrograms of total RNA was loaded and hybridized with mouse cDNA probes. Ribosomal RNA of 18S is shown in the lowest panel; B: The expression of differentiation marker genes of hepatocytes in adult (8 wk) transgenic mice. In the control liver of 8-wk-old mouse, G-6-Pase expression is low and TO expression is high. In contrast, G-6-Pase expression is high and TO expression is low in the transgenic liver. Ribosomal RNA of 18S is shown in the lowest panel; C: The expression of differentiation marker genes of hepatocytes in adult (24w) transgenic mice. Unlike the 8-wk-old mouse, expression levels of G-6-Pase and TO show small differences between the transgenic liver and the normal liver. Ribosomal RNA of 18S is shown in the lowest panel. D: Densitometry measurements of Northern blot analysis. Northern blot signals in Figure 3B and 3C were measured and normalized by the internal control bands (18S). The expression levels of G-6-Pase and TO in the liver of Tg-48 mouse were compared with the levels in the control liver. The ratios of band intensities (Tg-48/WT) were graphically shown. Two independent experiments were conducted in duplicate, and similar results were obtained.

DISCUSSION

A number of studies have suggested that HDGF is involved in the development of various organs[10,11,16,18,21]. We have demonstrated that HDGF is a unique growth factor, which is highly expressed in fetal liver and promotes fetal hepatocyte proliferation[16]. However, familial genes often compensate for the functions of other family members, and HDGF-null mice have been reported to show no obvious phenotype, perhaps as a result of the redundant functions of HDGF related genes[22]. We therefore generated the HDGF transgenic mice and examined the functional role of HDGF *in vivo* according to the gain-of-function method.

Although several other groups have also reported the involvement of HDGF in the development of various organs through its growth stimulating activity, little is known about the role of HDGF in cellular maturation. As for hepatocyte differentiation, Kamiya *et al*[20] established a primary culture system of murine fetal hepatocytes to investigate the mechanism that controls late fetal liver

development. In the culture system, the administration of Oncostain M and dexamethasone can induce hepatocyte differentiation and recapitulate the maturational process of hepatocytes ranging from mid-gestation to new-born stage. This culture system was used to clarify the involvement of HDGF in hepatocyte differentiation although down-regulation of HDGF could not induce the cellular differentiation process of the late gestation stage[16].

In the present study, the overexpression of HDGF under the control of the albumin promoter did not cause any apparent morphological abnormalities in the liver. However, the gene expression patterns showed the possibility that the maturational process of hepatocytes during the post-natal stage was disturbed. This result is consistent with the report by Lepourcelet *et al*[18], which documented that overexpression of HDGF in the mouse fetal gut explants retards epithelial differentiation, suggesting a suppressive role of HDGF in epithelial differentiation.

Several proteins strongly expressed in both tumors and fetal organs, such as carcinoembryonic antigen and AFP, are known as oncofetal proteins[23,24]. HDGF is expressed exclusively in both fetal and cancer tissues, indicating that HDGF can also be regarded as an oncofetal protein. Although several oncofetal proteins are clinically used as tumor markers, there are few proteins whose functional roles in cancer cells have so far been demonstrated. HDGF expression is strongly associated with the prognosis of many malignant diseases including pancreatic cancer, esophageal cancer, colorectal cancer, gastrointestinal stromal tumor, gastric cancer and heptocellular carcinoma (HCC)[25-31]. Recently, Lee *et al*[32] showed that individuals with HCC who shared a gene expression pattern with fetal hepatoblasts had a poor prognosis. The gene expression pattern that distinguished this subtype from other types of HCC contained the markers of oval cells (hepato-cholangio progenitor cells), thus suggesting that the HCC of this subtype may be derived from hepatic progenitor/stem cells. Two groups have shown that high expression of HDGF is closely related to the poor prognosis of HCC[30,31] and HDGF stimulates the growth of immature fetal hepatocytes[16]. Recently, we have found that HDGF is highly expressed in oval cells and promotes their proliferation (Iwamoto et al. in preparation), thereby suggesting the involvement of HDGF in the proliferation of immature hepatic cells. Therefore, HDGF may stimulate the proliferation of HCC cells derived from hepatic progenitor/stem cells and thereby cause the poor prognosis. HDGF expression may maintain the characteristics of immature cells and be associated with high growth activity of malignant cells. HDGF not only promotes hepatocyte proliferation but also inhibits their differentiation, indicating that HDGF is an oncofetal protein which participates both in the cellular growth and differentiation. Clarifying the functional role of HDGF would give us new insights into molecular mechanisms common to normal and malignant hepatic cell growth.

Since HDGF-null mice did not show any remarkable abnormalities, perhaps as a result of the compensation by HDGF-related genes, the down-regulation of HDGF should inhibit the growth of cancer cells without any serious side effects on normal organs. Therefore, HDGF is considered to be a candidate therapeutic target. Although little is known about the regulation of HDGF expression, we recently have found that Vitamin K2 negatively controls the transcription of HDGF in hepatoma cells[33]. However, the suppressive effects of the Vitamin K2 are limited and it is necessary to elucidate the whole regulation of the HDGF expression in hepatic cells, especially in hepatoma cells.

In conclusion, HDGF overexpressing transgenic mice showed the possible inhibitory role of HDGF on hepatocyte differentiation. The identification of both the regulation and signal transduction of HDGF makes it possible to obtain a better understanding of liver development, regeneration, and carcinogenesis.

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COMMENTS

Background

During liver development, immature hepatocytes differentiate and acquire many metabolic functions. However, few studies have documented the growth and differentiation of post-natal hepatocytes.

Research frontiers

Hepatoma-derived growth factor (HDGF) is a heparin-binding protein, which is involved in the hepatocyte proliferation during liver development. However, the role of HDGF in hepatocyte differentiation has not been elucidated. In this study, the authors show the inhibitory role of HDGF in the hepatocyte maturation by use of the transgenic mice.

Innovations and breakthroughs

In this article, transgenic mice were established which overexpressed HDGF in hepatocytes under the transcriptional control of the mouse albumin promoter/enhancer. This is the first report about the transgenic mouse of HDGF. Furthermore, this is the first study to clarify the functional role of HDGF in the hepatocyte maturation.

Applications

The results show the possibility that HDGF may maintain the characteristics of immature cells with high growth activity. This study might represent a future strategy for the prevention or treatment of the diseases by the targeting of HDGF.

Terminology

HDGF is a heparin-binding acidic glycoprotein consisted of 240 amino acids, which is identified from the conditioned media of HuH-7 hepatoma cells. HDGF plays an important role in the organ development and tissue repair. HDGF has been demonstrated to be a unique growth factor involved in liver development, regeneration and carcinogenesis.

Peer review

On the whole, this study holds novelty and importance in understanding the process of hepatocyte maturation. While the study seemed well conducted, it is not sure from the text whether repeated analysis of Western blot has been conducted and to what extent the G-6-Pase and TO expressions were raised or dropped with time. I suggest densitometry measurements of repeated Western blot analysis and to provide a bar chart on the normalized readings.

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Original Communication

A higher expression of hepatoma-derived growth factor in hepatocellular carcinoma cells and more tumor growth *in vivo*

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ABSTRACT

Hepatoma-derived growth factor (HDGF) is a unique nuclear targeting growth factor which plays an important role in both carcinogenesis and cancer progression. Exogenously supplied and endogenously over-expressed HDGF stimulates the proliferation of hepatocellular carcinoma (HCC) cells. In the present study, to clarify the effects of HDGF on HCC, HDGF over-expressing cells were cloned and their biological functions for the growth of HCC were investigated. An anchorage-independent colony formation assay, xenograft tumor formation experiment and a DNA chip analysis were all performed. In a human HCC cell line, HepG2 cells over-expressing HDGF (HepG2-HDGF) proliferate more rapidly than mock HepG2 (HepG2-neo) cells *in vitro*. According to an anchorage independent colony assay, HepG2-HDGF cells formed more and bigger colonies than HepG2-neo in soft agar. HepG2-HDGF cells generate tumors earlier and promote tumor growth more rapidly than HepG2-neo cells in nude mice. The tumors which develop from HepG2-HDGF cells show a more reddish color macroscopically and a richer in vasculature microscopically than the tumors from HepG2-neo cells. According to the DNA-chip analyses, both *in vitro* and *in vivo* up-regulated genes due to HDGF over-expression are demonstrated, including PDGF-A, matrix metalloproteinase-1, urokinase-type plasminogen activator, chitinase 3-like-2 and ankyrin, which are related to tumor growth and aggressive characteristics. These findings suggest that HDGF enhance tumor growth while also inducing the aggressive biological functions, through the expression of genes which are related to invasion, metastasis and angiogenesis *in vivo*. Therefore, HDGF is considered to play an important role in the progression of HCC as well as hepatocarcinogenesis.

KEYWORDS: HDGF, tumor, angiogenesis

INTRODUCTION

Hepatoma-derived growth factor (HDGF) is a heparin-binding growth factor, which has a mitogenic function on fibroblasts, smooth muscle cells, endothelial cells and hepatoma cells [1-7]. The trafficking to the nucleus is important to exert the mitogenic activity, and HDGF belongs to the group of the nuclear targeting growth factor [5, 6]. The down regulation of HDGF by anti-sense oligonucleotides and small interfering RNAs (siRNA) suppressed the proliferation of cancer cells [8, 9]. Furthermore, exogenously supplied HDGF stimulated the DNA synthesis and cellular proliferation of several cells including cancer cells, although its receptor has not yet been identified.

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