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HEPATOCYTE GROWTH FACTOR DOWN-REGULATES THE α-FETOPROTEIN GENE EXPRESSION IN PLC/PRF/5 HUMAN HEPATOMA CELLS

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Hepatocyte growth factor (HGF) is a potent mitogen for hepatocytes; however, in certain human hepatoma cell lines, the growth is inhibited by HGF. In the present study, the effect of HGF on the α-fetoprotein (AFP) gene expression was analyzed in PLC/PRF/5 human hepatoma cells. HGF did not inhibit cell proliferation, but dose-dependently suppressed AFP secretion at the concentrations of 10 ng/ml or less. By Northern blot analysis, the levels of AFP mRNA were suppressed by HGF, whereas the levels of β-actin mRNA used as a control did not show any significant changes. In the transient chloramphenicol acetyltransferase plasmid transfection assays, the AFP promoter activity was repressed by HGF, in contrast, the AFP enhancer activity was not affected by HGF. These results suggest that the AFP gene expression is down-regulated by HGF through the suppression of its promoter activity in human hepatoma cells.

Hepatocyte growth factor (HGF) was first isolated as a potent mitogen for mature hepatocytes from rat platelets (1, 2). Although HGF was originally described as hepatocyte specific, it is now shown that HGF has several alternative activities on a variety of cell types. In fact, HGF stimulates growth of melanocytes (3), renal tubular cells (4), and keratinocytes (5). HGF inhibits growth of certain sarcoma cells (6) as well as human hepatoma cells (7). Recently, several groups have shown that HGF is identical to scatter factor that promotes cell migration (8, 9). Thus, HGF, like other growth factors, is considered to have different effects in the different cells .

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Abbreviations: HGF, hepatocyte growth factor ; AFP, α-fetoprotein ; FBS, fetal bovine serum ; cDNA, complementary DNA ;CAT,chloramphenicol acetyltranseferase ; TGF, transforming growth factor.

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The α-fetoprotein (AFP) gene is expressed at a high level in embryo, but rapidly decreases to an almost undetectable level after birth (10). AFP expression is also activated in hepatoma cells (11) or transiently during liver regeneration (12). The human AFP gene is located on chromosome 4 (13), and the tissue-specific expression is regulated by approximately 5 kilobase pairs of its 5′ flanking sequence (14). Although AFP expression seems to be regulated by many growth factors and cytokines (15,21,26), little is known about the effect of HGF on the AFP gene expression.

In the present study, the regulatory mechanism of HGF in the AFP gene expression was analyzed in PLC/PRF/5 human hepatoma cells by means of Northern blotting and transient chloramphenicol acetyltransferase plasmid transfection assays.

MATERIALS AND METHODS

Cell Culture: The PLC/PRF/5 human hepatoma cell line was maintained in RPMI1640 with 10% fetal bovine serum (FBS) throughout the study.

Human recombinant HGF was purified from culture medium of the CHO cells transfected with plasmid containing human HGF cDNA (16). Cell growth and AFP secretion were analyzed using 24 well multiplates (Falcon plastics, Los Angles C.A.). PLC/PRF/5 cells (2×10^4cells) were placed into each well and incubated in 10 % FBS-RPMI1640 at 37°C in 5% CO_2. Two days later, the medium was replaced with 1 ml of the fresh medium containing various concentrations (0, 2, 5, 10 ng/ml) of HGF. The number of viable cells was counted at 24, 48 and 72h after incubation using the trypan blue dye exclusion method, and the amount of AFP in the medium was quantitatively assayed by a commercially available radioimmunoassay kit (Dainabot, Tokyo, Japan). The effect of HGF on cell proliferation was also evaluated by [^{3}H]thymidine incorporation. PLC/PRF/5 cells (1×10^4 cells) were placed on 96 well microplates (Falcon plastics, Los Angles C.A.) and incubated in 10% FBS-RPMI1640. Two days later, the medium was replaced with the fresh media containing various concentrations (0, 2, 5, 10 ng/ml) of HGF, and the cells were incubated at 37°C in 5% CO_2. [^{3}H]Thymidine (0.15 μCi/well) was added to the culture media 18h after incubation, and the cells were harvested on glass filters using a semiautomatic cell harvester (Labo Mash, Labo Sci., Tokyo, Japan) 24h after incubation. The radioactivity of each cell sample was determined using a liquid scintillation counter.

Northern Blot Analysis: Total cellular RNA was isolated from PLC/PRF/5 cells by the guanidinium isothiocyanate method (17). Total RNA (20 μg) was fractionated on a 1% formaldehyde agarose gel, transferred to a nylon membrane and hybridized with a [^{32}P]-labeled cDNA probe. AFP cDNA (pHAF-2) (18) or human β-actin cDNA (Wako Chem., Ltd., Osaka, Japan) were used as probes.

Cell Transfection and Chloramphenicol Acetyltransferase (CAT) Assays: The CAT plasmids used in this study were described previously (19,20). The pBR-CAT plasmid contains the CAT coding sequence and the simian virus 40 polyadenylation signal but no upstream regulatory sequences. pAF5.1-CAT, pAF1.0-CAT and pAF0.2-CAT contain 5.1 kilobase pairs (kb), 1.0 kb and 169 base pairs (bp) of the AFP5′-flanking sequence, respectively, linked to the CAT gene in pBR-CAT. pSVAF2.4-CAT contains the 2.4 kb full AFP enhancer region (-5.3 to -2.9 kb) inserted at the 5′ end of the CAT gene in pSV1′-CAT which contains the simian virus 40 TATA box but lacks most of the 72 bp repeat sequence. pAF5.1[Δ2.0]-CAT and pAF5.1[Δ2.7]-CAT contain both the 2.4 kb full AFP enhancer and promoter region but lacks 2.0 kb (-2.9 kb to -951 bp) and 2.7 kb (-2.9 kb to -169 bp) fragment of pAF5.1-CAT, respectively. pSV2-CAT plasmid (21) was also used as a control plasmid in this study.

Transfection was performed using 10 μg of plasmid DNA per flask (50 cm^2) by the lipofection method (22). After transfection, PLC/PRF/5 cells were incubated with the fresh media in the absence or presence of 10 ng/ml HGF. Two days later, the cells were harvested and lysed

by five cycles of freezing and thawing. The lysate was heated at 63°C for 10 min and the supernatant was used for determination of the CAT activity as previously described (23).

RESULTS

Effects of HGF on Cell Growth and AFP Secretion: When PLC/PRF/5 cells were incubated with varying concentrations of HGF (2-10 ng/ml), the number of viable cells did not differ between the control group and the HGF-treated group (Fig. 1). [^{3}H]Thymidine incorporation into PLC/PRF/5 cells was also not affected by HGF (Fig. 2). However, as shown in Fig. 3, AFP secretion was dose-dependently suppressed by HGF.

Decrease in the AFP mRNA Level by HGF: Total cellular RNA was isolated from the cells incubated for 48h in the absence or presence of HGF (2, 5, 10 ng/ml).As shown in Fig. 4, HGF caused a decrease in the AFP mRNA level in a dose-dependent manner, whereas the level of the β-actin mRNA was not significantly changed by HGF treatment. HGF-induced repression of the AFP mRNA level was greater than that of AFP secretion. Similar delays in the change of secreted AFP levels have been reported in other systems (21, 24), which likely reflect the time required for mRNA translation, protein processing, and secretion.

Suppression of the AFP Promoter Activity by HGF: In an attempt to analyze the molecular mechanism by which AFP expression is suppressed by HGF, CAT plasmid transfection experiments were carried out. CAT expression from pAF5.1-CAT which contains both the full AFP enhancer region and promoter region was suppressed by HGF. In contrast, CAT expression from pSVAF2.4-CAT which has the full AFP enhancer region but lacks the promoter region and CAT expression from pSV2-CAT used as a control plasmid did not show any significant changes by HGF (Fig. 5A). CAT expression from pAF1.0-CAT and pAF0.2-CAT containing only the AFP promoter region was low but suppressed by HGF treatment. Since CAT activities from these

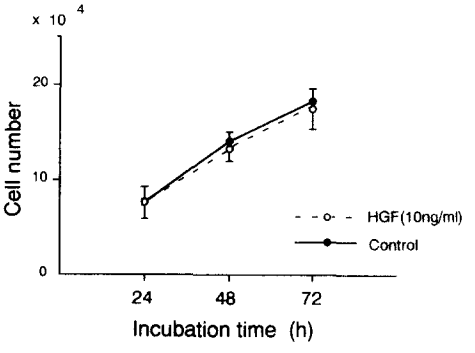

Figure 1. Effect of HGF on cell growth. The number of cells was counted at 24, 48 and 72 h after incubation with 10 ng/ml HGF (○) or without HGF (●). Results represent the mean ± SD (n=4).

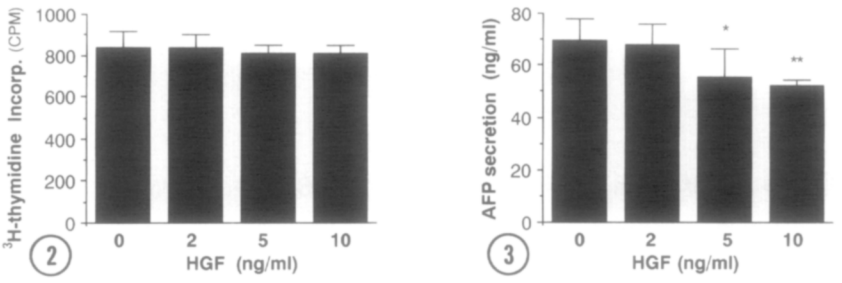

Figure 2. Effect of HGF on DNA synthesis. PLC/PRF/5 cells were incubated with varying concentration of HGF (0-10 ng/ml). [^{3}H] Thymidine incorporation was determined as described in Materials and Methods. Each point represents the mean ± SD of the values in 4 replicate assays of 3 experiments.

Figure 3. Inhibition of AFP secretion by HGF. Two days after HGF treatment, the amount of AFP in the culture media was analyzed using a commercially available radioimmunoassay kit. AFP secretion was expressed as ng/ml. Results represent the mean ± SD (n=4). *P < 0.05 vs control, **P < 0.01 vs control.

two plasmids were relatively low, pAF5.1[Δ2.0]-CAT and pAF5.1[Δ-2.7]-CAT plasmid which contain the 1.0 kb and the 169 bp fragment of the AFP promoter sequence, respectively, linked to the full AFP enhancer region were transfected. As expected, CAT activities from pAF5.1[Δ2.0]-CAT and pAF5.1[Δ2.7]-CAT plasmid were clearly suppressed by HGF (Fig. 5B).

DISCUSSION

AFP is characterized as an oncofetal glycoprotein and re-expressed in hepatoma cells. Recently, there has been a great deal of progress in characterization of cis- and trans- acting elements regulating the AFP gene expression (20,25,26). In addition to the promoter and enhancer regions of the AFP gene, the silencer elements which are possibly associated with a neonatal repression of the AFP gene expression have been identified to be located between the

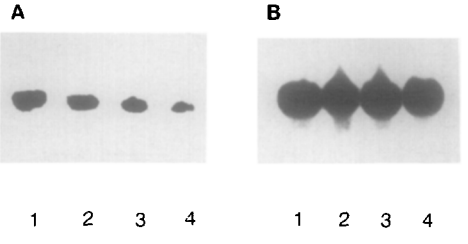

Figure 4. Decrease in the levels of the AFP mRNA by HGF. PLC/PRF/5 cells were treated with various concentrations of HGF, Control (lane 1), 2 ng/ml (lane 2), 5 ng/ml (lane 3), or 10 ng/ml (lane 4) ; Two days later, total RNA was isolated and analyzed for AFP mRNA (A) and β-Actin mRNA (B) by Northern blotting as described in Materials and Methods.

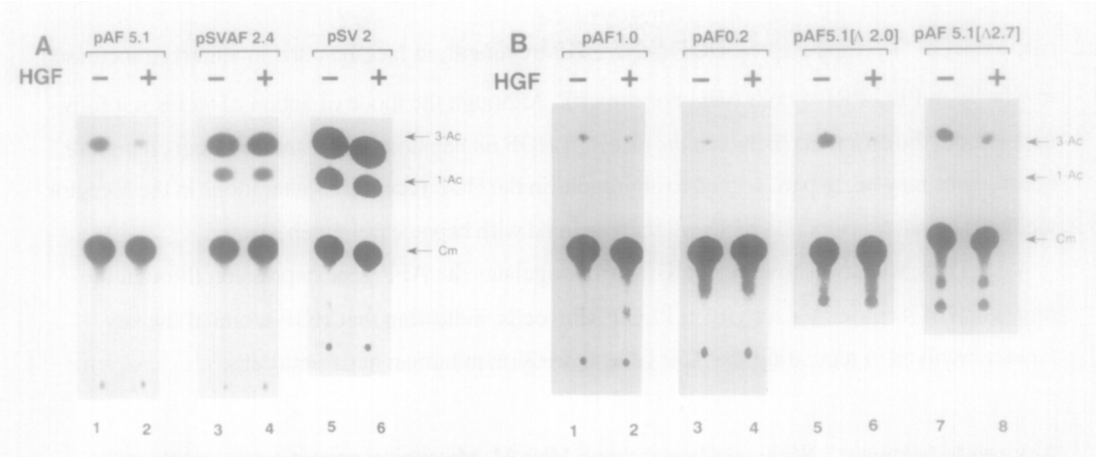

Figure 5. Effect of HGF on CAT expression from AFP-CAT chimeric genes. (A) PLC/PRF/5 cells were transfected with pAF5.1-CAT (lanes 1 and 2),pSVAF2.4-CAT (lanes 3 and 4), or pSV2-CAT (lanes 5 and 6) with (+) or without (-) HGF (10 ng/ml). Two days later, CAT activity was analyzed as described in Materials and Methods. The amounts of extract and incubation times were 50 μg of protein and 60 min (lanes 1 to 4), and 25 μg of protein and 60 min (lanes 5 and 6). (B) PLC/PRF/5 cells were transfected with pAF1.0-CAT (lanes 1 and 2), pAF0.2-CAT (lanes 3 and 4), pAF5.1[Δ2.0]-CAT (lanes 5 and 6), or pAF5.1[Δ2.7]-CAT (lanes 7 and 8) then incubated with (+) or without (-) HGF (10 ng/ml). The amounts of extract and incubation times were 50 μg of protein and 120 min (lanes 1 to 4) and 25 μg of protein and 60 min (lanes 5 to 8).
3-AC; 3 acetylchloramphenicol, 1-AC; 1 acetylchloramphenicol, Cm; Chloramphenicol.

enhancer and promoter region (20). Previous studies have demonstrated that transforming growth factor-β1 (TGF-β1) down-regulates the AFP gene expression through the repression of its promoter activity (21), whereas epidermal growth factor synergistically interacts with phorbol ester to suppress the AFP enhancer activity, resulting in a marked depression of the AFP gene transcription (26).

In the present study, HGF caused a decrease in the level of the AFPmRNA in PLC/PRF/5 human hepatoma cells at the concentrations of 10 ng/ml or less, where cell growth did not differ between the control group and the HGF-treated group. In the transient CAT plasmid transfection experiments, HGF did not affect the AFP enhancer activity, but clearly repressed the AFP promoter activity. We previously reported that TGF-β1 suppresses the AFP promoter activity; however, the mechanisms seem to be different between TGF-β1 and HGF, because TGF-β1 acts on the -1.0 kb to -170 bp fragment of the AFP promoter region, whereas HGF on the -169bp to +30 bp fragment.

HGF stimulates DNA synthesis in mature hepatocytes and plays an important role in liver regeneration (27). In contrast, several investigators revealed that HGF inhibits growth of certain hepatoma cells (28). In addition, HGF functions as a scatter factor in some cell lines including human hepatoma cell lines (29,30). Recent studies have shown that the protein encoded by the protooncogene known as c-*met* is a receptor for HGF (31), and that the elevation of intracellular

calcium [$(Ca^{2+})_i$] is induced by HGF (32). HGF treatment, in fact, resulted in a marked increase in $(Ca^{2+})_i$ in PLC/PRF/5 cells (data not shown). Although the mode of action of *met* is not fully understood, the difference between the effect of HGF on hepatoma cells and its effect on mature hepatocytes may be, in part, related to alterations in the HGF receptor or alterations in the receptor-mediated signal transduction pathway accompanied with hepatocarcinogenesis.

Thus, we have shown that HGF down-regulates the AFP gene expression through the repression of its promoter activity in PLC/PRF/5 cells, indicating that HGF is one of the key factors involved in regulating the AFP gene expression in human hepatoma cells.

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