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• BASIC RESEARCH •

Autocrine expression of hepatocyte growth factor and its cytoprotective effect on hepatocyte poisoning

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Abstract

AIM: To construct pEGFP-hepatocyte growth factor (HGF) expression vector, the to detect its expression in transfected human hepatocytes, and to investigate the influence of autocrine HGF expression on the proliferative potential and cytoprotective effects in human hepatocytes.

METHODS: Human HGF cDNA was ligated to the pEGFP vector. Recombinant plasmid was transfected into human hepatocyte line QZG with liposome. Expression of HGF protein was observed by fluorescence microscopy and immunohistochemistry. Hepatic cells were collected 24, 48, and 72 h after transfection to detect the number of [^{3}H]-TdR uptake in DNA. DNA synthesis was observed by using PCNA stain immunohistochemistry. Acute liver cell damage was induced by carbon tetrachloride. Cytoprotective effect was observed by examining the survival rate of hepatocytes and leakage of intracellular alanine transaminase (ALT) and potassium ions.

RESULTS: HGF identification of pEGFP-HGF by enzyme digestion showed that HGF fragment was cloned into *BamH* I and *Sal* I sites of pEGFP-N3. Expression of GFP in transfected hepatocytes was observed with fluorescence microscopy. The [^{3}H]-TdR uptake became 7 times as many as in the control group 96 h after transfection. After HGF transfection, the survival rate of hepatocytes poisoned by CCl_4 significantly increased (83% *vs* 61%, $P<0.05$), and the leakage of intracellular alanine transaminase and potassium ions decreased (586 nkat/L *vs* 1089 nkat/L, $P<0.01$; and 5.59 mmol/L *vs* 6.02 mmol/L, $P<0.01$ respectively). Culture of transfected hepatic cells promoted the proliferation of other non-transfected cells.

CONCLUSION: Transfected HGF is expressed in hepatic cells and has the activity of promoting cell division and protecting hepatic cells against poisoning.

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INTRODUCTION

Hepatocyte growth factor (HGF) was originally isolated as the most potent mitogen for primary hepatocytes. HGF has subsequently been revealed to be a multifunctional cytokine in a wide variety of epithelial cells, endothelial cells, and some mesenchymal cells during the past two decades[1-7]. In addition to mitogenic activity, HGF has some unique morphogenic activities such as an induction of epithelial tubulogenesis including in cells derived from the kidney, liver and mammary gland[8,9]. Characterization of scatter factor, which enhances epithelial cell motility, revealed that it had the same molecule as HGF. HGF is a heterodimeric glycoprotein composed of a 69 ku α-chain and a 34 ku β-chain, and has four homologous kringle domains in the α-chain[10]. The receptor capable of signal transduction of HGF is a c-met protooncogene product of heterodimeric tyrosine kinase[11,12]. Extensive studies on the physiological function of HGF have established that the growth factor functions as a potent hepatotrophic factor for liver injuries, but elevated plasma HGF levels have not been noted in patients with hepatic diseases[13-19].

HGF and its receptor MET, ordinarily constitute a paracrine signaling system in which cells of mesenchymal origin produce the ligand (HGF) which binds to its receptor (MET) that is predomanantly expressed in cells of epithelial origin[10]. While the effects of exogenous HGF on promoting normal hepatocyte growth have been well characterized, there appears to have a controversy on effect of autocrine HGF on hepatocytes. In this study, the effects of forced autocrine expression of HGF in hepatocytes were examined. huHGF was expressed by transducing hepatocytes with a vector containing huHGF cDNA. It was demonstrated that the forced expression of HGF in hepatocytes led to an increase in the population of transduced hepatocytes compared to non-transduced hepatocytes in the same cultures. The results suggest that autocrine expression presents an advantage for the proliferation and survival of hepatocytes poisoning.

MATERIALS AND METHODS

Materials

PBS-7 vector containing human HGF cDNA was donated by Professor Toshikazu Nakamura (Division of Biochemistry, Osaka University Medical School, JaPan). The transfer vector $pEGFPN_3$ containing a humanized green fluorescence protein (GFP) cDNA was purchased from Clontech Biology Company (USA).

Culture of hepatocytes

Human hepatocyte QZG (donated by the Department of Pathology, Fourth Military Medical University) was cultured in RPMI1640 medium (Gibco) supplemented with 150 mL/L fetal bovine serum (100 kU/L penicillin, 100 kU/L streptomycin) and incubated at 37 ℃ in humidified atmosphere of 50 mL/L CO_2 incubator.

Construction of pEGFP-HGF vectors

Construction of pEGFP-HGF vector was previously described[20]. The human HGF cDNA was isolated from pBluescript SK^- as a 2.3 kb *Bam*H I- *Sal* I fragment. The resulting cDNA fragment

was then inserted into the *Sal* I and *Bam*HI-sites of the pEGFP-N_3 vector.

Transduction of rat hepatocytes

Exponentially growing QZG cells were seeded into 35 mm tissue culture dishes and grown to 60-70% confluency in culture medium. Each culture dish was then transfected with 5 μg pEGFP-HGF constructs by using lipofectamine according to the instructions of the manufacturer (Gibco BRL, Eggenstein, Germany). Encapsulated pEGFP-HGF was incubated for 5 h on cells in serum free medium, then in medium containing 15 mL/L bovine serum. After a further incubation of the cells for 24 h, the culture medium was discarded and replaced by normal culture medium. Seventy-two hours after transfection, the QZG cells and their media were harvested for further analysis.

Analysis for HGF expression in hepatocyte

After transfected with pEGFP-HGF, the cells were examined for the presence of green fluorescence by using an inverted fluorescent light microscope. Immunocytochemical staining was performed by using HGF antibody on logarithmically growing cell lines. Firstly, the cells were plated onto coverslips, adhered overnight. Then, after rinsed three times with PBS, the cells were fixed in cold acetone for 8-10 min. Endogenous peroxidase was blocked with 10 mL/L hydrogen peroxide in absolute methanol for 30 min. The primary antibodies were applied for 2 h at 37 ℃ at 1:50 dilution in a humidified chamber. Then the typical SABC strategy followed.

Assay of DNA synthesis by ^{3}H-TdR incorporation

The cells were cultured in an appropriate medium for 24 h prior to beginning the experiment, and incubated with [^{3}H] thymidine (18.5 MBq/L, 37 kBq/well). After 6 h the cells were harvested with trysin/EDTA, and collected from the acetic fiber filter with a cellular collector and washed three times with PBS. The filter was dried overnight at 37 ℃, and transferred into a liquid scintillation counter (containing 10 g/L PO and 20 g/L POP in xylene) and cpm value was determined by the liquid scintillation counter (LS6500, Beckmen Co.)

Cell proliferation

The level of expression of proliferating cell nuclear antigen (PCNA) was used as a marker of cell proliferation. In the present study PCNA was measured by ABC immunohistochemical method.

Cytoprotective effects on CCl_4-intoxicated human hepatocytes

For assaying cytoprotective effect of autocrine HGF, hepatocyte QZG was poisoned by carbon tetrachloride (CCl_4). After treated with 15 mmol/L CCl_4 for 40 min, survival rate of cells was measured by trypan blue method. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in supernatant were measured by using a biochemical analyzer. Leakage of intracellular potassium ions was measured by using an ion analyzer.

In situ hybridisation

This was performed as previously described. Hepatocytes were transduced with pEGFP-HGF, trypsinized after 48 h and centrifuged onto slides (roughly 40 000 cells/cm^2). These slides were used for *in situ* hybridization. Results were evaluated by counting at least 3 viewing fields per slide at a magnification ×200.

Statistical analysis

The results are expressed as mean±SE. Statistical analysis was performed by the Student's *t*-test. $P<0.05$ was considered statistically significant.

RESULTS

Identification of recombinant pEGFP-HGF expression vector

The recombinant pEGFP-HGF vector was digested by *Bam*H I and *Sal* I. pEGFP-HGF contained a 2300-bp and a 4700-kb gene fragments separated by electrophoresis in 12 g/L agarose gel (Figure 1). It proved that the HGF gene was correctly inserted inpEGFP-HGF.

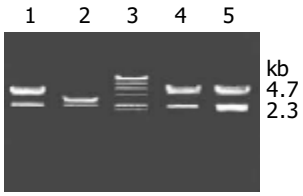

Figure 1 Identification of recombinant plasmids digested with restriction enzymes *Bam*H I and *Sal* I. 1, 4, 5: pEGFP-HGF plasmid digested with *Bam*H I and *Sal* I; 2: pBS-7 plasmid digested with *Bam*H I+*Sal* I; 3: λDNA / *Hin*dIII marker.

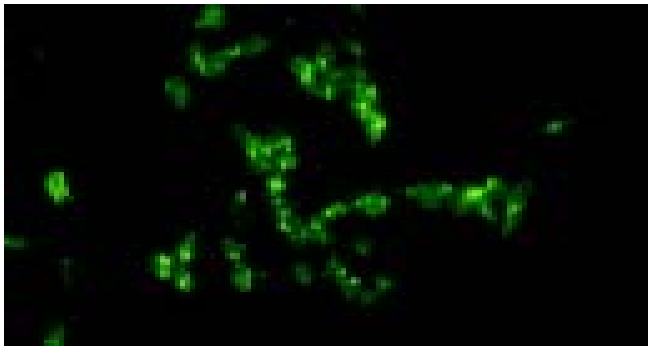

Figure 2 Green fluorescence in QZG cells transfected with pEGFP-HGF fusion constructs, Inverted fluorescent light microscope ×200.

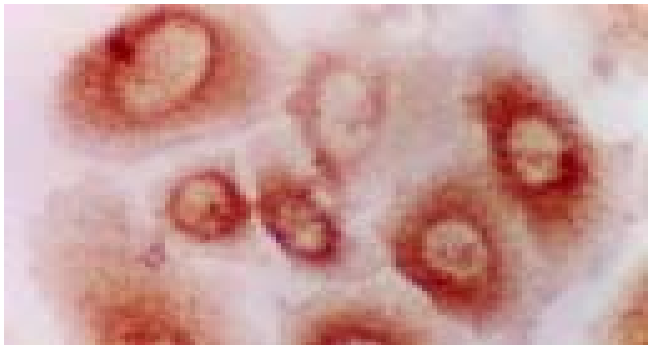

Figure 3 Expression of HGF protein in QZG cells transfected with pEGFP-HGF. SABC ×400.

Expression of pEGFP-HGF fusion proteins in QZG cell line

Green fluorescence was visible after transfection with pEGFP-HGF fusion constructs (Figure 2). The pEGFP-HGF fusion proteins were localized primarily in cytoplasm. The expression of HGF in the transfected QZG cells was also detected by immunohistochemical staining. A lot of brown granules were seen in HGF transfected QZG cell cytoplasms (Figure 3) proving that HGF gene could be expressed in QZG cells.

Effect on DNA synthesis

DNA synthesis as mirrored by [^{3}H]thymidine uptake of QZG cells was determined after transfection with pEGFP-HGF. DNA synthesis in QZG cells transfected by pEGFP-HGF was accelerated

in a time-dependent fashion increased by 7-fold compared to control group after 96 h transfection with pEGFP-HG F (Figure 4).

Cell proliferation

As shown in Figure 5, the expression rate of PCNA on QZG cells increased after transfection with pEGFP-HGF.

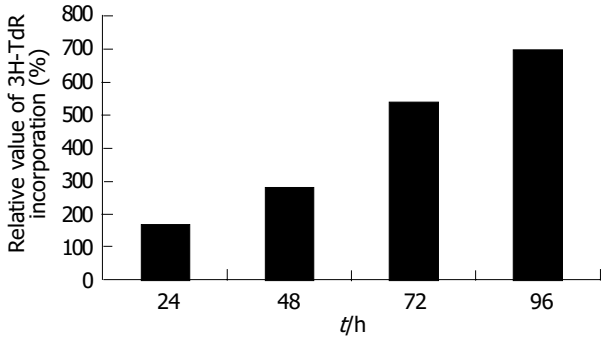

Figure 4 Relative values of ^{3}H-TdR incorporation in QZG cells transferred with pEGFP-HGF (relative to QZG cells transferred with pEGFP).

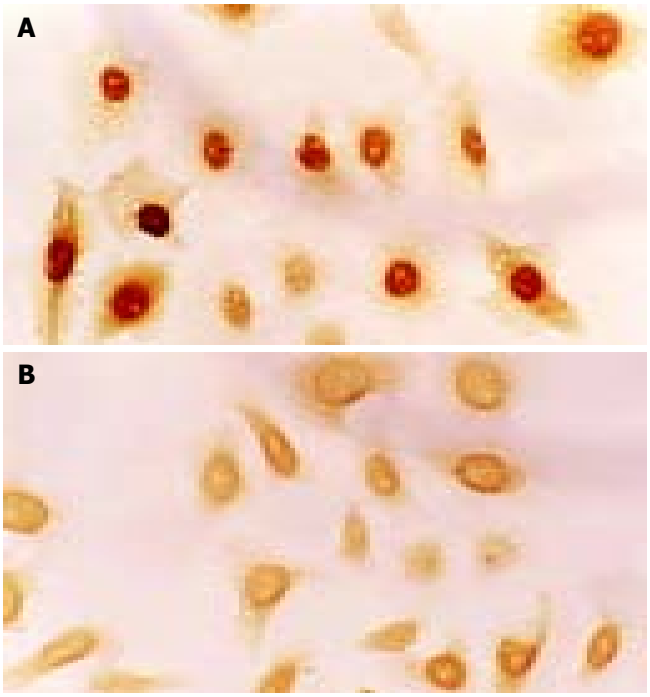

Figure 5 Expression of PCNA protein in QZG cells transferred with pEGFP-HGF (A) and transferred with pEGFP (B). ×200.

Table 1 Survival rate of hepatocytes and leakage of intracellular ALT and K^+

Group	Survival rate (%)	ALT (nkat/L)	K^+ (mmol/L)
QZG	96±2	403±70	5.21±0.04
pEGFP-HGF	98±1	429±80	5.18±0.02
QZG intoxicated by CCL_4	61±7[a]	1 089±223[b]	6.02±0.17[b]
pEGFP-HGF intoxicated by CCL_4	83±6[c]	586±124[d]	5.59±0.07[d]

[a]P<0.05, [b]P<0.01, *vs* QZG group; [c]P<0.05, [d]P<0.01, *vs* QZG intoxicated by CCL_4 group.

Cytoprotective effect on hepatocytes injuried by CCl_4

After pEGFP-HGF was transfected, the survival rate of QZG cells intoxicated by CCl_4 was significantly increased (83% *vs* 61%, t = 3.89, P<0.05). The leakage of intracellular alanine transaminase (ALT) (586 nkat/L *vs* 1 089 nkat/L, t = 13.07, P<0.01) and potassium ions (5.59 mmol/L *vs* 6.02 mmol/L, t = 12.91, P<0.01) was decreased (Table 1).

DISCUSSION

Hepatocyte growth factor (HGF) is well known as a pleiotropic substance with mitogenic, motogenic, morphogenic, and tumor suppressoion activities. HGF was found in the serum of partially hepatectomized rat and in rat platelets as the most potent stimulator of hepatocyte growth and DNA synthesis *in vitro*[21]. The mitogenic function of HGF was confirmed *in vivo*. In normal rats as well as partially hepatectomized rats, infusion of HGF stimulated a 6-fold increase in the mitotic labeling index of hepatocytes. It also stimulated protein synthesis, seen as increased hepatic messenger RNA content and serum albumin levels. HGF has been intensively investigated in terms of its beneficial effects on hepatic injury[22-24]. It has been documented that HGF reduced the hepatotoxicity of alpha-naphthylisothiocyanate, D-galactosamine, and carbon tetrachloride in rats[25,26]. Moreover, HGF increased survival of cirrhotic rats subjected to a major hepatic resection and treatment with dimethylnitrosamine[27-29]. These results suggest that HGF may play important roles in protection from and regeneration of hepatic injury and serve as a therapeutic agent.

HGF and its receptor MET, ordinarily constitute a paracrine signaling system in which cells of mesenchymal origin produce the ligand (HGF) which binds to its receptor (MET) that is predominantly expressed in cells of epithelial origin. In the present study, we demonstrated that HGF gene transfer into hepatocytes caused increased proliferation of hepatocytes by method of PCNA stain and increased DNA synthesis by method of [^{3}H]thymidine uptake. Forced HGF expression by cultured human hepatocytes had a mitogenic effect. The detection of HGF protein assessed by immunocytochemical staining confirmed the successful transfection of HGF gene.

Accumulated evidence has revealed that HGF has a cytoprotective effect on various types of liver cells. In hepatocytes, HGF attenuated the liver injury induced by lipopolysaccharide and D-galactosamine[30]. Moreover, HGF abrogated Fas-induced massive liver apoptosis and lethal hepatic failure by inducing Bcl-xL expression, with subsequent blockage of a Fas-mediated signaling pathway upstream of CPP32 in the liver, suggesting that HGF has an inhibitory effect on Fas-mediated apoptosis. HGF could also ameliorate hepatocellular dysfunction and posthepatectomy hyperbilirubinemia in an α-naphthylisothiocyanate-induced cholestasis model, in which the epithelia of bile ducts, as well as hepatocytes, became necrotic. This finding indicates that HGF could promote tissue repair of both epithelial cells and hepatocytes[31,32]. In the present study we demonstrated the cytoprotective effect of HGF transgene on hepatocyte injury by CCl_4. HGF transgene markedly increased the survival rate of hepatocytes against CCl_4 injury, decreased the leakage of intracellular alanine transaminase and potassium ions.

Since its introduction into cell biological research, the green fluorescent protein (GFP) of jellyfish *Aequorea victoria* has become a versatile tool for the analysis of protein function and dynamics at the cellular level. GFP, consisting of 238 amino acids, has been used as a tag for localization of a broad range of proteins in a wide variety of eukaryotic cells. A mutant of GFP, S65T with an excitation peak of 489 nm and an emission peak of 511 nm, could emit 4 to 6 times more fluorescence energy compared with wild-type GFP. GFP may be fused generically to

a target protein, and the fluorophore of GFP forms spontaneously in the presence of oxygen, thus rendering it an ideal probe for *in vivo* applications. The *in vivo* expression of GFP could obviate the fixation and permeablization of cells for immunofluorescence or the mirojijection of labeled proteins. GFP fusion proteins constitute a major advance in the study of the dynamics of intracelluar processes in living cells[33-37]. A major concern in the application of GFP as a fluorescent tag relates to whether the distribution of GFP fluorescence is identical to that of the protein to which it is fused. We have constructed GFP- HGF fusion gene and transfected into hepatocytes. The results indicate that the fusion protein can still be expressed in the nuclei of hepatocytes. Because liver disease, especially viral liver diseases are common in China, our study is of much more significance.

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