Autocrine expression of hepatocyte growth factor and its cytoprotective effect on hepatocyte 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 predominantly expressed in cells of epithelial origin[20]. 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 pEGFP-N3 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°C in humidified atmosphere of 50 mL/L CO2; 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 BamH I-Sal I fragment. The resulting cDNA fragment

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 [3H]-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 BamHI and SalI sites of pEGFP-N3. Expression of GFP in transfected hepatocytes was observed with fluorescence microscopy. The [3H]-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 CCl4 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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was then inserted into the Sal I and BamH I-sites of the pEGFP-N1 vector.

Transduction of rat hepatocytes
Exponentially growing QZG cells were seeded into 35 mm tissue culture dishes and grown to 60-70% confluence 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 hepatocytes
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 °C at 1:50 dilution in a humidified chamber. Then the typical SABC strategy followed.

Assay of DNA synthesis by ³H-TdR incorporation
The cells were cultured in an appropriate medium for 24 h prior to beginning the experiment, and incubated with [³H] 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 °C in a humidified chamber. Then the typical SABC strategy followed.

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 CCl₄-intoxicated human hepatocytes
For assaying cytoprotective effect of autocrine HGF, hepatocyte QZG was poisoned by carbon tetrachloride (CCl₄). After treated with 15 mmol/L CCl₄ for 40 min, survival rate of cells was measured by using a biochemical analyzer. Leakage of intracellular (AST) and alanine aminotransferase (ALT) in supernatant were measured by trypan blue method. Aspartate aminotransferase with 15 mmol/L CCl₄ for 40 min, survival rate of cells was measured by using a counter (LS6500, Beckmen Co.)

RESULTS
Identification of recombinant pEGFP-HGF expression vector
The recombinant pEGFP-HGF vector was digested by BamH 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 in pEGFP-HGF.

Assay of DNA synthesis by ³H-TdR incorporation
The results are expressed as mean±SE. Statistical analysis was performed by the Student’s t-test. P<0.05 was considered statistically significant.
in a time-dependent fashion increased by 7-fold compared to control group after 96 h transfection with pEGFP-HGF (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 transfected 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         | 61±7              | 1 089±223    | 6.02±0.17   |
| by CCL₄                 |                   |              |             |
| pEGFP-HGF intoxicated   | 83±6              | 586±124      | 5.59±0.07   |

\(^{a}P<0.05, {b}P<0.01, \text{vs QZG group}; {c}P<0.05, {d}P<0.01, \text{vs QZG intoxicated by CCL}_4\).  

**Cytoprotective effect on hepatocytes injured by CCL₄**

After pEGFP-HGF was transfected, the survival rate of QZG cells intoxicated by CCL₄ 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 suppression activities. HGF was found in the serum of partially hepatectomized rats 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 dimethylsulfoxide[^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 \[^{3}\]Hthymidine 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 CCL₄. HGF transgene markedly increased the survival rate of hepatocytes against CCL₄ 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 permeabilization of cells for immunofluorescence or the microinjection of labeled proteins. GFP fusion proteins constitute a major advance in the study of the dynamics of or the microinjection of labeled proteins. GFP fusion proteins are common in China, our study is of much more significance.

that the fusion protein can still be expressed in the nuclei of the distribution of GFP fluorescence is identical to that of the application of GFP as a fluorescent tag relates to whether in vivo a target protein, and the fluorophore of GFP forms spontaneously.

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