GH enhances proliferation of human hepatocytes grafted into immunodeficient mice with damaged liver

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Abstract

We investigated effects of human (h) GH on the proliferation of h-hepatocytes that had been engrafted in the liver of albumin enhancer/promoter driven-urokinase plasminogen activator transgenic/severe combined immunodeficiency disease (uPA/SCID) mice (chimeric mice). The h-hepatocytes therein were considered to be deficient in GH, because hGH receptor (hGHR) is unresponsive to mouse GH. Actually, hIGF-1 was undetectable in chimeric mouse sera. The aPA/SCID mice were transplanted with h-hepatocytes from a 6-year (6Y)-old donor, and were injected with recombinant hGH (rhGH). rhGH stimulated the repopulation speed of h-hepatocytes; and up-regulated hIGF-1, human signal transducers and activators of transcription (hSTAT) 3, and cell cycle regulatory genes such as human forkhead box M1, human cell division cycle 25A, and human cyclin D1. To confirm the reproducibility of these effects of rhGH, similar experiments were run using h-hepatocytes from a 46-year (46Y)-old donor. rhGH similarly enhanced their repopulation speed and up-regulated the expression of the above-tested genes, especially hIGF-1 and hSTAT1. The extent of the enhancement by rhGH was much less than that in 6Y-hepatocyte-chimeric mice most probably due to the difference in GHR expression levels between the two donors. In conclusion, this study clearly demonstrated that rhGH stimulates the proliferation of h-hepatocytes in vivo.

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Introduction

Using rodents as experimental animals, it has been shown that differentiated hepatocytes can re-enter into the cell cycle when stimulated by growth factors, cytokines, and hormones (Taub 1996, Michalopoulos & DeFrances 1997, Fausto 2000). Hepatocytes in culture usually lose their replication ability and normal phenotypes and, thus, have had limited usability in testing the effects of these factors on hepatocytes. In addition, their effects on human (h)-hepatocytes in vivo have not been studied due to the lack of a suitable animal model. Previously, we developed a method to yield humanized mice (chimeric mice) whose liver is mostly replaced with h-hepatocytes (Tateno et al. 2004). The h-hepatocytes were transplanted into immunodeficient mice with diseased liver, albumin enhancer/promoter driven-urokinase plasminogen activator transgenic/severe combined immunodeficiency disease (uPA/SCID) mice. The transplanted cells were engrafted in the liver, continuously replicated, and repopulated in the liver. The extent of repopulation was calculated as the ratio (replacement index, RI) of the h-hepatocyte-repopulated area to the total examined area. Recent technological improvements enabled us to yield children's hepatocyte-chimeric mice with RI>96%. The chimeric mice have been proven to be a useful animal model to examine biological and pathological features of h-hepatocytes in vivo (Tateno et al. 2004, Tsuge et al. 2005).

The regeneration capacity of rat liver decreases with age (Bucher et al. 1964, Stocker & Heine 1971), which is coincident with the fact that serum concentrations of growth
hormone (GH) and insulin-like growth factor 1 (IGF-1) diminish with age (Kelijman 1991, Corpas et al. 1993), suggesting the association of GH with liver regeneration. Actually, evidence has been accumulating that GH is involved in liver regeneration and accounts for an aspect of age-dependent regenerative response of the liver in rodents (Krupczak-Hollis et al. 2003). However, the effects of GH on the growth of h-hepatocytes have not been studied in vivo at all yet. hGH is capable of stimulating rodent cells, whereas rodent GH cannot stimulate human cells because of its inability to bind to hGH receptors (hGHRs; Souza et al. 1995). Furthermore, it should be noted that hGH is not circulating in h-hepatocyte-chimeric mice, which indicates that h-hepatocytes in chimeric mice are in GH-deficient conditions. These facts and considerations strongly suggest that a chimeric mouse will provide an opportunity to examine the effects of hGH on growth of h-hepatocytes in vivo.

In this study, we examined the effects of hGH on the proliferation of h-hepatocytes using chimeric mice. The treatment of chimeric mice with hGH increased the repopulation speed and RI of transplanted h-hepatocytes, and up-regulated the GH-related signaling molecules. The present study shows that a h-hepatocyte-chimeric uPA/SCID mouse is a useful in vivo model to examine the effects of growth factors, cytokines, and hormones on h-hepatocytes.

Materials and Methods

Animals

The uPA/SCID mice weighing 6.3–10.0 g were produced as previously described (Tateno et al. 2004). The zygosity of the uPA24 transgene was determined by a multiplex PCR as previously described (Meuleman et al. 2003). Homozygous uPA/SCID mice were used as hosts throughout this study.

Transplantation of hepatocytes and bromodeoxyuridine (BrdU)-labeling

Cryopreserved h-hepatocytes from a 6-year-old Caucasian girl (6YG) and 46-year-old Caucasian man (46YM) respectively were purchased from In vitro Technologies (Baltimore, MD, USA) and thawed as previously described (Tateno et al. 2004). Trypan blue-exclusion test showed that the viability of 6YG- and 46YM-hepatocytes was 71.5±4.3% (n=3) and 72.2±2.3% (n=3) respectively. The h-hepatocytes (7.5×10^5 cell) were transferred into the inferior splenic pole of uPA/SCID mice at 20–30 days after birth, through a small left-flank incision (Tateno et al. 2004). BrdU (Sigma Chemical Co.) was intraperitoneally injected into chimeric mice at a dose of 50 mg/kg body weight at 1 h before killing. Histological sections were prepared from the liver and stained with anti-BrdU antibodies as described below.

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<th>h GH and liver-deficient immunodeficient mice</th>
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rGH treatment

The 6YG- and 46YM-hepatocyte-chimeric mice were divided into two groups at 1 day post-transplantation; recombinant hGH (rGH; Wako Pure Chemical Industries Ltd, Osaka, Japan)-treated (rGH+, experimental) and –untreated (rGH−, control) groups. rGH was dissolved in water and used for animal injection. Animals of experimental groups were daily administered from day 1 after transplantation to 1 day before the day of killing with rGH by subcutaneous injection at 2.5 μg/10 μl per g body weight.

Measurement of human albumin (hAlb) and human IGF-1 concentrations in mouse blood or sera

hAlb concentration in blood of a chimeric mouse is correlated with RI of transplanted hepatocytes (Tateno et al. 2004). Blood (2 μl) was collected from the tail vein of h-hepatocyte-chimeric mice. The blood hAlb concentrations were determined with a latex agglutination assay (Eiken Immunological Laboratory, Tokyo, Japan) or a hAlb ELISA quantitation kit (Bethyl Laboratories Inc., Montgomery, TX, USA). As a measure of GH/IGF-1 signaling in the chimeric mice, serum human IGF-1 (hIGF-1) concentrations were determined using a hIGF-I ELISA kit (R&D Systems Inc., Minneapolis, MN, USA).

Immunohistochemistry and measurement of RI

Frozen sections were prepared from chimeric livers, fixed in −20 °C acetone for 5 min and incubated with anti-human cytokeratin 8 and 18 (hCK8/18) antibodies (dilution, 1:25; MP Biomedicals, Aurora, OH, USA). The hCK8/18 antibodies reacted with h-hepatocytes but not with mouse (m)–hepatocytes. Formalin-fixed paraffin sections of chimeric livers were incubated with mouse anti-BrdU antibodies (dilution, 1:10; DakoCytomation, Glostrup, Denmark) and goat anti-hAlb antibodies (dilution, 1:1000; Bethyl Laboratories). The primary antibodies were visualized with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) or peroxidase- and dextran-conjugated anti-mouse immunoglobulins (Dako Envision +; DakoCytomation) with 3',3'-diaminobenzidine (Sigma) as substrates. The sections were counterstained with Mayer’s hematoxylin. RI was calculated as the ratio of area occupied by hCK8/18-positive hepatocytes to the entire area examined on immunohistochemical sections of six lobes (Tateno et al. 2004). The ratios of BrdU-positive nuclei to hAlb-positive h-hepatocytes were determined by counting at least 1000 cells in 10 to 15 randomly selected vision fields in sections.

Quantification of mRNA in the livers of chimeric mice

Total RNAs were purified from liver tissues by an R.Naesy mini kit (Qiagen). Using 1 μg total RNA by PowerScript reverse transcriptase (Clontech Inc.) and Random Primer oligonucleotides (Invitrogen Corp.), cDNAs were
synthesized according to the manufacturer’s instruction. The mRNAs of genes shown in Table 1 were quantified in the liver tissues of chimeric mice by real-time RT-PCR (Tateno et al. 2004). Genes were amplified with a set of gene-specific primers shown in Table 1 and SYBR Green PCR mix (Applied Biosystems, Tokyo, Japan) in PRISM 7700 Sequence Detector (Applied Biosystems). We confirmed that these primers for h-genes amplified the h- but not the m-genes. Real-time RT-PCR was performed as follows: initial denaturation step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 or 62 °C for 1 min. All data were treated as previously described (Livak & Schmittgen 2001). The expression levels of the tested genes were normalized to the expression level of human glyceraldehyde 3-phosphate dehydrogenase (hGAPDH) gene and human hypoxanthine phosphoribosyltransferase 1 (hHPRT-1) gene.

Statistical analysis

Data were analyzed with StatView, 5.0 (SAS Institute Inc., Cary, NC, USA). Results are shown as the mean ± S.E.M. or s.i., and the significance of the difference between two groups under comparison was analyzed by Student’s t-test when data were normally distributed and otherwise by Welch’s test.

Results

Relationship of engraftment with repopulation of h-hepatocytes

In this study, we aimed to quantitatively assess the effect of rhGH on the extent of repopulation of h-hepatocytes in chimeric mice. It was considered that the number of engrafted h-hepatocytes depends on the number of the injected cells and affects the time length to reach the final (maximal) RI. Thus, we first examined the relationship between the number of originally engrafted h-hepatocytes and affects the time length to reach the final occupancy rate of assuming that there is a linear relationship between the number of the originally engrafted h-hepatocytes, and 55–61 days as a measure of the number of the repopulated h-hepatocytes. The levels of ‘19–22 day’-group (hAlb19–22) are plotted against those of ‘55–61 day’-group (hAlb55–61; Fig. 1). The graph consisted of two regions, a near linear region in which hAlb55–61 increased with hAlb19–22 in a near linear fashion and a region of near plateau in which the increase of hAlb19–22 did not meaningfully increase hAlb55–61. It can be said that the plateau level (6–10 mg/ml) represented the maximal level (the maximal RI) of the occupancy of h-hepatocytes in the experimental conditions we adopted. In the case of 7.5×10^5 cell transplantation, most chimeric mice showed hAlb19–22 < 0.5 mg/ml, and hAlb55–61 was increased with the increase of hAlb19–22. This result supported the above assumption that h-hepatocytes near linearly increased in number with the increase in the number of the originally engrafted h-hepatocytes and they did not reach the maximal repopulation state until 55–61 days post-transplantation. However, it should be noted here that the hAlb level does not correctly reflect the number of the repopulated h-hepatocytes as we showed in the previous study (Tateno et al. 2004) and also in the present study.

In contrast, in the case of 10^4–3×10^5 cell transplantation, most of the chimeric mice showed hAlb19–22 > 0.5 mg/ml and reached the plateau level (>6 mg/ml) at 55–61 days, suggesting that there is an appropriate number of h-hepatocytes to be injected to obtain a high-engraftment rate (the number ratio of the engrafted h-hepatocytes to the injected h-hepatocytes). It is considered that the number of 7.5×10^5 was smaller than this appropriate number. It appeared in 10^4–3×10^5 h-hepatocyte transplantation experiments that the proliferation of

Table 1 Oligonucleotide primers used in PCR amplification of growth hormone (GH)-related genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’–3’)</th>
<th>Reverse primer (5’–3’)</th>
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<tr>
<td>hGHR</td>
<td>TCACATCAAGGTGAATTCACAC</td>
<td>GGGAGTGGCGAGCGTTG</td>
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<tr>
<td>hIGF-1</td>
<td>GCCTCCGGAATCTGATATA</td>
<td>CATCTGTGAGATTTAGCTGAC</td>
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<tr>
<td>hSTAT1</td>
<td>TTGCGAGCAAACGAGAAGCAAG</td>
<td>AATATTCCAACAGGTCCAAA</td>
</tr>
<tr>
<td>hSTAT3</td>
<td>GACACAAATCCCAAGAAGAT</td>
<td>GAGGAGTCTGCTGGGAACG</td>
</tr>
<tr>
<td>hFoxM1</td>
<td>GCATCTTCGCTCTCTGTTG</td>
<td>GCAATTCGAGATACTGAGT</td>
</tr>
<tr>
<td>hCdc25A</td>
<td>CAAAGAGAGGGAAGCAGATTC</td>
<td>CATGAGTGTATCTCTTCCG</td>
</tr>
<tr>
<td>hCdk1 B1</td>
<td>CCTGATGAACTACATAGG</td>
<td>CTGGAGAGGAGAGCTGAGG</td>
</tr>
<tr>
<td>hCdk1 D1</td>
<td>TGTGAATTTTTACAAATC</td>
<td>CATGGAGAGGAGAGCTGAGG</td>
</tr>
<tr>
<td>hCdk2</td>
<td>AAACATCAAGGTACAATTC</td>
<td>GGGTGAATCAGTATTCCTCAG</td>
</tr>
<tr>
<td>hGAPDH</td>
<td>CACCTCTGAGGTTGGT</td>
<td>CATGTTAGAAATCGGTGAG</td>
</tr>
<tr>
<td>hHPRT1</td>
<td>TGTCAGCGCATTATAC</td>
<td>CAGTTAGAGTTAGCAGC</td>
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SCID mice were transplanted with 10.0 × 10^5 6YG-hepatocytes (open circles). hAlb levels at 19–22 days post-transplantation (hAlb_19–22) were plotted against those at 55–61 days (hAlb_55–61). The hAlb_55–61 in the 7.5 × 10^4 cell-transplantation experiment were lineally correlated with those at the hAlb_19–22 (r^2 = 0.86, solid line). The hAlb_55–61 in the 10.0 × 10^5 cell-transplantation experiment were also lineally correlated with hAlb_19–22 in the range <0.5 mg/ml of hAlb (r^2 = 0.87, dotted line).

h-hepatocytes terminated around 60 days post-transplantation when the hAlb level reached 6 mg/ml irrespective of hAlb_19–22, probably due to the contact inhibition among the h-hepatocytes, the toxicity of h-hepatocytes for the mouse when hAlb exceeded 6 mg/ml, and other yet unknown reasons. In 10.0 × 10^5 cell-transplantation experiments, there were several mice that showed hAlb_19–22 <0.5 mg/ml. An apparent similar relationship between hAlb_19–22 and hAlb_55–61 was seen in these animals as in 7.5 × 10^3 h-hepatocyte transplantation experiments. Therefore, we empirically concluded that h-hepatocytes increased in number in an apparently quasilinear fashion after the engraftment from hAlb_19–22 <0.5 mg/ml to hAlb_55–61 < 6 mg/ml. Thus, we utilized chimeric mice with hAlb_19–22 <0.5 mg/ml for examining the effects of hGH on the proliferation of h-hepatocytes in the following experiments.

It has been generally recognized that hGH is capable of stimulating rodent cells, whereas rodent GHSs are not able to bind to GHRs on h-hepatocytes (Souza et al. 1995). Six uPA/SCID mice were transplanted with 10.0 × 10^5 6YG-hepatocytes/mouse. Half of them were treated with rGH. The blood hAlb levels were monitored throughout the experimental period (up to 55 days) after transplantation (Fig. 2A). The concentrations of hAlb rapidly increased and exceeded 0.5 mg/ml (0.5–1.36 mg/ml) around 20 days post-transplantation in all mice irrespective of the treatment of rGH and reached over 6 mg/ml around 50 days after transplantation. There were no differences in hAlb levels between rGH^- and rGH^+ chimeric mice. At the end of this experiment, mice were killed for determining RI on immunohistological sections prepared from their liver tissues (Fig. 2B). Also, there were no differences in RIs between the two groups. Serum hIGF-1 levels were determined for mice from rGH^- and rGH^+ groups. hIGF-1 was not detected in the animals of rGH^- groups whose hAlb concentrations were 8.2 ± 1.1 mg/ml (n = 3). In contrast hIGF-1 was detectable (11.9 ± 11.2 mg/ml, n = 3) in mice of rGH^+ groups whose hAlb concentrations were 7.9 ± 1.9 mg/ml (n = 3). Thus, the absence of hIGF-1 in sera of mice in rGH^- groups is explainable at least in part by assuming that h-hepatocytes did not respond to rGH in the chimeric mice, and when rGH was given, h-hepatocytes responded to it and up-regulated hIGF-1 expression.

Enhancement of the repopulation of h-hepatocytes by hGH

Nine and eight uPA/SCID mice were transplanted with 7.5 × 10^5 6YG- and 10.0 × 10^5 46YM-hepatocytes/animal, then five and four of them were treated with rGH respectively. Chimeric mice with both 6YG- and 46YM-hepatocytes showed variable hAlb levels, which were 0.01–0.6 and 0.0005–0.3 mg/ml at 20 days post-transplantation respectively. Three rGH^+ and rGH^- mice with 0.01–0.05 mg/ml hAlb each were selected from 6YG- and 46YM-chimeric mice. The rGH enhanced the increase of hAlb levels in both groups (Fig. 3). rGH^- animals slowly increased the values after 20 days post-transplantation, whereas rGH^+ mice rapidly increased them in 6YG-chimeric mice (Fig. 3A). The transplanted cells in rGH^- 46YM-mice also slowly grew as in 6YG-mice (Fig. 3B). The rGH also accelerated the repopulation in these mice, though its effect was considerably lower when compared with 6YG-hepatocyte mice.

The chimeric mice transplanted with 6YG- and 46YM-hepatocytes shown in Fig. 3A and B respectively were killed at 70 and 76 days post-transplantation for histological examinations respectively. There were no differences between rGH^+ and rGH^- mice in body weight and liver size at the time of killing. h-Hepatocytes were immunohistochemically identified as hCK8/18-positive cells (Fig. 3C through F). Distributions of the immunopositive 6YG- and 46YM-cells in rGH^- animals are shown in Fig. 3C and E respectively. h-Hepatocyte colony

Figure 2 Repopulation of h-hepatocytes in uPA/SCID mice. Each uPA/SCID mouse was transplanted with 10.0 × 10^3 6YG-hepatocytes. Animals in rGH^+ group were daily injected with rGH and hAlb concentrations in host blood were monitored (A). RIs of rGH^- (open bar) and rGH^+ 6YG-chimeric mice (closed bar) were determined at 55 days post-transplantation (B). Values represent the mean ± S.E.M. of three different mice.

Figure 1 Relationship between hAlb concentrations in early and late time points after transplantation. uPA/SCID mice were transplanted with 7.5 (closed circles) and 10.0 × 10^5 6YG-hepatocytes (open circles). hAlb levels at 19–22 days post-transplantation (hAlb_19–22) were plotted against those at 55–61 days (hAlb_55–61). The hAlb_55–61 in the 7.5 × 10^3 cell-transplantation experiment were lineally correlated with those at the hAlb_19–22 (r^2 = 0.86, solid line). The hAlb_55–61 in the 10.0 × 10^5 cell-transplantation experiment were also lineally correlated with hAlb_19–22 in the range <0.5 mg/ml of hAlb (r^2 = 0.87, dotted line).
sizes were larger in 6YG- than in 46YM-hepatocytes. The size was greatly increased in rhGH-cmice for both 6YG- (Fig. 3D) and 46YM-hepatocytes (Fig. 3F). Histological sections were prepared from six liver lobes of 6YG-chimeric mice at 70 days post-transplantation (C and D) and from those of 46YM-chimeric mice at 76 days post-transplantation (E and F). The sections were stained with anti-hCK8/18 antibodies. Sections ‘C and E’ and ‘D and F’ were from rhGH- and rhGH+ mice respectively. The cytoplasm of hCK8/18-positive h-hepatocytes is stained brown. Scale bar in F: 20 μm (G and H). RI was calculated as the ratio of the brown colored areas to the examined total ones for 6YG- (G) and 46YM-chimeric mice (H). Open and closed bars show rhGH- and rhGH+ mice respectively. Values represent the mean ± S.E.M. of three different mice. Asterisks indicate significant differences (* P<0.05, Student’s t-test).

Effects of rhGH on DNA synthesis of h-hepatocytes in chimeric mice

We investigated whether rhGH stimulated the DNA synthesis of h-hepatocytes in chimeric mice. uPA/SCID mice were transplanted with 6YG- or 46YM-hepatocytes, injected with...
rHGH, and were exposed with BrdU before killing at 2 weeks post-transplantation. BrdU-positive h-hepatocytes were often distributed in the peripheral regions of the colonies (Fig. 4A for 6YG-hepatocytes). The BrdU-labeling index of 6YG-hepatocytes in rHGH+ mice was 2.2-fold higher (P<0.05) than that in rHGH− mice (Fig. 4B), indicating that GH induced the entry of h-hepatocytes into the S-phase of the cell cycle. The index of rHGH+ 46YM-mice was 1.4-fold higher than that of rHGH− ones, but the difference was not significant.

Expression of hepatocyte growth-associated genes in chimeric livers

Previously, we showed the BrdU-labeling index of h-hepatocytes (9-month-old Caucasian boy) in chimeric mice at 1, 3, and 5 weeks post-transplantation was ~9, 5, and 2% respectively, and thereafter gradually decreased to <0.5% at 10–11 weeks (Emoto et al. 2005). Taking the present results shown in Fig. 4 and the above cited previous ones together, we considered that transplanted h-hepatocytes become most proliferative around 1 week post-transplantation, and, thus, hepatocyte growth-associated genes become activated then. However, there were not enough h-hepatocytes yet to yield sufficient RT-PCR amplification. Thus, we examined the effect of rHGH on the expression of hepatocyte growth-associated genes in h-hepatocytes of chimeric livers at 2 weeks point post-transplantation. Chimeric mice were treated with rHGH as in Fig. 4 and were killed at 2 weeks post-transplantation to determine mRNA levels of 10 genes by real-time RT-PCR: hGHR, hIGF-1, human signal transducers and activators of transcription (hSTAT) 1, hSTAT3, human forkhead box (hFox) M1, human cell division cycle (hCdc) 25A, h-cyclin B1, h-cyclin D1, human cyclin-dependent kinases (hCdk) 1, and hCdk2. The expression levels were normalized to that of hGAPDH gene. The ratios of the expression under rHGH+ to that under rHGH− are depicted as graphs for 6YG- (Fig. 5A) and 46YM-mice (Fig. 5B). rHGH did markedly increase hIGF-1 mRNA in both 6YG- (Fig. 5A) and 46YM-hepatocyte-chimeric mice (Fig. 5B; P<0.05, Student’s t-test or Welch’s test). The stimulation rate (9.1-fold) in 6YG-hepatocyte mice was much higher than that (2.6-fold) in 46YM-hepatocyte ones. The effects of rHGH were generally much prominent in chimeric mice bearing 6YG-hepatocytes as compared with that in those bearing 46YM-ones. In 6YG-hepatocyte-chimeric mice, rHGH significantly increased the expressions of mRNAs of hSTAT3, hFoxM1, hCdc25A, and h-cyclin D1 (P<0.05, Student’s t-test, Fig. 5A). The expression levels for mRNAs of hSTAT1, h-cyclin B1, hCdk1, and hCdk2 were higher in the rHGH+ group than in rHGH− group in 6YG-hepatocyte-chimeric mice, although the difference in the ratio between the two groups was not significant. Similarly, mRNAs of hSTAT1 and hSTAT3 were induced by rHGH in 46YM-hepatocyte-chimeric mice, although the difference was not significant (Fig. 5B). In contrast to the expression in 6YG-chimeric mice, rHGH did not induce mRNAs of hFoxM1, hCdc25A, h-cyclin B1, h-cyclin D1, hCdk1, and hCdk2 in 46YM-chimeric mice (Fig. 5B). 6YG-hepatocytes expressed hGHR mRNA at a 19.5-fold higher level than 46YM-hepatocytes in rHGH− group, suggesting differences in the responsiveness of these growth-related genes to rHGH might be due to the difference in GHR expression levels between the two donors. Similar results were obtained when the data were normalized by hHPRT-1 as another housekeeping gene.

Discussion

Hepatocytes of uPA/SCID mice undergo severe injury and, thus, genes of cytokines involved in liver regeneration are activated (Mars et al. 1995, Michalopoulos & DeFrances...
In contrast to such abundant studies on rodents, there have been no studies on the effects of GH on h-hepatocytes in vivo, because we considered that h-hepatocytes in chimeric mice are in GH-deficient conditions. This consideration was supported by the present study because we showed that hIGF-1 was actually undetectable in the chimeric mouse sera. We expected that rhGH treatment might enhance the proliferation of h-hepatocytes in uPA/SCID mice. Chimeric mice were yielded bearing h-hepatocytes from two donors whose sex and age were different, 6YG and 46YM, and were treated with rhGH. As a result, we were able to demonstrate for the first time that rhGH stimulates the proliferation of h-hepatocytes in vivo. This conclusion was reproducibly obtained from the two independent experiments using hepatocytes from different donors, although the extent of the stimulation was much higher for 6YG-hepatocytes than for 46YM-hepatocytes. This difference of GH-stimulation between the two donors might be explainable by the difference of GHR-expression level between them as shown in this study.

Studies on the molecular mechanisms of the action of GH are currently progressing in rodents. c-fos gene is an immediate early responsive gene to GH, which is mediated by STAT1 and STAT3 (Gronowski & Rotwein 1994, Gronowski et al. 1995, Herrington et al. 2000). Our study showed that rhGH increased the expression of hSTAT1 and hSTAT3 mRNAs in h-hepatocytes in chimeric mice. GH stimulates the growth of target cells through GHRs and its endocrine IGF-1 (Daughaday & Rotwein 1989). GH stimulates the synthesis and the secretion of IGF-1 by hepatocytes (Sjogren et al. 1999, Yakar et al. 1999). Secreted IGF-1 binds to the IGF-IR, which activates the expression of cell cycle-related genes such as cyclin D1 through ERK pathway (Desbois-Mouthon et al. 2006). Our present study showed that rhGH enhanced the expression of hIGF-1 and cyclin D1 mRNAs in the liver of chimeric mice. Therefore, it is concluded that GH stimulates the growth of h-hepatocytes through activating GH/IGF-1/IGF-IR/ERK signaling. These results suggest that the stimulation of growth of hepatocytes by GH is induced through similar mechanisms in both rodents and humans. Studies remain to be done on the protein phosphorylation or the activation of the signaling cascades after the rhGH-stimulation using currently developed h-hepatocyte-chimeric mice.

It was shown in rodents that GH increases the FoxM1 level (Krupczak-Hollis et al. 2003), which stimulates the cell cycle progression at both the G1/S- and G2/M-phase transitions (Wang et al. 2001, 2002a,b, 2005, Major et al. 2004). Progression through the cell cycle is regulated by the temporal activation of multiple families of Cdk. Cdc25A, Cdc25B, and Cdc25C with phosphatase activities are involved in the activation of Cdns in a way that these enzymes dephosphorylate catalytic units of Cdns (Sebastian et al. 1993). Upon S-phase progression, Cdc25A phosphatase activates Cdk2-cyclin E by dephosphorylating inhibitory Cdk2 residues (Massague 2004). Progression through the
G2/M transition requires the activation of the Cdk1–cyclin B complex through dephosphorylation and the activation of Cdk1 by the Cdc25B and Cdc25C phosphatases, the latter of which is activated by Polo-like kinase 1 phosphorylation (Barr et al. 2004). It is noteworthy that rGH up-regulated mRNAs of hFoxM1, hCdc25A, h-cyclin B1, h-cyclin D1, hCdk1, and hCdk2 in 6YG-hepatocytes, but not 46YM-counterparts. Thus, it can be said that GH activates cell cycle progression of h-hepatocytes as known in rodents. The phosphorylation levels of Janus activating kinase 2 and GHR complex were decreased with age of rats (Xu et al. 1995). In the present study we showed that 6YG-hepatocytes expressed hGHR mRNA at much higher levels than 46YM-hepatocytes. This apparent age-dependent GH-expression level of h-hepatocytes should be tested in further studies with sufficient samples of donor hepatocytes for statistical treatments of the obtained results.

In this study we demonstrated usefulness of a h-hepatocyte-chimeric uPA/SCID mouse as an in vivo model to study effects of GH on the proliferation of h-hepatocytes. h-Hepatocyte-chimeric mice were also yielded using another type of immunodeficient and liver-injured mice obtained by crossing uPA-transgenic mice with mice whose recombinant type of immunodeficient and liver-injured mice obtained by crossing uPA-transgenic mice with mice whose recombinant activation gene-2 (RAG-2) had been deleted (Dandri et al., 2001). It is worthy of examining in the future whether the effects of r/GH on h-hepatocytes observed in the present study can be reproduced in this uPA/RAG-2 mouse model. As clearly demonstrated for GH–GHR binding in the present study, h-hepatocytes in mice could be deficient for other growth factors and cytokines due to problems in interspecies ligand–receptor interaction. However, this limitation of h-hepatocyte-chimeric mice will provide us opportunities to study the mechanism of their interactions in vivo using chimeric mice in place of human body as exemplified for r/GH on h-hepatocytes in this study.

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References


Major ML, Lepe R & Costa RH 2004 Forkhead Box M1B (FoxM1B) transcriptional activity requires binding of Cdk/cyclin complexes for phosphorylation-dependent recruitment of p300/CBP co-activators. Molecular and Cellular Biology 24 2649–2661.


Stocker E & Heine WD 1971 Regeneration of liver parenchyma under normal and pathological conditions. Beiträge zur Pathologie 144 400–408.


Wang X, Krupczak-Hollis K, Tan Y, Dennewitz MB, Adami GR & Costa RH. 2002b Increased hepatic Forkhead Box M1B (FoxM1B) levels in old-aged mice stimulated liver regeneration through diminished p27kip1 protein levels and increased Cdc25B expression. *Journal of Biological Chemistry* **277** 44310–44316.


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