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<td>Fujiwara, Shinsuke; Fujioka, Hikaru; Tateno, Chise; Taniguchi, Ken; Ito, Masahiro; Ohishi, Hiroshi; Utoh, Rie; Ishibashi, Hiromi; Kanematsu, Takashi; Yoshizato, Katsutoshi</td>
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A novel animal model for \textit{in vivo} study of liver cancer metastasis

Shinsuke Fujiwara, Hikaru Fujioka, Chise Tateno, Ken Taniguchi, Masahiro Ito, Hiroshi Ohishi, Rie Utoh, Hiromi Ishibashi, Takashi Kanematsu, Katsutoshi Yoshizato

\textbf{Abstract}

\textbf{AIM:} To establish an animal model with human hepatocyte-repopulated liver for the study of liver cancer metastasis.

\textbf{METHODS:} Cell transplantation into mouse livers was conducted using alpha-fetoprotein (AFP)-producing human gastric cancer cells (h-GCCs) and h-hepatocytes as donor cells in a transgenic mouse line expressing urokinase-type plasminogen activator (uPA) driven by the albumin enhancer/promoter crossed with a severe combined immunodeficient (SCID) mouse line (uPA/SCID mice). Host mice were divided into two groups (A and B). Group A mice were transplanted with h-GCCs alone, and group B mice were transplanted with h-GCCs and h-hepatocytes together. The replacement index (RI), which is the ratio of transplanted h-GCCs and h-hepatocytes that occupy the examined area of a histological section, was estimated by measuring h-AFP and h-albumin concentrations in sera, respectively, as well as by immunohistochemical analyses of h-AFP and human cytokeratin 18 in histological sections.

\textbf{RESULTS:} The h-GCCs successfully engrafted, repopulated, and colonized the livers of mice in group A (RI = 22.0% ± 2.6%). These mice had moderately differentiated adenocarcinomatous lesions with disrupted glandular structures, which is a characteristics feature of gastric cancers. The serum h-AFP level reached 211.0 ± 142.2 g/mL (range, 7.1-324.2 g/mL). In group B mice, the h-GCCs and h-hepatocytes independently engrafted, repopulated the host liver, and developed colonies (RI = 12.0% ± 6.8% and 66.0% ± 12.3%, respectively). h-GCC colonies also showed typical adenocarcinomatous glandular structures around the h-hepatocyte-colonies. These mice survived for the full 56 day-study and did not exhibit any metastasis of h-GCCs in the extrahepatic regions during the observational period. The mice with an h-hepatocyte-repopulated liver possessed metastasized h-GCCs and therefore could be a useful humanized liver animal model for studying liver cancer metastasis \textit{in vivo}.

\textbf{CONCLUSION:} A novel animal model of human liver cancer metastasis was established using the uPA/SCID mouse line. This model could be useful for \textit{in vivo} testing of anti-cancer drugs and for studying the mechanisms of human liver cancer metastasis.
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Key words: Urokinase-type plasminogen activator/severe combined immunodeficient mouse; Mouse with humanized liver; Liver cancer metastasis; Alpha-fetoprotein-producing gastric cancer cells

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INTRODUCTION

Tumor metastasis, which is defined by a process in which tumor cells originating from an organ invade another anatomically distant organ, is the leading cause of cancer-related mortality[1-3]. One of the major target organs for cancer metastasis is the liver[4-7], and therefore there is increasing need for animal models that accurately mimic the pathophysiological situations in human liver and are suitable for investigating the mechanisms of hepatic cancer metastasis. In fact, several studies have attempted to transplant metastatic h-tumor cells into the livers of the immuno-compromized mice, such as athymic nude mice[8], which cannot generate T cells, severe combined immunodeficient (SCID) mice that lack mature B and T cells[9,10], and NOD/SCID/Il2Rγnull (NOG) mice[11-13], which are deficient in T, B, and natural killer cells, and have impaired dendritic cells. In these animal models, the transplanted h-tumor cells invade the hepatic parenchyma, which is composed of mouse hepatocytes that are phylogenetically distant from h-hepatocytes and are known to exhibit biological and pathological features that are different from the human counterpart.

Heckel et al[14] established transgenic mice expressing urokinase type plasminogen activator (uPA) under the control of the albumin (Alb) enhancer/promoter and found that the m-hepatocytes were constitutively damaged due to constant exposure to the expressed uPA. In another study, a mouse line possessing a humanized liver (chimeric mouse) was generated by transplanting healthy and normal h-hepatocytes into the liver of the immuno-and liver-compromised mouse, which was created by mating the uPA-Tg mouse with the SCID mouse (uPA/SCID mouse)[15,16].

We previously developed chimeric mice where the liver was stably and reproducibly replaced with h-hepatocytes and found that the occupancy ratio or replacement index (RI) in the parenchyma was quite high (> 90%) in best cases[17]. Human hepatocytes in the chimeric liver were stably and reproducibly replaced with h-hepatocytes and liver-compromized mouse, which was created by mating the uPA-Tg mouse with the SCID mouse (uPA/SCID mouse). In the present study, we established a chimeric mouse as a novel experimental model that sufficiently mimics the pathophysiological micro-environment in h-liver for studying liver cancer metastasis.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of the National Hospital Organization, Nagasaki Medical Center, the Hiroshima Prefectural Institute of Industrial Science and Technology Ethics Board, and the PhoenixBio Ethics Board. This study was conducted in accordance with their guidelines.

Animals

The uPA/SCID mice were generated and used as transplant hosts once they reached an age of 24-32 d old as previously described[18]. The mice were maintained in the laboratory in a specific pathogen-free environment in accordance with the guidelines of the Hiroshima Prefectural Institute of Industrial Science and Technology Ethics Board as well as the PhoenixBio Ethics Board.

Cancer cells

Human gastric cancer cells (h-GCCs) were purchased from the Japanese Collection of Research Biosources (Osaka, Japan) and used as liver metastatic cancer cells. These cells are adenocarcinoma cells derived from human gastric cancer cells that produce alpha-fetoprotein (AFP) and have a high affinity for liver tissue[19,20]. The cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma Chemical Co., St. Louis, MO, United States) containing 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO, United States) in an atmosphere of 95% air and 5% CO2 at 37 °C.

Cell transplantation into the uPA/SCID

Human GCCs were suspended at a concentration of 1 × 107 cells/mL and placed on ice until transplantation. Cryopreserved h-hepatocytes derived from a 6-year-old African female were purchased from BD Biosciences (San Jose, CA, United States), thawed in a 37 °C water bath, rapidly diluted with culture medium at 4 °C, and washed twice to remove the cryopreservation solution. The cell viability was assessed by a trypan blue exclusion test. The uPA/SCID mice were anesthetized with ether and then were intrasplenically injected with the h-hepatocytes as previously described[21]. Blood samples, 5 μL each, were periodically collected from the host tail-vein for...
determining concentrations of human albumin (h-Alb) and human AFP (h-AFP) using an h-Alb enzyme-linked immunosorbent assay quantification kit (Bethyl Laboratories Inc., Montgomery, TX) and an h-AFP enzyme immunoassay test kit (Hope Laboratories, Belmont, CA, United States), respectively.

Histological and immunohistochemical evaluation of the m-liver

Liver tissue specimens were removed from the transplanted mice, paraffin-embedded, sectioned at a 4 μm thickness, and stained with hematoxylin and eosin (H and E). Human hepatocyte-colonies were identified by staining the sections with mouse monoclonal antibodies against human-specific cytokeratin 18 (h-CK18) (DAKO, Glostrup Denmark). Human GCCs in the m-liver were identified by h-AFP staining with a polyclonal Ab (Novoceastra Laboratories Ltd., United Kingdom). The sections were treated with a biotinylated, goat anti-rabbit IgG for h-CK18 and rabbit anti-m-IgG (DAKO, Glostrup Denmark) for h-AFP. All of the tissue specimens or cells were counterstained with H and E.

**Determination of h-hepatocytes and h-GCCs repopulation of the uPA/SCID m-liver**

Serial liver sections were double immunostructures for h-CK18 and h-AFP to identify h-hepatocytes/h-GCCs and h-GCCs, respectively. The extent of repopulation of h-hepatocytes and h-GCCs in the chimeric mouse liver was determined as the RI, which is the occupational ratio of the transplanted cells in the examined area of histological sections, as previously described[12]. The RI of h-hepatocytes (RI_{h-hepatocytes}) in the uPA/SCID m-liver was determined using h-CK18 as a marker to histologically identify h-hepatocytes. When appropriate, the RI for h-GCCs (RI_{h-GCCs}) was referred to as the metastatic index (MI_{h-GCCs}) in this study. Human hepatocytes and h-GCCs were identified on histological sections as the h-CK18-positive (h-CK18⁺) and h-AFP-negative (h-AFP⁻) cells and the h-CK18⁻ and h-AFP⁺ cells, respectively. The RI_{h-hepatocytes} and MI_{h-GCCs} of the m-livers were calculated as the ratio of the “h-CK18⁻/h-AFP⁺” and “h-CK18⁻/h-AFP⁻” areas to the entire examined area of the sections, respectively.

### Table 1  Serum concentrations of human albumin and human alpha-fetoprotein in host mice at 56 d post-transplantation

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Transplanted cells</th>
<th>No. of animals</th>
<th>Serum concentration</th>
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<tr>
<td></td>
<td>h-GCCs</td>
<td>4</td>
<td>h-Alb (mg/mL)</td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
<td>UD</td>
</tr>
<tr>
<td>B</td>
<td>h-GCCs and h-hepatocytes</td>
<td>6</td>
<td>0.03-9.1</td>
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<td></td>
<td></td>
<td></td>
<td>(3.1 ± 3.5)</td>
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The numerals represent the range of the concentrations and those in the parentheses indicate the mean ± SD. h-GCCs: Human gastric cancer cells; h-Alb: Human albumin; h-AFP: Human alpha-fetoprotein; h-hepatocytes: Human hepatocytes; UD: Undetectable.

RESULTS

**Experimental groups**

The uPA/SCID mice were divided into two groups (A and B groups). Four uPA/SCID mice in group A were each injected with $1 \times 10^6$ h-GCCs. Six mice in group B were co-transplanted with $7.5 \times 10^6$ h-hepatocytes and h-GCCs each. The blood h-Alb and h-AFP concentrations were periodically monitored after cell transplantation. The mice were euthanized at the termination of the experiments and their livers, spleens, and lungs were microscopically examined to identify any metastasis of h-GCCs.

**Group A experiment**

Human GCCs were transplanted into the livers of uPA/SCID mice and euthanized 56 d after transplantation. Human GCC colonies were macroscopically distinguishable from the host m-liver cells as brown colored regions (Figure 1A). Histological examinations showed that these areas contained h-GCC colonies and host m-liver cells composed of m-parenchymal and m-nonparenchymal cells (Figure 1B). The whitish or pale regions observed in Figure 1A were composed of only m-liver cells. The specimens were also stained for h-AFP to define h-GCCs (Figure 1C and D). Human GCCs formed colonies with well-developed glandular structures, which is a characteristic feature of gastric cancer. The serum concentrations of h-AFP increased to $211.0 \pm 142.2$ g/mL (range 7.1-324.2 g/mL, Table 1), which reflected the repopulation of h-GCCs in the liver, since serum h-AFP was undetectable in uPA/SCID mice without transplantation of h-GCCs (data; not shown). The MI of h-GCCs (MI_{h-GCCs}) was 22.0% ± 2.6% at the termination of the experiment 56 d post-transplantation.

**Group B experiment**

Both h-hepatocytes and h-GCCs were simultaneously transplanted into six uPA/SCID mice. The serum concentrations of h-Alb and h-AFP monitored after the cell transplantation (Figure 2). These protein levels were variable among individual mice, and three mice (No. 1-3) had substantially elevated h-Alb levels over the 56-d study. In addition, these mice exhibited RI_{h-hepatocytes} > 70% based on the correlation graph between h-Alb concentrations and RI_{h-hepatocytes}. These hosts also had markedly elevated h-AFP concentrations. In particular, mice No. 1 and 2 showed the highest h-Alb levels (approximately 9.1 mg/mL) and h-AFP concentrations (approximately 126.1 mg/mL) at 56 d post-transplantation (Table 1; Figure 2). As shown in Figure 3A, mouse 1 had the highest h-Alb and h-AFP levels, and the liver was composed of brown and whitish regions indicated by the thick and thin arrows, respectively, which corresponded to the colonies composed of both h-hepatocytes and h-GCCs or m-liver cells, respectively. The brown region in the liver shown in Figure 3A was sectioned and stained with H and E (Figure 3B), anti-h-CK18 Abs to identify both h-hepatocytes and...
h-GCCs (Figure 3C), and the anti-h-AFP Ab to identify h-GCCs (Figure 3D). A comparison of Figure 3B and C showed that most of the section from Figure 3B was occupied with h-CK18+ cells, which corresponded to the cells in the less eosinophilic areas of the H and E section. Human CK18-m-liver cells were located in eosinophilic areas in the H and E section, which were sporadically distributed as clusters with variable forms among large engrafted h-cell colonies. h-GCCs formed moderately differentiated adenocarcinomas with disrupted glandular structures, which is a characteristic feature of gastric cancer. Morphometric analyses using these h-CK18- and h-AFP-stained serial sections indicated that the RI of hepatocytes in group B mice was 66.0% ± 12.3% (n = 6) and 12.0% ± 6.8% (n = 6), respectively. The mice in
group B survived for the entire 56 d study. Extrahepatic sites and organs, such as the peritoneal cavity and kidney, were also examined for the presence of metastatic h-GCC lesions. The metastatic h-GCCs were not found in the extrahepatic regions during the observational period, indicating that the cells did not metastasize to any other regions.

**DISCUSSION**

An ideal animal model for liver metastasis of h-cancer cells should possess at least two key features. First, the transplanted cancer cells need to invade and colonize in the host liver. Second, the liver of the host model has to provide the human cells with appropriate pathophysiological microenvironments that recapitulate the h-liver in vivo. Most of the conventional models to date manifest the first feature, but none of them have been able to sufficiently recapitulate the microenvironment of the h-liver in vivo. In the present study, we established a unique and novel model that possessed both of these features.

In our study, we successfully engrafted the liver with
h-GCCs in the group A mice, and the cells formed relatively large colonies, with the MI as high as 25% at 56 d post-transplantation. However, such a considerably high MI could be a result of effects from either the donor or host side of the model. We chose h-AFP h-GCCs as a metastatic cancer cell line, since previous studies reported that patients with AFP gastric cancer showed a higher liver MI than those with AFP cells; more than 70% of the patients developed liver metastasis.[18,19]. These AFP cancer cells express c-Met[19], which is the receptor for human hepatocyte growth factor (HGF), and therefore it is plausible that the cells have a high affinity for liver tissue under conditions where the levels of activated HGF in these tissues become high.[19,20]. In the present study, we utilized the uPA/SCID mice as hosts, which possessed a uPA transgene product that continuously damages the hepatocytes. In this model, the host hepatocytes generate pro-inflammatory environments in the liver, which stimulates the mobilization and expression of HGF in the liver tissues, including hepatocytes.

The role of uPA is an important aspect in this model. The host m-hepatocytes express unusually high levels of uPA, which is thought to induce severe damage in the replicative ability of m-hepatocytes through the activation of plasminogen, fibrinogen, and other proteins within the rough endoplasmic reticulum (RER) involved in proteolysis that lead to functional defects of the RER[21]. In addition, uPA is secreted from m-hepatocytes into the plasma[10], indicating that it circulates to liver tissues through sinusoidal capillaries and activates the conversion of blood plasminogen to plasmin. Therefore, the host liver tissue may provide h-GCCs with a pre-metastatic-like microenvironment. In fact, previous studies have indicated that uPA and its receptor (uPAR) play critical roles in the extravasation of tumors[22-24]. Therefore, the injected h-GCCs are prone to extravasate liver tissues through the portal vein and sinusoid because of the uPA-induced fragility of vascular and sinusoidal endothelia and subsequently engraft liver tissues through an affinity for c-Met. Once the h-GCCs invade liver tissues, they can relatively easily propagate due to c-Met signaling in the host parenchyma, and can consequently replace m-hepatocytes as a result of the uPA-mediated damage. These conditions are also convenient for engraftment and proliferation of normal, healthy h-hepatocytes, as shown in this study when co-transplanted with h-GCCs.

The co-transplantation of h-hepatocytes with h-GCCs also resulted in the development of metastatic colonies in the mice similar to the transplantation of h-GCCs alone. In this type of transplantation experiment, large variances in serum concentrations of replacement marker proteins (h-Alb and h-AFP) were observed. The h-AFP kinetic curves were different from those of h-Alb and exhibited an increase of the serum observed. The h-AFP kinetic curves were different from those of h-Alb and exhibited an increase of the serum.

Interactions between the invading cancer cells and the accepting host cells. There seemed to be two groups of animals within the experimental groups, one that more easily accepted xenogeneic cells and another that demonstrated resistance. However, we have consistently observed similar variances in h-Alb levels among individual mice when we generated h-hepatocyte chimeric mice[12], though inbred mice were used as hosts. These variances are accidental in nature and might originate from some differences in manipulation procedures for transplantation as well as uncontrollable differences in the phenotypes of the uPA Tg mice[10]. Despite these variances at the individual level, experimental group B of this study clearly demonstrated that we were able to reproducibly create mice whose livers were co-repopulated with healthy, normal h-hepatocytes and h-GCCs. Both h-hepatocytes and h-GCCs have high affinities for liver tissue, which drives engraftment of the liver and results in the generation of a humanized liver with metastatic cancer cells. We also found that the Rl-hepatocytes (66.0% ± 12.3%) was significantly higher than MI h-GCC (12.0% ± 6.8%), which may be a reflection of the difference in the inherent replication rates of the cells and adaptability to the host liver tissues. Our results indicate that h-hepatocytes are, as a whole, superior to h-GCCs in colony growth.

Relevant and reproducible animal models are indispensable tools for deducing the mechanisms of liver metastasis and pharmacokinetics of anti-cancer drugs, and several models have been developed to meet these practical needs, though they are quite limited[12,23-30]. Preclinical tests of anti-cancer drugs for their effectiveness and toxicity in relevant animal models are required prior to application in humans[10]. Toxicity data from non-primate species have been quite poor at predicting outcomes in subsequent human clinical trials, since there are significant differences in the metabolic activities of the hepatocytes between humans and rodent[22-34]. Therefore, animal models with a humanized liver are more physiologic and will provide better tools for analyzing the pharmacokinetics of anti-cancer drugs as well as studying cancer metastasis[35-37]. To our knowledge, no intrahepatic metastatic cancer model with a humanized liver has been available to date[23-30,35-37]. The m-liver in the present study was chimeric and was composed of normal h-hepatocytes and m-hepatocytes. Previous studies have reported that the h-hepatocytes in these chimeric livers are functional and secreted a variety of hepatic proteins, such as Alb, α1 antitrypsin, apolipoprotein A, apolipoprotein E, several clotting factors, and complement proteins present in h-plasma[10]. Transplanted h-hepatocytes also retain normal pharmacological responses, which makes the chimeric mouse model useful for studying the metabolism of compounds that cannot be easily administered to healthy volunteers[13,14,16]. In vitro studies using these mice showed their utility in evaluating the metabolism of drugs catalyzed by both phase I and phase II enzymes[13,15,19,60]. Since the liver functions of
the chimeric mice described in this study have not yet been characterized, future studies are needed to assess the model for anti-cancer drug testing. Taking together, the h-hepatocyte-chimeric mice may provide a useful bridge for studying human liver-related diseases because of the similarities with humans in physiological function and drug kinetics.

In conclusion, we have established a unique and novel animal model for studying liver cancer metastasis. The chimeric liver of the uPA/SCID mouse containing both human cancer cells and hepatocytes could be utilized as an appropriate model for in vivo testing of the efficacy and human-type metabolisms of candidate drugs for anti-cancer treatment as well as studying the mechanisms of liver cancer metastasis.

ACKNOWLEDGMENTS

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COMMENTS

Background

One of the major target organs for cancer metastasis is the liver, and therefore, there has been increasing needs for animal models that can sufficiently mimic the pathophysiological situation in human liver and that are suitable for investigating the mechanisms of hepatic cancer metastasis.

Research frontiers

An ideal animal model for liver metastasis of human cancer cells should possess at least two key features. First, the transplanted cancer cells need to invade and colonize the liver of the host. Second, the liver of the host model has to provide the human cells with appropriate pathophysiological microenvironments that recapitulate the human liver in vivo. In the present study, the authors established a unique and novel animal model with both of these features.

Innovations and breakthroughs

A liver-humanized mouse was generated by transplanting healthy and normal h-hepatocytes into urokinase type plasminogen activator/severe combined immunodeficient (uPA/SCID) mice (immuno- and liver-compromized mice), and the liver was stably and reproducibly replaced with human hepatocytes. This is the first report of a novel experimental model that sufficiently mimics the pathophysiological situation of human liver.

Applications

The chimeric liver of the uPA/SCID mouse containing both human cancer cells and hepatocytes could be utilized as an appropriate model for in vivo testing of anti-cancer drugs as well as studying the mechanisms of liver cancer metastasis.

Terminology

The uPA/SCID mouse is a transgenic mouse line that expressed uPA under the control of the albumin enhancer/promoter which constitutively damages the hepatocytes due to constant exposure to uPA. A liver-humanized mouse (chimeric mouse) was generated by transplanting healthy and normal human hepatocytes into mouse liver of the uPA/SCID mouse (immuno- and liver-compromized mouse), which had been generated by mating the uPA-Tg mouse with the SCID mouse. This mouse model sufficiently mimics the pathophysiological situation in human liver.

Peer review

This study tries to establish an animal model with hepatocyte-repopulated liver for in vivo study of liver cancer using uPA/SCID mouse, which could be useful for studying liver cancer metastasis. The authors transplanted uPA/SCID mouse either with human gastric cancer cells (h-GCCs) or h-GCCs with h-hepatocytes and observed that both colonies can repopulate mouse liver. The study is well conducted, the manuscript is well-written and the figures are of good quality.

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