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BRIEF ARTICLE

A novel animal model for *in vivo* study of liver cancer metastasis

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Abstract

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

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.

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 *in vivo*.

CONCLUSION: A novel animal model of human liver cancer metastasis was established using the uPA/SCID mouse line. This model could be useful for *in vivo* testing of anti-cancer drugs and for studying the mechanisms of human liver cancer metastasis.

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[1,2]. One of the major target organs for cancer metastasis is the liver[1-3], 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[4], which cannot generate T cells, severe combined immunodeficient (SCID) mice that lack mature B and T cells[5-7], and NOD/SCID/$_c^{null}$ (NOG) mice[8,9], 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*[10] 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-compromized mouse, which was created by mating the uPA-Tg mouse with the SCID mouse (uPA/SCID mouse)[10,11].

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[12]. Human hepatocytes in the chimeric m-liver have been intensively and extensively characterized based on normal hepatic phenotypes, such as expression profiles of cytochrome P450, the major xenobiotic-metabolizing enzymes, drug-metabolizing capacities, and hepatitis virus infectivity[11,13-15]. Based on these studies, which indicate that a chimeric m-liver can appropriately recapitulate the characteristics of h-liver, we hypothesized that the chimeric mouse as an animal model can be used to investigate the underlying mechanisms of tumor metastasis into the liver where the parenchyma is largely composed of normal and healthy h-hepatocytes.

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 Phoenix-Bio 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[14,15]. 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[16-18]. 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% CO_2 at 37 ℃.

Cell transplantation into the uPA/SCID

Human GCCs were suspended at a concentration of 1 × 10^7 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 ℃ water bath, rapidly diluted with culture medium at 4 ℃, 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[12]. Blood samples, 5 μL each, were periodically collected from the host tail-vein for

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

Experimental groups	Transplanted cells	No. of animals	Serum concentration	
			h-Alb (mg/mL)	h-AFP (mg/mL)
A	h-GCCs	4	UD	7.1-324.2 (211.0 ± 142.2)
B	h-GCCs and h-hepatocytes	6	0.03-9.1 (3.1 ± 3.5)	0.3-126.1 (54.3 ± 60.7)

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.

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 (Novocastra 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 immunostained 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 ($RI_{h\text{-}hepatocytes}$) in the uPA/SCID m- liver was determined using h-CK18 as a maker to histologically identify h-hepatocytes. When appropriate, the RI for h-GCCs ($RI_{h\text{-}GCCs}$) was referred to as the metastatic index ($MI_{h\text{-}GCCs}$) 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 $RI_{h\text{-}hepatocytes}$ and $MI_{h\text{-}GCC}$ 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.

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 1×10^6 h-GCCs. Six mice in group B were co-transplanted with 7.5×10^5 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.

RESULTS

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 ± 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 ($MI_{h\text{-}GCC}$) 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 $RI_{h\text{-}hepatocytes} > 70\%$ based on the correlation graph between h-Alb concentrations and $RIs_{h\text{-}hepatocytes}$[12]. 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 the 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

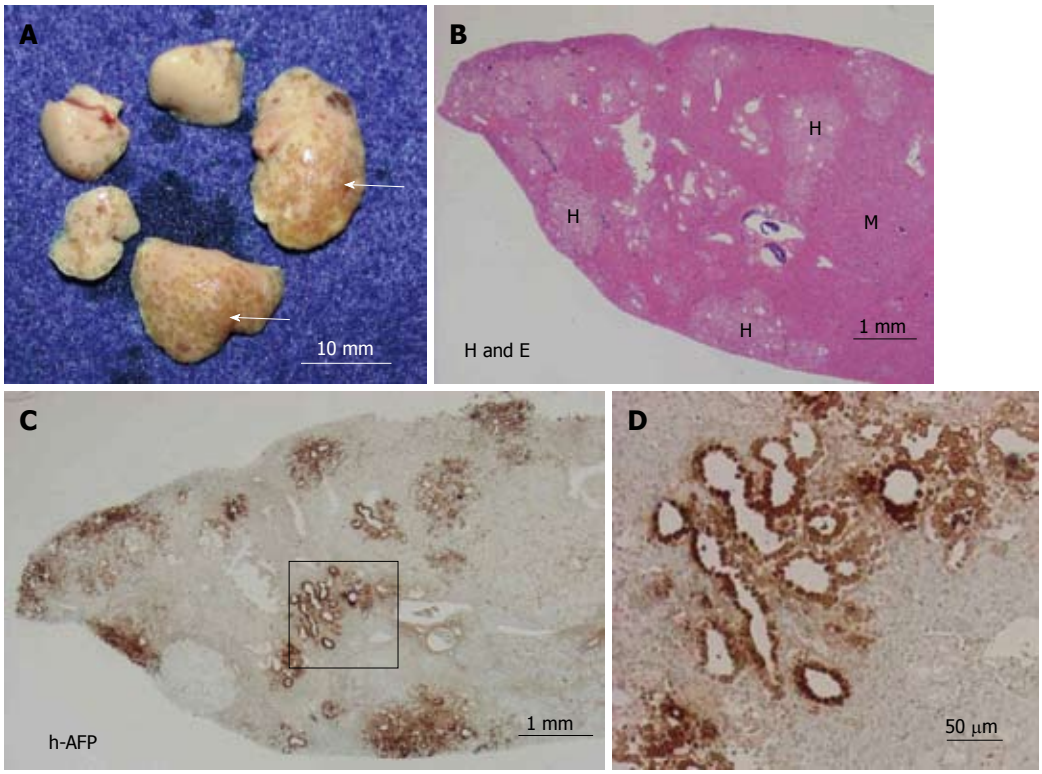

Figure 1 Macro- and microscopic images of the liver from group A mice. A: The urokinase-type plasminogen activator/severe combined immunodeficient mouse mice were transplanted with human gastric cancer cells (h-GCCs) and euthanized 56 d later, at which time the livers were isolated and photographed; B: The arrows in A point to concentrated regions of h-GCC colonies, and the sections were stained with hematoxylin and eosin (H and E). H and M in B represent h-GCC colonies and m-liver cell regions, respectively; C: The sections were stained with anti-human alpha-fetoprotein (h-AFP) antibodies; D: The square region in C is enlarged and shown.

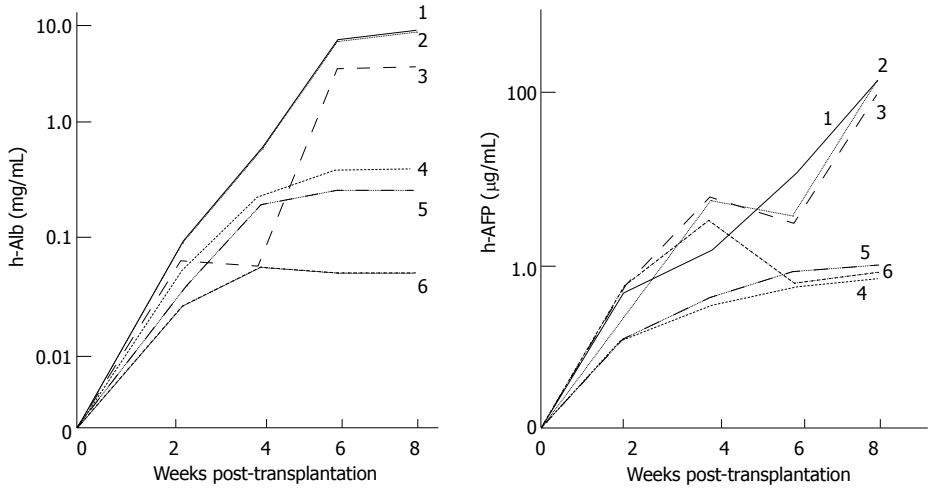

Figure 2 Changes in the serum concentrations of human albumin and human alpha-fetoprotein in group B-mice. Six mice (No.1-6) were co-transplanted with h-hepatocytes and human gastric cancer cells. The serum levels of human albumin (h-Alb) (left panel) and human alpha-fetoprotein (h-AFP) (right panel) were periodically monitored after the cell transplantation.

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. Human-AFP^{+} h-GCC-colonies were distinguished by comparing Figure 3B-D. These colonies were surrounded with less eosinophilic h-hepatocytes (Figure 3D) that were swollen and clearer (Figure 3B and C). Magnified views of the brown area obtained from another serial sections of the liver shown in Figure 3A are shown in Figure 4A (H and E) and Figure 4B (h-AFP-stain). Human 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 $RI_{h\text{-}hepatocyte}$ and $MI_{h\text{-}GCC}$ in group B mice was 66.0% ± 12.3% (n = 6) and 12.0% ± 6.8% (n = 6), respectively. The mice in

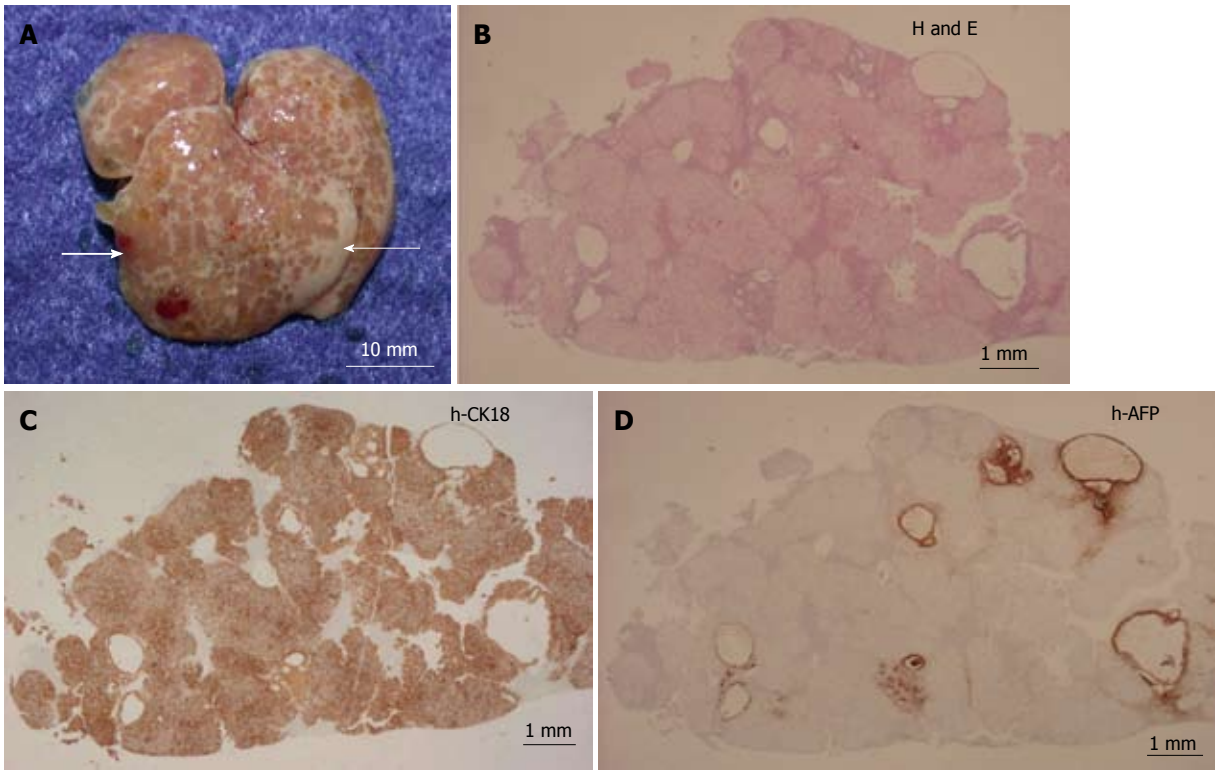

Figure 3 Macroscopic image of the liver of mouse No. 1 from Figure 2 at 56 d post-transplantation. A: The thick and thin white arrows point to h-cells [human hepatocytes (h-hepatocytes) and human gastric cancer cells (h-GCCs)] and m-liver cell regions, respectively; B: The liver was sectioned and stained with hematoxylin and eosin (H and E); C: The liver was sectioned and stained with anti-h-CK18; D: The liver was sectioned and stained with anti-human alpha-fetoprotein (h-AFP) antibodies. The h-AFP + (h-GCC) colonies were surrounded by less eosinophilic h-hepatocytes.

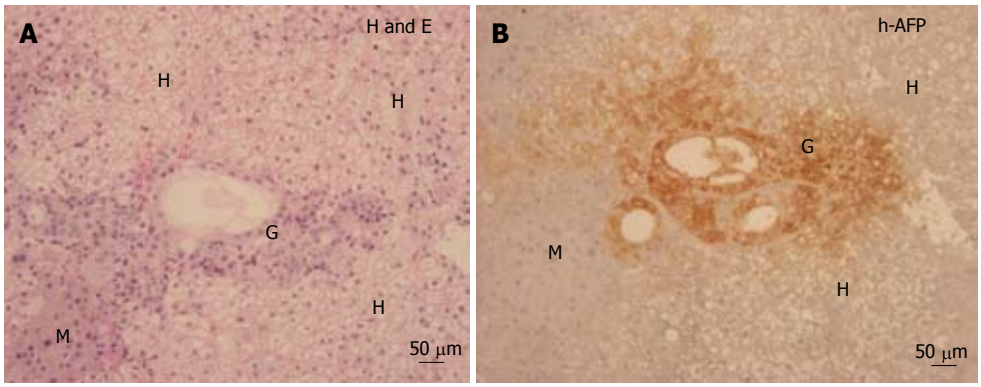

Figure 4 Magnified images of hepatic histology from group B mice. A: A serial section of the liver in Figure 3 was subjected to hematoxylin and eosin (H and E); B: A serial section of the liver in Figure 3 was subjected to human alpha-fetoprotein (h-AFP) staining. H, G and M represent the areas occupied by human-hepatocytes, human gastric cancer cells (h-GCCs), and host m-liver cells, respectively. h-GCCs composed moderately differentiated adenocarcinoma with disrupted glandular structures.

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 [4-6]. In the present study, we established a unique and novel 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 tissues under conditions where the levels of activated HGF in these tissues become high [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 pro-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 level through "three steps": initial increase, followed by a plateau or decline, and then a sharp increase. This complex h-AFP kinetic pattern suggests the presence of 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 $RI_{h\text{-}hepatocyte}$ (66.0% ± 12.3%) was significantly higher than $MI_{h\text{-}GCC}$ (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[2,25-30]. Preclinical tests of anti-cancer drugs for their effectiveness and toxicity in relevant animal models are required prior to application in humans[31]. 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[32-34]. 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[25,30,35-37]. 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, -1 antitrypsin, apolipoprotein A, apolipoprotein E, several clotting factors, and complement proteins present in h-plasma[38]. 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[14,15]. *In vivo* studies using these mice showed their utility in evaluating the metabolism of drugs catalyzed by both phase I and phase II enzymes[13-15,39,40]. 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.

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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 the *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 h-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 transfected 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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