(4) VA-coupled liposomes carrying the ROCK inhibitor significantly ameliorated systemic hypotension in comparison with VA-free liposomes carrying the ROCK inhibitor. These results provide evidence that the VA-coupled liposomal ROCK inhibitor selectively delivers the ROCK inhibitor to the liver and can attenuate IR injury in steatotic livers without detrimental adverse effects.

To confirm the specific delivery of VA-coupled liposomes to the liver in vivo, we administered VA-Lip-GFP to rats with steatotic livers. Using immunohistochemical analysis, we observed that VA-coupled liposomes were predominantly taken up by the liver and that the nonspecific uptake of VA-Lip-Y observed in the spleen occurred in a VA receptor–independent manner mediated by the reticuloendothelial system. These results are consistent with previous studies indicating that VA-coupled cationic liposomes are specifically taken up by the liver.[17] Furthermore, we showed that GFP accumulation in normal liver tissue sections included the desmin-stained area. These results suggest that VA-coupled cationic liposomes are specifically taken up by HSCs. However, the extent to which HSCs take up VA-Lip-Y remains unclear and requires further study.

The collagen gel contraction assay and the morphological studies showed that liposomal formation for Y-27632 produced biological effects at a dose of 0.1 μM liposome, whereas for nonliposomal Y-27632, a dose of 10 μM was necessary. These results indicated that liposomal Y-27632 was approximately 100-fold more effective than free Y-27632. The coupling of VA to the liposome as a ligand of HSCs had effects similar to those of the VA-free liposome in our in vitro study (Fig. 2B,C). Sato et al.[17] reported that a transduction time of 30 minutes should be used to distinguish between the receptor-mediated transduction efficacy of vitamin A-coupled liposomes with small interfering RNA and the nonspecific transduction efficacy of liposomes with small interfering RNA because the nonspecific lipofection rate will increase and become more similar to receptor-mediated transduction with longer transduction times. These morphological and contraction assays lasted for 24 and 2 hours, respectively. This result suggests that HSCs can take up liposomes without involving VA receptors.

VA-free liposomes carrying the ROCK inhibitor showed protective effects against IR injury in steatotic livers similar to those of VA-coupled liposomes carrying the ROCK inhibitor. These results suggest that in the steatotic liver, the ROCK inhibitor works not only by direct inhibition of HSCs[14,19] but also by other mechanisms such as the activation of nitric oxide synthase in SECs[23] or the inhibition of neutrophil infiltration.[24] Although this new drug, VA-Lip-Y, increases selective HSC targeting, other mechanisms may also contribute to improve IR injury. Indeed, it has been shown that microcirculatory disturbances are induced in steatotic livers and that steatotic livers lack swollen SECs, an early marker of SEC injury.[25]

Impaired hepatic microcirculation has been proposed as the main contributing factor for the low tolerance of steatotic livers for IR injury.[26] We have previously shown that improvements in microcirculatory disturbances by the ROCK inhibitor can aid in preventing IR injury in steatotic livers.[14] In the current study, the blood flow in the steatotic liver after IR significantly recovered with VA-Lip-Y and Lip-Y treatments in comparison with Y-27632 treatment. Additionally, the portal perfusion pressure after IR was lowered by Y-27632 or Lip-Y and VA-Lip-Y treatments (Fig. 6C). These results indicated that the ROCK inhibitor prevented HSC activation and relaxed HSC contraction to decrease the resistance of intrahepatic microcirculation. Therefore, these improvements in hepatic microcirculation may contribute to a reduction in IR injury in the steatotic liver. Furthermore, a significant improvement in the hepatic blood flow and a significant lowering of the portal perfusion pressure were observed in VA-Lip-Y–treated rats versus Lip-Y–treated rats (Fig. 6A,B). These results suggest that VA-coupled liposomes increase Y-27632 accumulation in HSCs. On the other hand, the liver blood flow after ischemia in rats treated with 10 mg/kg Y-27632 was significantly lower than that in untreated rats. This reduction in the hepatic blood flow may be attributed to Y-27632–induced severe hypotension (Fig. 6A,B). After reperfusion, the liver blood flow in the 10 mg/kg Y-27632–treated rats gradually increased, and this may have ameliorated IR injury, whereas the liver blood flow in the untreated rats did not increase during the quantification of the hepatic blood flow.

Targeting HSCs is an elegant approach to bypassing the problem of extrahepatic effects caused by nonhepatic ROCK inhibition. In the current study, the degree of hypotension induced by VA-Lip-Y was significantly less than that induced by Y-27632 at 10 mg/kg or by Lip-Y (Fig. 6D). This result indicates that VA-coupled liposomes increased the selectivity for livers and reduced nonspecific delivery to systemic blood vessels. However, the systemic injection of VA-coupled liposomes still induced mild hypotension (Fig. 6D). This mild hypotension induced by the venous injection of VA-Lip-Y was also thought to be due partially to the bolus injection of the drug. This mild hypotension may be alleviated by a 1-hour venous drip injection of VA-Lip-Y. Similarly, when the ROCK inhibitor fasudil is administered by venous drip infusion for 1 hour, it does not induce systemic hypotension.[23] Fasudil also induces detrimental systemic hypotension by bolus venous injection. Therefore, studies are required to investigate whether a 1-hour venous drip of the drug can prevent mild hypotension. In summary, VA-coupled liposomes used for the delivery of ROCK inhibitors can prevent IR injury in a rat model of a steatotic liver and ameliorate detrimental adverse effects.

REFERENCES

1. McCormack L, Petrowsky H, Jochum W, Furrer K, Clavien PA. Hepatic steatosis is a risk factor for postoperative complications after major hepatectomy: a matched case-control study. Ann Surg 2007;245:923-930.

2. Gomez D, Malik HZ, Bonney GK, Wong V, Toogood GJ, Lodge JP, Prasad KR. Steatosis predicts postoperative morbidity following hepatic resection for colorectal metastasis. Br J Surg 2007;94:1395-1402.
3. Trevisani F, Colantoni A, Caraceni P, Van Thiel DH. The use of donor fatty liver for liver transplantation: a challenge or a quagmire? J Hepatol 1996;24:114-121.
4. Hui AM, Kawasaki S, Makuuchi M, Nakayama J, Ikegami T, Miyagawa S. Liver injury following normothermic ischemia in steatotic rat liver. Hepatology 1994;20:1287-1293.
5. Caraceni P, Ryu HS, Subbotin V, De Maria N, Colantoni A, Roberts L, et al. Rat hepatocytes isolated from alcohol-induced fatty liver have an increased sensitivity to anoxic injury. Hepatology 1997;25:943-949.
6. Zhang JX, Bauer M, Clemens MG. Vessel- and target cell-specific actions of endothelin-1 and endothelin-3 in rat liver. Am J Physiol 1995;269(pt 1):G269-G277.
7. Hennenberg M, Biecker E, Trebicka J, Jochem K, Zhou Q, Schmidt M, et al. Defective RhoA/Rho-kinase signaling contributes to vascular hypocontractility and vasodilation in cirrhotic rats. Gastroenterology 2006;130:838-854.
8. Trebicka J, Leifeld L, Hennenberg M, Biecker E, Eckhardt A, Fischer N, et al. Hemodynamic effects of urotensin II and its specific receptor antagonist palosuran in cirrhotic rats. Hepatology 2008;47:1264-1276.
9. Trebicka J, Hennenberg M, Laleman W, Shelest N, Biecker E, Schepke M, et al. Atorvastatin lowers portal pressure in cirrhotic rats by inhibition of RhoA/Rho-kinase and activation of endothelial nitric oxide synthase. Hepatology 2007;46:242-253.
10. Schepke M, Heller J, Paschke S, Thomas J, Wolff M, Neef M, et al. Contractile hyporesponsiveness of hepatic arteries in humans with cirrhosis: evidence for a receptor-specific mechanism. Hepatology 2001;34:884-888.
11. Bosch J, García-Pagán JC. Complications of cirrhosis. I. Portal hypertension. J Hepatol 2000;32(suppl 1):141-156.
12. Hennenberg M, Trebicka J, Sauerbruch T, Heller J. Mechanisms of extrahepatic vasodilation in portal hypertension. Gut 2008;57:1300-1314.
13. Zhou Q, Hennenberg M, Trebicka J, Jochem K, Leifeld L, Biecker E, et al. Intrahepatic upregulation of RhoA and Rho-kinase signalling contributes to increased hepatic vascular resistance in rats with secondary biliary cirrhosis. Gut 2006;55:1296-1305.
14. Kuroda S, Tashiro H, Igarashi Y, Tanimoto Y, Nambu J, Oshita A, et al. Rho inhibitor prevents ischemia-reperfusion injury in rat steatotic liver. J Hepatol 2012; 56:146-152.
15. Ogawa T, Tashiro H, Miyata Y, Ushitora Y, Fudaba Y, Kobayashi T, et al. Rho-associated kinase inhibitor reduces tumor recurrence after liver transplantation in a rat hepatoma model. Am J Transplant 2007;7:347-355.
16. Etienne-Manneville S, Hall A. Rho GTPases in cell biology. Nature 2002;420:629-635.
17. Sato Y, Murase K, Kato J, Kobune M, Sato T, Kawano Y, et al. Resolution of liver cirrhosis using vitamin A–coupled liposomes to deliver siRNA against a collagen-specific chaperone. Nat Biotechnol 2008;26:431-442.
18. Kikuchi A, Aoki Y, Sugaya S, Serikawa T, Takakuwa K, Tanaka K, et al. Development of novel cationic liposomes for efficient gene transfer into peritoneal disseminated tumor. Hum Gene Ther 1999;10:947-955.
19. Mizunuma K, Ohdan H, Tashiro H, Fudaba Y, Ito H, Asahara T. Prevention of ischemia-reperfusion-induced hepatic microcirculatory disruption by inhibiting stellate cell contraction using rock inhibitor. Transplantation 2003;75:579-586.
20. Elinav E, Ali M, Bruck R, Brazowski E, Phillips A, Shapira Y, et al. Competitive inhibition of leptin signaling results in amelioration of liver fibrosis through modulation of stellate cell function. Hepatology 2009;49:278-286.
21. Sohail MA, Hashmi AZ, Hakim W, Watanabe A, Zipprich A, Groszmann RJ, et al. Adenosine induces loss of actin stress fibers and inhibits contraction in hepatic stellate cells via Rho inhibition. Hepatology 2009;49:185-194.
22. van der Heijden M, Versteilen AM, Sipkema P, van Nieuw Amerongen GP, Musters RJ, Groeneveld AB. Rho-kinase-dependent F-actin rearrangement is involved in the inhibition of PI3-kinase/Akt during ischemia-reperfusion-induced endothelial cell apoptosis. Apoptosis 2008;13: 404-412.
23. Anegawa G, Kawanaka H, Yoshida D, Konishi K, Yamaguchi S, Kinjo N, et al. Defective endothelial nitric oxide synthase signaling is mediated by rho-kinase activation in rats with secondary biliary cirrhosis. Hepatology 2008;47:966-977.
24. Takeda K, Jin MB, Fujita M, Fukai M, Sakurai T, Nakayama M, et al. A novel inhibitor of Rho-associated protein kinase, Y-27632, ameliorates hepatic ischemia and reperfusion injury in rats. Surgery 2003;133:197-206.
25. McCuskey RS, Ito Y, Robertson GR, McCuskey MK, Perry M, Farrell GC. Hepatic microvascular dysfunction during evolution of dietary steatohepatitis in mice. Hepatology 2004;40:386-393.
26. El-Badry AM, Moritz W, Contaldo C, Tian Y, Graf R, Clavien PA. Prevention of reperfusion injury and microcirculatory failure in macrosteatotic mouse liver by omega-3 fatty acids. Hepatology 2007;45:855-863.

IJC
International Journal of Cancer

Fatty liver creates a pro-metastatic microenvironment for hepatocellular carcinoma through activation of hepatic stellate cells

Yoshihiro Mikuriya, Hirotaka Tashiro, Shintaro Kuroda, Junko Nambu, Tsuyoshi Kobayashi, Hironobu Amano, Yuka Tanaka and Hideki Ohdan

Department of Gastroenterological and Transplant Surgery, Applied Life Sciences, Institute of Biomedical & Health Sciences, Hiroshima University, Japan

Fatty liver (FL) is associated with development of hepatocellular carcinoma (HCC). However, whether FL itself promotes the progression of HCC is unclear. We recently found that hepatic stellate cells (HSCs) were prominently activated in the steatotic liver. Here, we investigated whether steatotic livers promote HCC progression and whether HSCs of steatotic liver are associated with HCC progression. We implanted rat HCC cells into diet-induced steatotic livers in rats *via* portal vein injection. Thereafter, HSCs and HCC cells were co-implanted subcutaneously into nude rats. Migration and proliferation of HCC cells were measured, and activation of ERK and Akt in these cells was determined by western blotting. Chemokines secreted from HSCs and HCC cells were also evaluated by ELISA. Steatotic livers significantly promoted HCC metastasis compared with non-steatotic livers. Additionally, co-implantation of HCC cells with HSCs from steatotic livers produced significantly larger tumors in recipient rats as compared to those induced by HCC cells co-implanted with HSCs from normal livers (NLs). HSCs isolated from steatotic livers, compared with HSCs isolated from NLs, secreted greater amounts of interleukin-1α, vascular endothelial growth factor, and transforming growth factor-β. These cytokines may enhance the proliferation and migration of HCC cells by increasing the phosphorylation of ERK and Akt in HCC cells. Moreover, we noted that the Rho-kinase inhibitor deactivated activated HSCs and attenuated HCC progression. In conclusion, the rat steatotic liver microenvironment favors HCC metastasis, and this effect appears to be promoted by activated HSCs in the steatotic liver.

Non-alcoholic fatty liver disease (NAFLD) is one of the most common hepatic disorders in developed countries. The epidemic of obesity in developed countries has increased along with its attendant complications, including metabolic syndrome and NAFLD. Recently, there is increasing evidence that NAFLD, including the more aggressive non-alcoholic steatohepatitis (NASH), is associated with hepatocellular carcinoma (HCC).[1–3] Diabetes and obesity are established independent

Key words: HCC, hepatic stellate cell, fatty liver, Rho-kinase

Abbreviations: CDD: choline-deficient diet; CM: conditioned media; DMEM: Dulbecco's modified Eagle's medium; ELISA: enzyme-linked immunosorbent assay; FL: fatty liver; FBS: fetal bovine serum; HCC: hepatocellular carcinoma; HGF: hepatocyte growth factor; HSCs: hepatic stellate cells; HSCFL: hepatic stellate cells isolated from fatty liver; HSCNL: hepatic stellate cells isolated from normal liver; HFD: high-fat diet; HIF: hypoxia inducible factor; IL-1α: interleukin-1α; IQGAP1: IQ motif containing GTPase activating protein 1; MMPs: matrix metalloproteinases; MTT: methyl thiazolyl tetrazolium; MAPK: mitogen-activated protein kinase; NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatotic hepatitis; NL: normal liver; ROCK: Rho-associated kinase; SASP: senescence-associated secretory phenotype; SDF-1: stromal derived factor-1; TIMP-1: tissue inhibitor metalloproteinase-1; TGF-βRII: TGF-β receptor II; TGF-β: transforming growth factor-β; VEGF: vascular endothelial growth factor

Additional Supporting Information may be found in the online version of this article.

Brief description: Although fatty liver is associated with hepatocarcinogenesis, it is unclear whether fatty liver promotes hepatocellular carcinoma (HCC) progression. Through and in vivoimodels, we investigated whether steatotic liver promotes HCC progression and whether steatotic liver hepatic stellate cells (HSCs) are associated with HCC progression. Activated fatty liver HSCs significantly contributed to HCC proliferation and migration, and exhibited increased secretion of interleukin-α, vascular endothelial growth factor, and transforming growth factor-β in the tumor microenvironment.

Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology; **Grant number:** 21591748

DOI: 10.1002/ijc.29096

History: Received 8 Dec 2013; Accepted 10 July 2014; Online 23 July 2014

Correspondence to: Hirotaka Tashiro, Department of Gastroenterological and Transplant Surgery, Applied Life Sciences, Institute of Biomedical & Health Sciences, Hiroshima University, 1-2-3, Kasumi, Hiroshima 734-8551, Japan, Tel.: +81-82-257-5222, Fax: +81-82-257-5224, E-mail: htashiro@hiroshima-u.ac.jp

What's new?
Fatty liver is associated with hepatocellular carcinoma (HCC), but its exact role has been unclear. In this study, the authors found that HCC metastasis was increased in rats whose livers were steatotic. They also found that when hepatic stellate cells (HSCs) from rats with steatotic livers were co-injected with HCC cells into normal rats, the resulting tumors were significantly larger than when normal HSC cells were used. The increased levels of cytokines secreted by activated fatty-liver HSCs may enhance proliferation and migration of HCC.

risk factors for the development of HCC,[3] and obesity is reported to be an independent risk factor for HCC recurrence after curative treatment, such as hepatectomy in patients with NASH.[4] It has been reported that obesity and fatty liver (FL) promote a chemical carcinogen-induced hepatocarcinogenesis.[5,6] However, the functional impact of FL on the progression and metastasis of HCC remains largely unexplored.

Hepatic stellate cells (HSCs) are key contributors to liver fibrosis and portal hypertension.[7–9] Recently, these cells were postulated to form a component of the pro-metastatic liver microenvironment because they can transdifferentiate into highly proliferative and motile myofibroblasts, which have been implicated in desmoplastic reactions and metastatic growth.[10,11] Moreover, HSC activation has been shown to correlate with the severity of hepatic steatosis.[12,13] Therefore, activated HSCs in FL may enhance the progression of HCC, but this possibility has not been fully explored. Therefore, we investigated whether FL in rats has a microenvironment that can promote the progression of HCC, and whether the HSCs in FL enhance the progression of HCC.

Material and Methods

Animals

Four-week-old male Buffalo and F344 nude rats were purchased from Clea Japan, (Tokyo, Japan), and F344 rats were purchased from Charles River Breeding Laboratories (Osaka, Japan). Four-week-old rats were fed a choline-deficient diet (CDD) (Oriental Yeast Co., Tokyo, Japan) for 6 weeks or a high-fat diet (HFD) for 16 weeks (F2HFD2, 82% kcal fat; Oriental Yeast Co.) to promote the development of FL. All animal experiments were performed according to the guidelines set by the United States National Institutes of Health (1996).

Cell lines

The rat HCC cell line McA-RH7777 was obtained from the American Type Culture Collection (Rockville, MD). The rat HCC cell lines C1 and L2 were kindly provided by Dr. K. Ogawa, National Institute of Health Sciences (Tokyo, Japan). The McA-RH7777 cell line originated in Buffalo rats, whereas the C1 and L2 cell lines originated in F344 rats. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 at 37°C.

Isolation of HSCs

HSCs were isolated from rat livers according to previously described procedures.[14] The purity of the cells was estimated through ordinal light and fluorescence microscopic examinations and by indirect enzyme immunoreactivity with an anti-desmin antibody (Dako, Versailles, France).

Conditioned medium

Conditioned medium (CM) was harvested from cultured HSCs after incubation in serum-free DMEM for 48 hr. At the end of the incubation period, the medium was stored at −80°C until use.

Proliferation assay

HCC cells were seeded at 5,000 cells per well in 96-well plates and cultured overnight in DMEM supplemented with 10% FBS. The medium was then changed to serum-free DMEM and CM. Incubations continued for 24 hr before the addition of 3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide [methyl thiazolyl tetrazolium (MTT), nonradioactive proliferation assay; Promega Corp, Madison, WI] for 4 hr. Cellular MTT was solubilized with acidic isopropanol, and the optical density was measured at 570 nm by using a 96-well plate reader. The survival fraction was then quantified.

Migration assay

For studies of HCC cell migration, 8-μm-pore size Transwell chambers (Corning, NY) were used. In total, 5×10^5 HSCs were seeded into the lower chamber that was coated with collagen type I in 1 mL of medium containing 10% FBS, and cultured for 48 hr. No HSCs were added to the control wells. The medium was changed to 750 μL of RPMI supplemented with 0.1% bovine serum albumin, and 2×10^4 HCC cells in 200 μL of RPMI with 0.1% bovine serum albumin were added to the upper chamber. After incubation at 37°C in 5% CO_2 for 24 hr, the non-migrating cells on the upper surface of the membrane were removed with a cotton swab. Cells were fixed in 4% paraformaldehyde and stained with propidium iodide solution (Dojindo, Kumamoto, Japan). Migrating cells were counted at 200× magnification in nine adjacent microscope fields for each membrane.

Enzyme-linked immunosorbent assay

The amount of vascular endothelial growth factor (VEGF), tissue inhibitor of metalloproteinases 1 (TIMP-1), matrix metalloproteinase-9 (MMP-9), transforming growth factor-β1 (TGF-β1), and interleukin 1α (IL-1α) were quantified using ELISA kits, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Western blot analysis

Cells were cultured in DMEM without 10% FBS overnight. Thereafter, the cells were incubated in the presence of CM for 10 min before being homogenized in lysis buffer (Cell Lysis Buffer; Cell Signaling Technology, Danvers, MA). Western blot analysis was performed as described previously.[14] Antibodies to β-actin were purchased from Abcam (Tokyo, Japan). Antibodies to Akt, p-Akt, anti-p44/42, mitogen-activated protein kinase (MAPK), and anti-phospho-p44/42 MAPK antibodies were purchased from Cell Signaling Technology (Beverly, MA). The phosphorylation levels were normalized to the levels of total Akt or MAPK protein expression.

Experimental model of intrahepatic HCC metastasis

HCC cells (5×10^6 cells or 5×10^5 cells/body) were implanted into the livers of rats *via* portal vein injection. In rats that were fed on a CDD, normal diets were given after the injection of the HCC cells, whereas in rats fed with a HFD, the HFD was continued until the study was completed. At the end of the experiment, rats were humanely sacrificed. The area of the liver occupied by tumor was calculated by averaging the percentage of the liver area occupied by tumor in microscopic sections continuously cut at 5-mm intervals.

Confocal immunofluorescence and histology

Phalloidin staining of isolated HSCs was performed as described previously.[14] Samples were observed under a conventional fluorescence microscope or a laser confocal microscope. For histological analysis, formalin-fixed liver tissue sections were cut, stained with hematoxylin–eosin, and examined microscopically. To assess the grade of the steatosis, sections were stained with oil red O.

HSC/tumor cell co-implantation model

C1 cells (5×10^6 cells/body) were implanted into the subcutis of F344 nude rats, either alone or in combination with HSCs isolated from F344 rats (5×10^6 cells/body). Successful implantation of HSCs was determined *via* frozen section analysis of the co-implantation of fluorescently labeled HSCs and HCC cells. The HSCs were fluorescently labeled with red fluorescent linker dye (PKH26 Red Fluorescent Cell Linker Kit; Sigma, Sigma-Aldrich, St Louis, MO) according to the manufacturer's instructions. At the end of the experiment, rats were humanely sacrificed. The largest tumor diameter and the tumor weight were measured 4 weeks after implantation. The survival of HSCs and cellular proliferation were assessed by immunohistochemical analysis of desmin and Ki-67 (BD Pharmingen, San Jose, CA), respectively.

Treatment of HSCs by Y-27632

The specific Rho-associated kinase (ROCK) inhibitor, Y-27632, was purchased from Wako (Osaka, Japan). Activated HSCs were deactivated by incubation with Y-27632 (10 μM) in serum-free DMEM for 3 hr.

Statistical analysis

The survival rates of rats were compared using the Kaplan–Meier method and were analyzed using the log-rank test. The tumor engraftment rates were compared by the chi-square test. One-way analysis of variance was used for multiple comparisons. All the data are expressed as the average (±SE). p Values less than 0.05 were considered statistically significant. Statistical analyses were performed with the SPSS software, version 16 (SPSS Japan, Tokyo, Japan).

Technical and material details of the cytokine assay are given in the Supporting Information Materials.

Results

CDD induced FL, activation of HSCs and increased secretion of cytokines

Feeding on a CDD for 6 weeks resulted in severe steatotic changes (60% macrosteatosis) (Supporting Information Fig. S1*a*). The purity of the isolated HSCs was estimated by fluorescence microscopic examination and by indirect enzyme immunoreactivity with an anti-desmin antibody, and was found to be >90% (Supporting Information Fig. S1*b*). HSCs isolated from FL (HSCFL) of rats fed on a CDD for 6 weeks had significantly increased stress fiber formation compared to HSCs isolated from normal liver (HSCNL) (Supporting Information Fig. S1*c*). We assessed the mediators secreted by cells in monoculture by performing cytokine arrays on CM samples. TIMP-1 and VEGF were detected in CM harvested from HSCFL, whereas they were not detected in CM harvested from HSCNL (Supporting Information Fig. S1*d*–S1*f*).

CDD-induced FL has a permissive microenvironment for HCC metastasis

To assess the effects of FL on HCC metastasis, we first implanted McA-RH7777 cells (5×10^5 cells/body) *via* the portal vein into the livers of syngeneic Buffalo rats. One of seven rats with normal livers (NLs) developed a small, single nodular HCC; all seven rats survived more than 8 weeks after inoculation of the HCC cells. In contrast, all ten rats with FL fed on a CDD for 6 weeks developed diffusely distributed tumors, and five of ten died of HCC within 8 weeks (Figs. 1*a*–1*c*). Volumes of the HCC tumors were significantly greater in rats with FL than in rats with NL (Fig. 1*d*).

In other experiments, L2 cells (5×10^6 cells/body) were injected into the portal veins of F344 rats that were fed on either a CDD or a normal diet. In all eight rats fed on a CDD for 6 weeks, multiple nodular liver tumors developed within 8 weeks, and four of these rats also developed pulmonary metastases. In contrast, none of the five rats fed on a normal diet developed tumors (Supporting Information Figs. S2*a* and S2*b*).

HSCFL stimulate HCC cell proliferation and migration *in vitro*

First, we investigated whether HSCFL could induce the proliferation of McA-RH7777 and C1 HCCs. When these cells were cultured with CM harvested from syngeneic HSCFL,

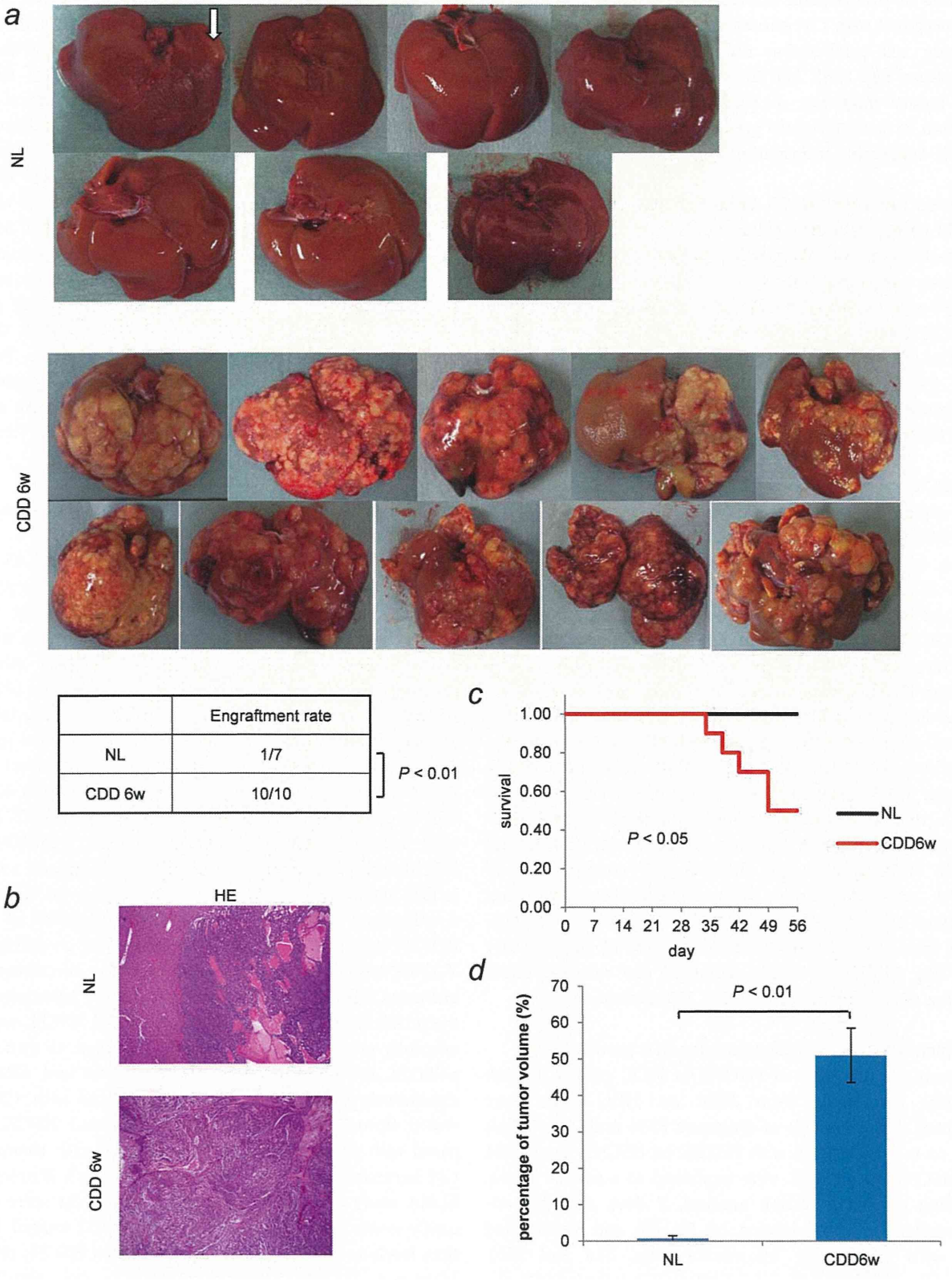

Figure 1. Choline deficient diet-induced FL has a permissive microenvironment for HCC metastasis. (*a*) Macrography of liver tumors in NL and FL. One of seven rats with NL developed a single nodular tumor (arrow) after inoculation of HCC cells (McA-RH7777 cells, 5×10^5 cells/body), whereas all ten rats with FL developed diffusely distributed liver tumors. (*b*) Hematoxylin-eosin-stained images of liver tumors in NL and FL. (*c*) Kaplan–Meier curve documenting the survival of rats inoculated with HCC cells. (*d*) The graph shows the percentage of tumor volume (tumor volume/liver volume). Results are presented as means (±SE).

their rate of proliferation increased, whereas when the cells were incubated with CM harvested from HSCNL, no increase in tumor cell proliferation occurred (Fig. 2*a*, Supporting Information Fig. S3*a*). We then examined whether HSCFL could induce migration of HCCs. HSCFL promoted HCC migration to a significantly greater extent than did HSCNL (Fig. 2*b*, Supporting Information Fig. S3*b*).

Factors secreted from HSCFL enhanced activation of MAPK and Akt pathways in HCC cells

To determine the signaling pathways that may be involved in the tumor-promoting effects of HSCFL, we examined McA-RH7777 cells treated with CM harvested from a monoculture of HSCFL for activation of Akt and MAPK by western blotting. The phosphorylation levels of Akt and ERK were significantly increased in cells treated with CM harvested from monoculture of HSCFL in comparison with CM harvested from HSCNL (Fig. 2*c*).

HCC-HSC cross-talk is bidirectional

We analyzed the secretion of VEGF, TGF-β1, IL-1α, MMP-9 and TIMP-1 by HCC cells and HSCs in monoculture and co-culture, using ELISA kits. Only an additive effect of VEGF and TGF-β1 was noted, and their levels were significantly increased in the CM harvested from co-culture of HCC cells and HSCFL compared with the levels in CM harvested from co-culture of HCC cells and HSCNL (Figs. 2*d* and 2*e*). Although HSCNL, HSCFL, and HCC cells barely secreted IL-1α and MMP-9, both factors were secreted in CM harvested from co-culture of HCC cells and HSCNL. Furthermore, the concentrations of IL-1α and MMP-9 were significantly increased in CM harvested from co-culture of HCC cells and HSCFL, due to a synergistic effect, compared to concentrations in CM harvested from co-culture of HCC cells and HSCNL (Figs. 2*f* and 2*g*). However, the concentration of TIMP-1 was significantly lower in CM harvested from co-culture of HCC cells and HSCFL compared to CM harvested from monoculture of HSCFL because of an inhibitory effect (Fig. 2*h*). These results suggested that cytokine secretions are altered through HCC-HSC interactions.

Co-implantation with HSCFL promotes HCC growth *in vivo*

We examined the effect of HSCFL on HCC growth *in vivo*. C1 cells, originating from F344 rat HCC cells, were implanted to the subcutis of syngeneic F344 nude rats, either alone or in combination with HSCFL or HSCNL from F344 rats. HSCNL and HSCFL were implanted to a similar extent, according to frozen section analysis, 2 days after the co-implantation of HSCs labeled by PKH26 and non-labeled HCC cells (Supporting Information Figs. S4*a* and S4*b*). When C1 cells (5×10^6 cells/body) were implanted into the subcutis of syngeneic F344 nude rats, three of the nine rats implanted with HCC cells alone and six of nine rats implanted with HCC cells and HSCNL developed tumors at the site of implantation, but all nine rats implanted with HCC cells and HSCFL developed tumors. Moreover, the rats implanted with HSCFL in addition to HCC cells developed significantly larger tumors than those implanted with HCC cells and HSCNL (Figs. 3*a*–3*d*). The presence of desmin-positive cells was determined by immunohistochemical analysis. The number of desmin-positive cells in the tumors of rats co-transplanted with HSCFL was comparable with those co-transplanted with HSCNL, whereas only a few desmin-positive cells were noted in tumors of rats implanted with C1 cells alone (Supporting Information Figs. S4*c* and S4*e*). In addition, we used immunohistochemistry to examine the expression of nuclear Ki-67, a cellular proliferation marker. The number of Ki-67-positive cells was significantly greater in the tumors of rats co-transplanted with HSCNL than in the tumors of rats transplanted with C1 cells alone. Furthermore, the number of Ki-67-positive cells was significantly increased in the tumors of rats co-transplanted with HSCFL compared with those co-transplanted with HSCNL (Supporting Information Figs. S4*d* and S4*f*).

Rho-kinase inhibitor attenuates HCC progression through deactivation of HSC-FL

We have previously shown that HSCFL exhibited increased stress-fiber formation and F-actin expression as compared to HSCNL,[14] and increased stress-fiber formation and F-actin expression in HSCFL were suppressed by treatment with the ROCK inhibitor, Y-27632. However, HSCFL were re-activated within 48 hr of Y-27632 administration (Fig. 4*a*). In this study, we investigated whether the ROCK inhibitor can deactivate activated HSCFL and suppress tumor progression in co-culture. First, we investigated the effect of Y-27632-treated HSCFL on the proliferation of McA-RH7777 cells by performing the MTT assay. When McA-RH7777 cells were cultured with CM harvested from Y-27632-treated HSCFL, cell proliferation was suppressed compared with that in CM harvested from untreated HSCFL (Fig. 4*b*). Thereafter, a migration assay showed that the migration of McA-RH7777 cells was significantly suppressed in co-culture with Y-27632-treated HSCFL compared with co-culture with untreated HSCFL (Fig. 4*c*). Furthermore, the enhancement in the proliferation and migration by Y-treated HSCFL were significantly greater than those of HSCNL (Figs. 4*b* and 4*c*). In addition, the phosphorylation levels of Akt and ERK were significantly decreased in HCC cells treated with CM harvested from monoculture of Y-27632-treated HSCFL compared with phosphorylation levels in HCC cells treated with CM harvested from untreated HSCFL (Fig. 4*d*). Moreover, an ELISA study showed that the levels of TGF-β1 were significantly lower in CM harvested from Y-27632-treated HSCFL than levels in CM harvested from untreated HSCFL (Fig. 4*e*). Moreover, the concentration of IL-1α was significantly decreased in CM harvested from a co-culture of HCC and HSCFL treated with Y-27632, compared to that in CM harvested from a co-culture of HCC and untreated HSCFL (Fig. 4*f*). However, the levels of VEGF and TIMP-1 in CM

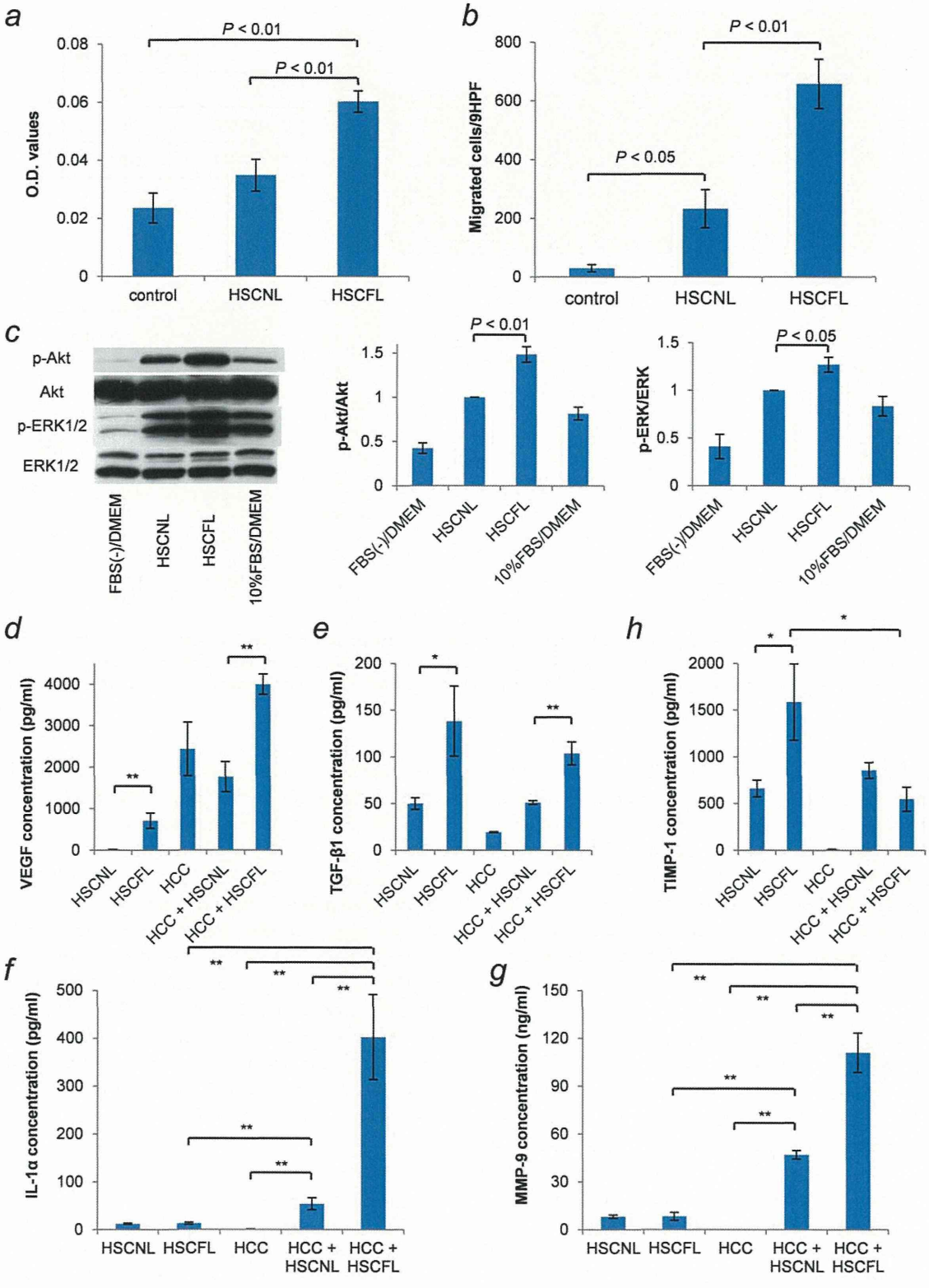

Figure 2. HSCFL promoted HCC progression *in vitro*. (*a* and *b*) HSC-FL promoted HCC proliferation and migration. (*c*) Protein lysates from McA-RH7777 cells treated with conditioned media from HSCNL or HSCFL were analyzed for Akt and ERK1/2 activation. The phosphorylation levels were normalized to total protein expression. (*d–h*) Cytokine secretion from McA-RH7777 cells and HSCs in monoculture and co-culture, using ELISA kits. Data shown are presented from more than 5 independent experiments. *$p < 0.05$, **$p < 0.01$.

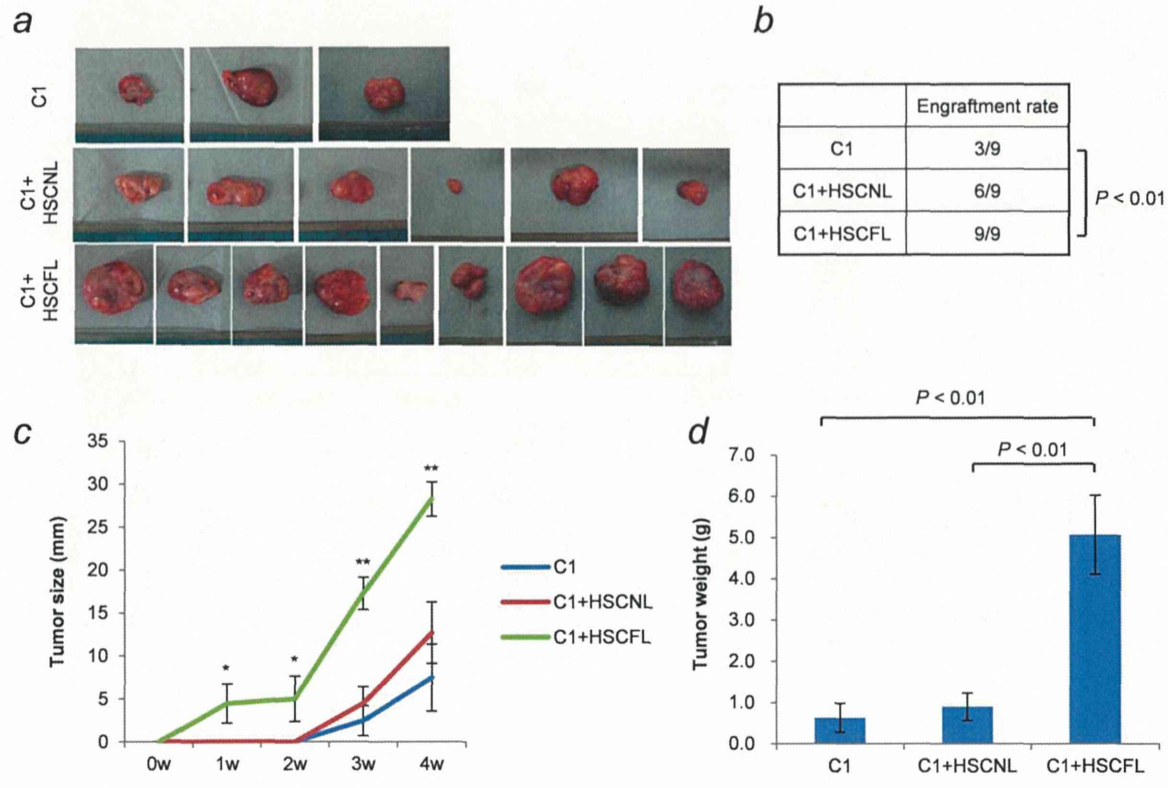

Figure 3. HSCFL promoted HCC progression *in vivo*. (*a*) Macrography of subcutaneous tumors in each group. (*b*) The table shows the engraftment rate in each group. (*c*) Tumor sizes were measured weekly. Data are presented as means (±SE). *$p < 0.05$, **$p < 0.01$ compared to the C1+HSCNL group (*d*) The graph shows the average tumor weight 4 weeks after co-implantation. Data are presented as means (±SE).

harvested from Y-27632-treated HSCFL and the levels of MMP-9 and TIMP-1 in CM harvested from a co-culture of HCC and HSCFL treated with Y-27632 were similar to those in CM harvested from untreated HSCFL and in CM harvested from a co-culture of HCC and untreated HSCFL, respectively (Supporting Information Figs. S5*a*–S5*d*). These results indicated that Rho-kinase inhibitor did not inhibit the overall features of HSC, but partially inhibited the activation of HSC.

High fat diet-induced FL also has a permissive microenvironment for HCC metastasis

We assessed the effect of HFD-induced FL (non-CDD-induced FL) on HCC metastasis. Rats fed on a HFD for 16 weeks developed microvesicular steatosis [approximately 20–30% steatosis, (Supporting Information Fig. S6*a*)], although the HFD-induced FL appeared less fibrotic compared to CDD-induced FL (Supporting Information Fig. S6*b*). Furthermore, the serum levels of AST were significantly lower in rats with HFD-induced FL than in those with CDD-induced FL (Supporting Information Fig. S6*c*). All the six rats fed on a HFD for 16 weeks developed several nodular tumors at 8 weeks after the inoculation of McA-RH7777 cells (5×10^5 cells/body). In contrast, one of the seven rats fed on a normal diet for 16 weeks developed several small tumors (Figs. 5*a* and 5*b*). Volumes of the HCC tumors were significantly greater in rats with HFD-induced FL than in rats with NL (Fig. 5*c*).

HSC derived from HFD-induced FL stimulate HCC cell proliferation and migration *in vitro*

We investigated whether HSCFL of rats fed on a HFD for 24 weeks (HSCHFD) could induce proliferation and migration of HCC cells. HSCHFD promoted HCC migration to a significantly greater extent than did HSC isolated from NL of rats fed on a normal diet for 24 weeks (Fig. 5*d*). In addition, HSCHFD promoted HCC proliferation to a significantly greater extent than did HSC isolated from NL of rats fed on a normal diet for 24 weeks (Fig. 5*e*).

Discussion

A rapidly growing literature indicates that NAFLD, including NASH, is associated with HCC.[1–3] However, whether NAFLD itself promotes the progression and metastasis of HCC is unclear. Therefore, we investigated whether FL either promotes or suppresses HCC progression. Our results have shown that both CDD- and HFD-induced FL have pro-metastatic microenvironments in the model of portal vein HCC cell inoculation. Furthermore, HSCs were activated in

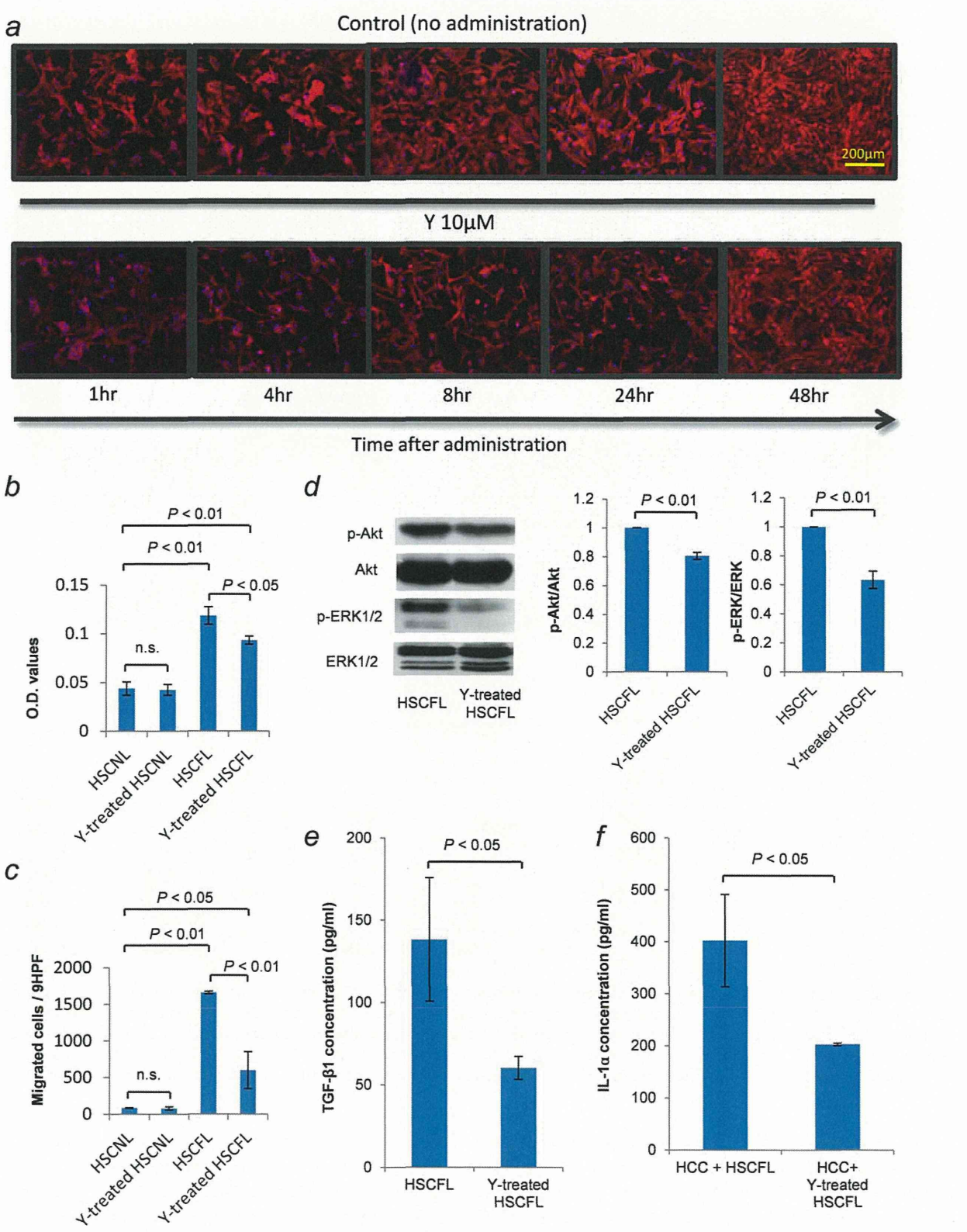

Figure 4. The Rho-kinase inhibitor, Y-27632, attenuated the HCC-promoting effect of HSCFL. (*a*) Stress fiber and F-actin expression in HSCFL and Y-27632-treated HSCFL (Y-treated HSCFL). (*b*) Conditioned media harvested from Y-treated HSCFL (Y-treated HSCFL-CM) significantly suppressed the proliferation of McA-RH7777 compared with that of untreated HSCFL-CM. (*c*) Migration of McA-RH7777 under the stimulation of Y-treated HSCFL was significantly suppressed compared with that of untreated HSCFL. (*d*) The phosphorylation levels of Akt and ERK were significantly suppressed in HCC cells treated with Y-treated HSCFL-CM, compared to those in HCC cells treated with untreated HSCFL-CM. (*e*) Y-27632 suppressed TGF-β1 secretion of HSCFL. (*f*) Y-27632 suppressed IL-1α secretion on co-culture of HCC and HSCFL.

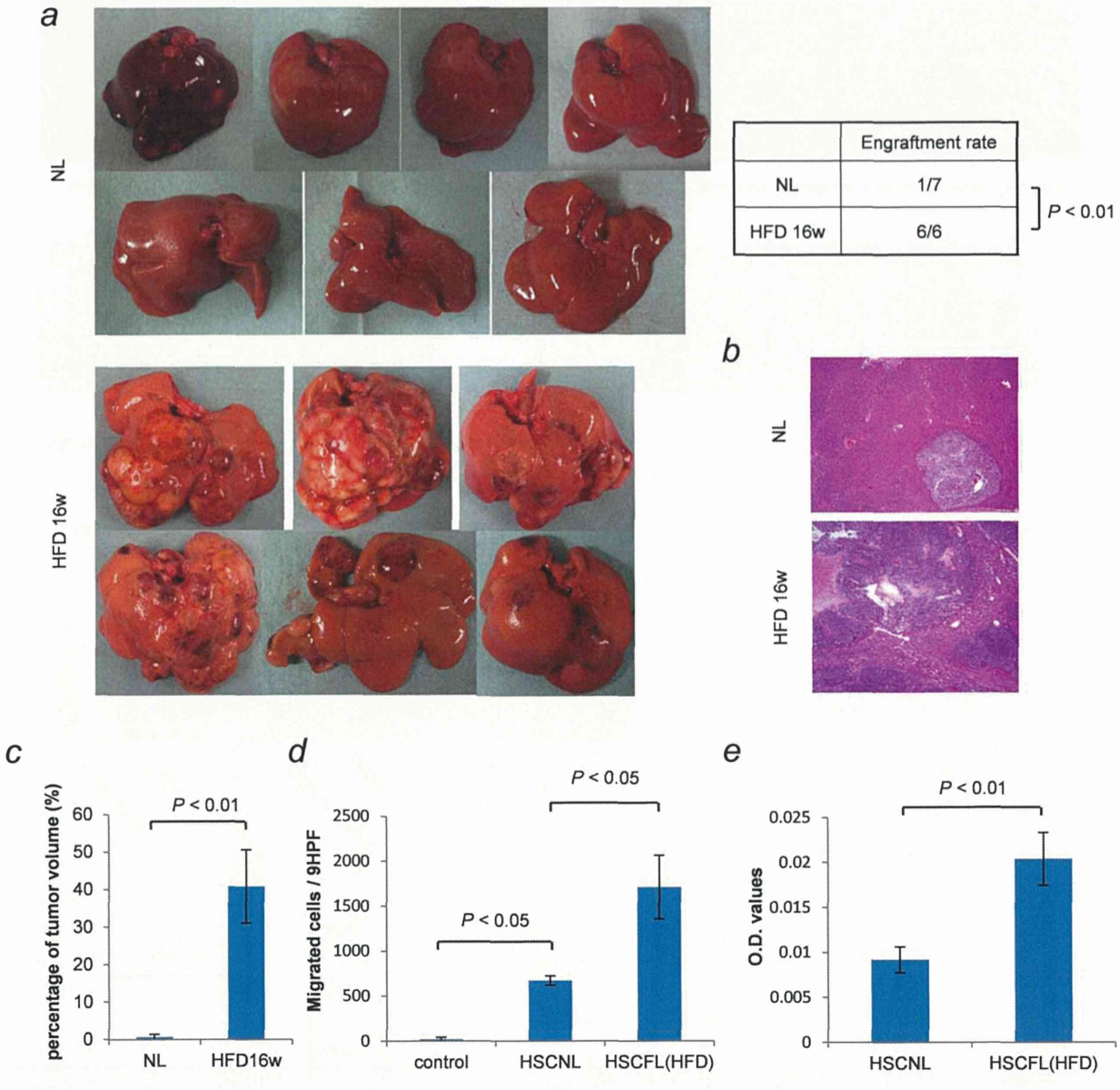

Figure 5. High fat diet-induced FL also provides a permissive microenvironment for HCC metastasis and progression. (*a*) Macrography of liver tumors in NL and high fat diet (HFD)-induced FL. Only one of seven rats with NL developed several nodular tumors after inoculation of HCC cells (McA-RH7777 cells, 5×10^5 cells/body), whereas all six rats with FL induced by feeding of a HFD for 16 weeks developed multiple nodular liver tumors. (*b*) Hematoxylin–eosin-stained images of liver tumors in NL and HFD-induced FL. (*c*) The graph shows the percentage of tumor volume in the liver. (*d* and *e*) HSCs derived from HFD-induced FL stimulated HCC migration and proliferation *in vitro*.

both CDD- and HFD-induced FL, and these activated HSCs enhanced the proliferation and migration of HCC cells. In addition, Y-27632, a Rho-kinase inhibitor, partially reduced the progression of HCC through deactivating activated HSCs. These results indicated that the steatotic liver microenvironment favors HCC progression and metastasis through the activation of HSCs.

In the current study, we have shown that CDD-induced FL activates HSCs to enhance the proliferation and migration of HCC in co-culture and co-implantation models through the secretion of paracrine signaling molecules such as VEGF. HSCs can transdifferentiate into highly proliferative and motile myofibroblasts during the activation process that follows liver injury.[8] In addition, we noted that the HSCs derived from HFD-induced FL, which appeared less fibrotic compared with CDD-induced FL, promoted the migration and proliferation of HCC cells, even though the HSCs may not be fully activated. Free fatty acids such as oleate and

palmitate reportedly stimulate the activation of fibrosis-related genes (*i.e.*, TGF-β, TIMP-1) in HSCs.[15] Activated HSCs produce growth factors and cytokines, such as TGF-β, hepatocyte growth factor (HGF), stromal-derived factor-1 (SDF-1), and IL-1, to stimulate the proliferation, adhesion, and migration of cancer cells.[16,17] It has been postulated that HSCs are a component of the prometastatic liver microenvironment.[10,11] Neaud *et al.*[18] showed that myofibroblasts increased the invasiveness of HCC cells by secreting HGF. Recently, Liu *et al.* demonstrated that the IQ motif containing GTPase-activating protein 1 (IQGAP1) binds to TGF-β receptor II (TGF-βRII) and suppresses TGF-βRII-mediated signaling in HSCs, thus preventing myofibroblastic differentiation. IQGAP1 deficiency in HSCs promoted myofibroblast activation, tumor implantation, and metastatic growth *via* upregulation of paracrine signaling molecules, including SDF-1/CXCL12 and HGF.[19] Our results are consistent with that report. Yoshimoto *et al.*[6] showed that senescence-associated secretory phenotype (SASP)[20] plays crucial roles in promoting obesity-associated HCC development in mice. In the current study, some of the SASP factors, such as IL-1α, CXCR2-binding chemokines, and TGF-β were increased in activated HSCs. These activated HSCs may be senescing, and this effect may be related to the promotion of metastatic growth of HCCs, but further study of this possibility is necessary. Sancho-Bru *et al.*[21] have examined the effect of hepatocarcinoma cells on HSCs in a co-culture system, and have reported the interaction of HSCs and HCC cells. In that study, co-culture of the cells reduced the expression of fibrogenic factors, such as procollagen-αI(I). Those results may be consistent with our results indicating the presence of decreased levels of TIMP-1 and increased levels of MMP-9 in CM harvested from co-culture of HCC cells and HSCs. Coulouarn *et al.* also showed that hepatocyte-HSC cross-talk generated a permissive proangiogenic microenvironment by inducing VEGF and MMP9 expression in HSCs.[22] Our results indicate that HCCs and HSCs have bidirectional cross-talk; that is, this interaction is proangiogenic and tumorigenic, but also antifibrogenic. These paradoxical results may have important implications for the progression of HCC. It is speculated that the remodeling of the extracellular matrix, along with the formation of new vessels, contributes to the invasiveness of HCC.

We have also shown that a ROCK inhibitor converted activated HSCs to inactivated HSCs, thereby suppressing the progression of HCC. The Rho signaling pathway and actomyosin system are reportedly involved in the motility and invasion of various cells, including cancer cells.[23–25] It is known that Rho signaling is involved in HSC activation, and a specific ROCK inhibitor, Y-27632, inhibited the activation of HSCs by regulating the formation of actin fibers and focal adhesion.[25–27] Our results are consistent with those reports. However, the production of TGF-β and IL-1α was suppressed by Y-27632, whereas HSCs reverted by treatment with Y-27632 still secreted several cytokines, including VEGF and MMP-9. These data indicated that HSCs did not fully revert to a quiescent state, but rather retained a preactivated intermediate state.[28] Several reports, including ours,[29] have also shown that treatment of tumor-bearing rats with a ROCK inhibitor suppressed peritoneal dissemination of cancer cells and intrahepatic metastasis of HCC cells.[30,31] This suggested that the ROCK inhibitor is implicated in suppressing HCC progression through not only a direct action on HCC (by inhibiting actomyosin contractility of HCC cells), but also through another indirect action within the cancer stroma, which includes HSCs.

Several limitations to our study should be considered. In the present study, we used hepatoma cells derived from rats. The animals develop large tumors within a few weeks of HCC inoculation, which diffusely infiltrate the liver. This may lead to the development of a different tumor microenvironment and different interactions between HCC and HSCs compared to those present in tumors that arise endogenously. Furthermore, we have shown that the progression of HCC in FL is associated with activated HSCs. However, the activation of HSCs is probably one of several mechanisms associated with the tumorigenic environment in FL. It has been shown that fatty change in hepatocytes induces hypoxic environments in the liver.[32] Indeed, fat droplet accumulation in the cytoplasm of hepatocytes is associated with an increase in cell volume, which may result in partial or complete obstruction of the hepatic sinusoidal space and reduction in sinusoidal blood flow. A state of chronic cellular hypoxia persists in FL, which induces hypoxia-inducible factor (HIF).[33] HIF can induce a vast array of gene products controlling energy metabolism, neovascularization, survival, and cell migration, and is recognized as a strong promoter of tumor growth.[34] The sinusoidal endothelial cells are injured in cases of FL.[32] The number of adherent leukocytes in the injured sinusoid cells induced by a methionine-and CDD was found to be significantly increased, compared with that in normal sinusoid cells. The injured sinusoid cells in the FL may promote tumor cell arrest and extravasation into the hepatic parenchyma. Additional studies of the other mechanisms by which a FL promotes tumor progression are needed. We have also shown that FL with approximately 10–20% steatosis, which was induced by a long-term feeding (16 weeks) of a HFD, promoted the progression of HCC. However, it remains unexplored whether FL with less than 10–20% steatosis promotes the progression of HCC.

In conclusion, our results indicate that the rat steatotic liver microenvironment favors HCC metastasis. This effect appears to be promoted through the activation of HSCs in the steatotic liver.

Acknowledgements

The authors like to thank K. Ogawa, National Institute of Health Sciences (Tokyo, Japan), for the generous gift of the HCC cell lines, as well as M. Kiyokawa and Y. Ishida for their excellent technical assistance.

References

1. Baffy G, Brunt EM, Caldwell SH. Hepatocellular carcinoma in non-alcoholic fatty liver disease: an emerging menace. *J Hepatol* 2012;56:1384–91.
2. Starley BQ, Calcagno CJ, Harrison SA. Nonalcoholic fatty liver disease and hepatocellular carcinoma: a weighty connection. *Hepatology* 2010;51: 1820–32.
3. Stickel F, Hellerbrand C. Non-alcoholic fatty liver disease as a risk factor for hepatocellular carcinoma: mechanisms and implications. *Gut* 2010; 59:1303–7.
4. Ohki T, Tateishi R, Shiina S, et al. Visceral fat accumulation is an independent risk factor for hepatocellular carcinoma recurrence after curative treatment in patients with suspected NASH. *Gut* 2009;58:839–44.
5. Park EJ, Lee JH, Yu GY, et al. Dietary and genetic obesity promote liver inflammation and tumorigenesis by enhancing IL-6 and TNF expression. *Cell* 2010;140:197–208.
6. Yoshimoto S, Loo TM, Atarashi K, et al. Obesity-induced gut microbial metabolite promotes liver cancer through senescence secretome. *Nature* 2013;499:97–101.
7. Reynaert H, Thompson MG, Thomas T, et al. Hepatic stellate cells: role in microcirculation and pathophysiology of portal hypertension. *Gut* 2002;50:571–81.
8. Friedman SL. Mechanisms of hepatic fibrogenesis. *Gastroenterology* 2008;134:1655–69.
9. Mederacke I, Hsu CC, Troeger JS, et al. Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its aetiology. *Nat Commun* 2013;4:2823.
10. Kang N, Gores GJ, Shah VH. Hepatic stellate cells: partners in crime for liver metastases? *Hepatology* 2011;54:707–13.
11. Hernandez-Gea V, Toffanin S, Friedman SL, et al. Role of the microenvironment in the pathogenesis and treatment of hepatocellular carcinoma. *Gastroenterology* 2013;144:512–27.
12. Washington K, Wright K, Shyr Y, et al. Hepatic stellate cell activation in nonalcoholic steatohepatitis and fatty liver. *Hum Pathol* 2000;31:822–8.
13. Reeves HL, Burt AD, Wood S, et al. Hepatic stellate cell activation occurs in the absence of hepatitis in alcoholic liver disease and correlates with the severity of steatosis. *J Hepatol* 1996;25:677–83.
14. Kuroda S, Tashiro H, Igarashi Y, et al. Rho inhibitor prevents ischemia-reperfusion injury in rat steatotic liver. *J Hepatol* 2012;56:146–52.
15. Bechmann LP, Zahn D, Gieseler RK, et al. Resveratrol amplifies profibrogenic effects of free fatty acids on human hepatic stellate cells. *Hepatol Res* 2009;39:601–8.
16. Holt AP, Haughton EL, Lalor PF, et al. Liver myofibroblasts regulate infiltration and positioning of lymphocytes in human liver. *Gastroenterology* 2009;136:705–14.
17. Amann T, Bataille F, Spruss T, et al. Activated hepatic stellate cells promote tumorigenicity of hepatocellular carcinoma. *Cancer Sci* 2009;100: 646–53.
18. Neaud V, Faouzi S, Guirouilh J, et al. Human hepatic myofibroblasts increase invasiveness of hepatocellular carcinoma cells: evidence for a role of hepatocyte growth factor. *Hepatology* 1997;26: 1458–66.
19. Liu C, Billadeau DD, Abdelhakim H, et al. IQGAP1 suppresses TbetaRII-mediated myofibroblastic activation and metastatic growth in liver. *J Clin Invest* 2013;123:1138–56.
20. Kuilman T, Peeper DS. Senescence-messaging secretome: SMS-ing cellular stress. *Nat Rev Cancer* 2009;9:81–94.
21. Sancho-Bru P, Juez E, Moreno M, et al. Hepatocarcinoma cells stimulate the growth, migration and expression of pro-angiogenic genes in human hepatic stellate cells. *Liver Int* 2010;30:31–41.
22. Coulouarn C, Corlu A, Glaise D, et al. Hepatocyte-stellate cell cross-talk in the liver engenders a permissive inflammatory microenvironment that drives progression in hepatocellular carcinoma. *Cancer Res* 2012;72:2533–42.
23. Burridge K, Wennerberg K. Rho and Rac take center stage. *Cell* 2004;116:167–79.
24. Uehata M, Ishizaki T, Satoh H, et al. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* 1997;389:990–4.
25. Sanz-Moreno V, Gaggioli C, Yeo M, et al. ROCK and JAK1 signaling cooperate to control actomyosin contractility in tumor cells and stroma. *Cancer Cell* 2011;20:229–45.
26. Iwamoto H, Nakamuta M, Tada S, et al. A p160ROCK-specific inhibitor, Y-27632, attenuates rat hepatic stellate cell growth. *J Hepatol* 2000;32: 762–70.
27. Murata T, Arii S, Nakamura T, et al. Inhibitory effect of Y-27632, a ROCK inhibitor, on progression of rat liver fibrosis in association with inactivation of hepatic stellate cells. *J Hepatol* 2001;35: 474–81.
28. Troeger JS, Mederacke I, Gwak GY, et al. Deactivation of hepatic stellate cells during liver fibrosis resolution in mice. *Gastroenterology* 2012;143: 1073–83 e22.
29. Ogawa T, Tashiro H, Miyata Y, et al. Rho-associated kinase inhibitor reduces tumor recurrence after liver transplantation in a rat hepatoma model. *Am J Transplant* 2007;7:347–55.
30. Itoh K, Yoshioka K, Akedo H, et al. An essential part for Rho-associated kinase in the transcellular invasion of tumor cells. *Nat Med* 1999;5:221–5.
31. Takamura M, Sakamoto M, Genda T, et al. Inhibition of intrahepatic metastasis of human hepatocellular carcinoma by Rho-associated protein kinase inhibitor Y-27632. *Hepatology* 2001;33: 577–81.
32. McCuskey RS, Ito Y, Robertson GR, et al. Hepatic microvascular dysfunction during evolution of dietary steatohepatitis in mice. *Hepatology* 2004;40:386–93.
33. Nath B, Levin I, Csak T, et al. Hepatocyte-specific hypoxia-inducible factor-1alpha is a determinant of lipid accumulation and liver injury in alcohol-induced steatosis in mice. *Hepatology* 2011;53: 1526–37.
34. Pouyssegur J, Dayan F, Mazure NM. Hypoxia signalling in cancer and approaches to enforce tumour regression. *Nature* 2006;441:437–43.

OncoImmunology 4:1, e983765; January 2015; Published with license by Taylor & Francis Group, LLC

Impact of multiplicity of functional KIR-HLA compound genotypes on hepatocellular carcinoma

Naoki Tanimine and Hideki Ohdan*

Gastroenterological and Transplant Surgery; Institute of Biomedical & Health Sciences; Hiroshima University; Hiroshima, Japan

Keywords: hepatocellular carcinoma, human leukocyte antigen, killer immunoglobulin-like receptor, natural killer cell recurrence

Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; HCC, hepatocellular carcinoma; HLA, human leukocyte antigen; KIR, killer immunoglobulin-like receptors.

Natural killer (NK) cells are potential immune components against hepatocellular carcinoma (HCC) after curative hepatectomy. Patients at high risk of HCC recurrence can be identified by quantifying NK cell licensing. Therefore, therapeutic strategies that manipulate NK cell activity may possibly improve the prognosis of HCC patients.

NK cells are key components of the innate immune system that act against infectious and neoplastic cells. NK cell activation is dependent upon the inhibitory-activating receptor equilibrium. Killer cell immunoglobulin-like receptors (KIRs) are the most polymorphic among these receptors. They contribute to receptor–ligand interactions that determine NK cell responses by recognizing specific human leukocyte antigen (HLA) class I allotype ligands.

In 2006, Anfossi et al. reported an educational mechanism for human NK cells that involves self-specific inhibitory KIR and cognate HLA ligand interactions. This mechanism leads NK cells to acquire a higher resting response capacity,[1] consistent with the NK cell "licensing" mechanism in mice.[2] The polymorphic genes for KIRs and their cognate HLA ligands generate diverse immune responses. Ligand specificities for five inhibitory KIRs and the different strengths of licensing effects through different KIR–HLA ligand interactions have been defined. Thereafter, Yu et al. demonstrated that NK cells, which express multiple inhibitory KIRs for self-HLA ligands within variegated NK cell repertoires, show a synergistic effect of licensing, i.e., the expression of progressively higher numbers of self-reactive inhibitory KIRs is correlated with increased effector capacity.[3] This quantitative effect of NK cell licensing has been verified consistently in mice by analyzing the functional influence of the multiple interactions of self-major histocompatibility complex (MHC)-specific inhibitory receptors.[4] Recently, Beziatz et al. also showed a linear effect of KIR gene copy number variation and HLA ligands on the overall functional responsiveness of the NK cell repertoire by analyzing KIR–HLA genotypes of healthy human volunteers.[5] Despite the accumulating evidence suggesting a quantitative effect of NK cell licensing in basic studies, its clinical impact on neoplastic diseases has never been demonstrated. This year, we were the first to report the impact of multiplicity of functional KIR–HLA compound genotypes on HCC recurrence after curative hepatectomy.[6] The presence of *KIR2DL1-C2*, *KIR2DL2-C1*, *KIR3DL1-BW4*, or *KIR3DL2-A3/11*, functional compound genotypes that intrinsically license NK cells, did not markedly affect HCC recurrence. However, the cumulative risk of recurrence in patients with at least three compound genotypes (highly licensed patients) was significantly lower than that in patients with one or two compound genotypes (poorly licensed patients), suggesting that the effect of NK cell licensing on HCC recurrence is quantitative. This result matches the previously postulated theory that the number and type of host MHC class I alleles quantitatively tune the responsiveness of individual NK cell subsets expressing the corresponding KIR.[5] Highly licensed patients may equip NK cells with more vigorous immune-surveillance activity to act as potent constitutive immune effectors against both intrahepatic metastasis and *de novo* carcinogenesis, as compared to poorly licensed patients (**Fig. 1**).

We have previously shown that the distribution of NK cells is anatomically biased, e.g., NK cells are quite abundant in human livers.[7] Functionally, liver NK cells display a significantly higher cytotoxic activity against neoplastic cells, including against HCC, through a tumor necrosis factor-related apoptosis-inducing ligand-mediated mechanism,

*Correspondence to: Hideki Ohdan; Email: hohdan@hiroshima-u.ac.jp
Submitted: 10/30/2014; Accepted: 10/31/2014
http://dx.doi.org/10.4161/2162402X.2014.983765

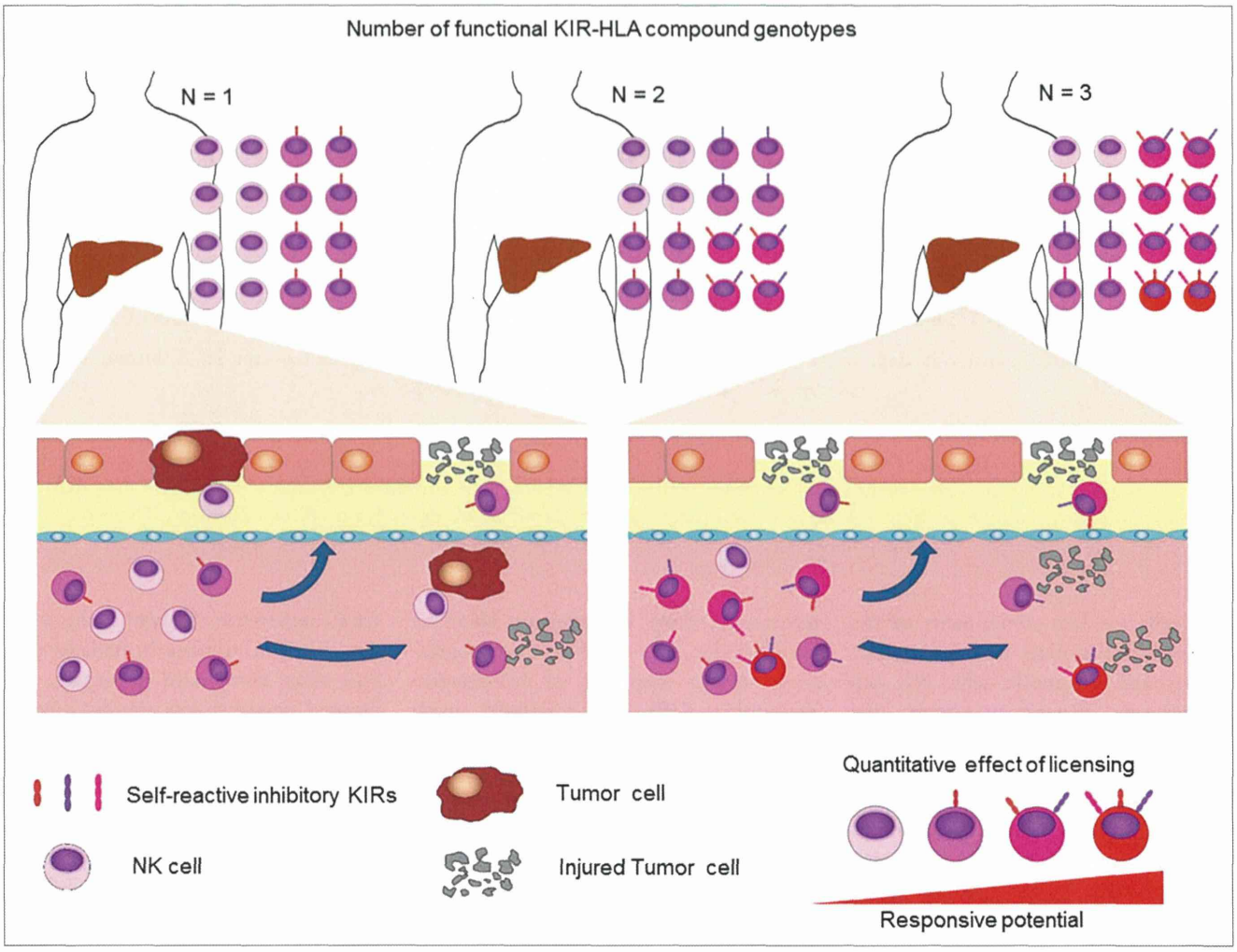

Figure 1. NK cell licensing depends on the number of functional KIR–HLA compound genotypes. The quantitative effects of NK cell licensing influence the overall functionality of NK cell-mediated tumor surveillance. Increasing the number of functional KIR–HLA compound genotypes stochastically induces the generation of NK cell repertoires, which progressively express self-reactive inhibitory KIRs. The difference in NK cell repertoires potentially generates different NK cell surveillance functions, e.g., abundant recruitment of highly licensed NK cells prevents carcinogenesis through attacks on circulating metastatic cells in sinusoid or *de novo* neoplastic cells.

than peripherally circulating NK cells. Based on this fact and the evidence of a quantitative effect of NK cell licensing, we wondered whether the multiplicity of functional KIR–HLA compound genotypes might exclusively influence intrahepatic carcinogenesis or metastasis, regardless of their origin. It would be of great interest to define the impact of the quantitative effect of NK cell licensing on intrahepatic cholangiocarcinoma, liver metastasis of colorectal cancer, or other malignancies.

We demonstrated the predictive value of the multiplicity of functional KIR–HLA compound genotypes for HCC recurrence after curative hepatectomy. One potential strategy to compensate for the genetic susceptibility to HCC recurrence would be an immune therapy that manipulates the NK cell activity. To this end, the clinical efficacy of adoptive immunotherapy with interleukin-2 and anti-CD3 monoclonal antibody-activated autologous peripheral lymphocytes, such as NK cells, has been evaluated; prolonged relapse-free survival in HCC patients following resection of the primary tumor was analyzed. [8] However, the details of the mechanisms underlying such effects remain unclear. As liver NK cells display more vigorous cytotoxicity against HCC than peripheral NK cells, we recently proposed a novel type of adjuvant immunotherapy for preventing HCC recurrence in liver transplant recipients. In this immunotherapy, transplant recipients are intravenously injected with lymphokine-activated killer cells, including activated NK cells derived from liver allografts.[7] A clinical phase I trial revealed the feasibility and safety of this immune therapy.[9] To define the long-term benefits of this approach in terms of the control of HCC recurrence after liver transplantation, a phase II trial, which will investigate the influence of the KIR–HLA genotypes in donors and recipients of liver transplants, is currently under consideration.

Antibody targeting agents for cancer treatment use both compliment-mediated

cytotoxicity and antibody-dependent cell-mediated cytotoxicity (ADCC) to lyse antibody-coated cells. Since NK cells contribute to ADCC, therapies combining NK cells and antibody targeting have the added advantage of local activation of NK cells at the tumor site via CD16 activation. A previous report demonstrated that unlicensed NK cells formed the predominant subset of NK cells, which have potent ADCC due to their lack of inhibitory receptors for self.[10] Hence, the impact of NK cell licensing on the therapeutic outcome of antibody targeting agents may need to be elucidated through further studies.

Disclosure of Potential Conflicts of Interest

The authors have declared no conflicts of interest.

References

1. Anfossi N, Andre P, Guia S, Falk CS, Roetynck S, Stewart CA, Breso V, Frassati C, Reviron D, Middleton D et al. Human NK cell education by inhibitory receptors for MHC class I. Immunity 2006; 25:331-42; PMID:16901727; http://dx.doi.org/10.1016/j.immuni.2006.06.013
2. Kim S, Poursine-Laurent J, Truscott SM, Lybarger L, Song YJ, Yang L, French AR, Sunwoo JB, Lemieux S, Hansen TH et al. Licensing of natural killer cells by host major histocompatibility complex class I molecules. Nature 2005; 436:709-13; PMID:16079848; http://dx.doi.org/10.1038/nature03847
3. Yu J, Heller G, Chewning J, Kim S, Yokoyama WM, and Hsu KC. Hierarchy of the human natural killer cell response is determined by class and quantity of inhibitory receptors for self-HLA-B and HLA-C ligands. J Immunol 2007; 179:5977-89; PMID:17947671; http://dx.doi.org/10.4049/jimmunol.179.9.5977
4. Brodin P, Lakshmikanth T, Johansson S, Karre K, and Hoglund P. The strength of inhibitory input during education quantitatively tunes the functional responsiveness of individual natural killer cells. Blood 2009; 113:2434-41; PMID:18974374; http://dx.doi.org/10.1182/blood-2008-05-156836
5. Beziat V, Traherne JA, Liu LL, Jayaraman J, Enqvist M, Larsson S, Trowsdale J, and Malmberg KJ. Influence of KIR gene copy number on natural killer cell education. Blood 2013; 121:4703-7; PMID:23637128; http://dx.doi.org/10.1182/blood-2012-10-461442
6. Tanimine N, Tanaka Y, Kobayashi T, Tashiro H, Miki D, Imamura M, Aikata H, Tanaka J, Chayama K, and Ohdan H. Quantitative effect of natural killer cell licensing on hepatocellular carcinoma recurrence after curative hepatectomy. Cancer Immunol Res 2014; 2(12):1142-7; PMID:25135909; http://dx.doi.org/10.1158/2326-6066.CIR-14-0091
7. Ishiyama K, Ohdan H, Ohira M, Mitsuta H, Arihiro K, and Asahara T. Difference in cytotoxicity against hepatocellular carcinoma between liver and periphery natural killer cells in humans. Hepatology 2006; 43:362-72; PMID:16440347; http://dx.doi.org/10.1002/hep.21035
8. Takayama T, Sekine T, Makuuchi M, Yamasaki S, Kosuge T, Yamamoto J, Shimada K, Sakamoto M, Hirohashi S, Ohashi Y et al. Adoptive immunotherapy to lower postsurgical recurrence rates of hepatocellular carcinoma: a randomised trial. Lancet 2000; 356:802-7; PMID:11022927; http://dx.doi.org/10.1016/S0140-6736(00)02654-4
9. Ohira M, Ishiyama K, Tanaka Y, Doskali M, Igarashi Y, Tashiro H, Hiraga N, Imamura M, Sakamoto N, Asahara T et al. Adoptive immunotherapy with liver allograft-derived lymphocytes induces anti-HCV activity after liver transplantation in humans and humanized mice. J Clin Invest 2009; 119:3226-35; PMID:19805910; http://dx.doi.org/10.1172/JCI38374
10. Tarek N, Le Luduec JB, Gallagher MM, Zheng J, Venstrom JM, Chamberlain E, Modak S, Heller G, Dupont B, Cheung NK et al. Unlicensed NK cells target neuroblastoma following anti-GD2 antibody treatment. J Clin Invest 2012; 122:3260-70; PMID:22863621; http://dx.doi.org/10.1172/JCI62749

ELSEVIER

Risk Factors for Development of New-Onset Diabetes Mellitus and Progressive Impairment of Glucose Metabolism After Living-Donor Liver Transplantation

T. Abe, T. Onoe, H. Tahara, H. Tashiro, K. Ishiyama, K. Ide, M. Ohira, and H. Ohdan

ABSTRACT

Background. New-onset diabetes mellitus (NODM) has a negative impact on graft and patient survivals. Hepatitis C virus (HCV) infection, high body mass index, increased donor and recipient ages, and calcineurin inhibitor (CNI) type have been identified as risk factors for the development of NODM. We aimed to elucidate the risk factors for the development of NODM and those for progressive glucose intolerance in adult living-donor liver transplant (LDLT) recipients.

Methods. We collected data from 188 primary liver transplant recipients (age > 16 years) who underwent LDLT from June 1991 to December 2011 at Hiroshima University Hospital. Risk factors for NODM and progressive impairment of glucose metabolism in pre-transplantation diabetes mellitus (DM) recipients were examined.

Results. Pre-transplantation DM was diagnosed in 32 recipients (19.3%). The overall incidence of NODM was 6.0% (8/134 recipients). Multivariate analysis revealed that old recipient age (≥55 years) is a unique predictive risk factor for developing NODM. The incident of pre-transplantation DM was significantly higher in recipients with HCV infection than in those without HCV. A high pre-transplantation triglyceride level was an independent risk factor for progressive impairment of glucose tolerance among 32 LDLT recipients with pre-transplantation DM. All of the NODM patients were being treated with tacrolimus at the time of diagnosis. Switching the CNI from tacrolimus to cyclosporine allowed one-half of the patients (4/8) to withdraw from insulin-dependent therapy. NODM and post-transplantation glucose intolerance had no negative impact on patient and graft outcomes.

Conclusions. Older age of the recipient (≥55 years) was a significant risk factor for NODM. Hypertriglyceridemia in the recipients with DM is an independent risk factor for post-transplantation progressive impairment of glucose metabolism. NODM had no negative impact on outcomes in the LDLT recipients.

NEW-ONSET DIABETES MELLITUS (NODM) is recognized as a common complication in organ transplantation, one of the risk factors that affect graft and patient survival in solid organ transplantation. NODM contributes to the risk for infection, cardiovascular disease, and neurologic complications [1]. The prevalence of NODM after liver transplantation is 18%–50% [2–5]. Risk factors for the development of NODM include increased age, ethnicity, family history of type 2 diabetes, obesity, hepatitis C virus (HCV) and cytomegalovirus infections, glucocorticoid treatment, and the use of other immunosuppressive medications

From the Department of Gastroenterological and Transplant Surgery, Applied Life Sciences, Institute of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan (T.A., T.O., H. Tahara, H. Tashiro, K. Ishiyama, K. Ide, M.O., H.O.); and Institute for Clinical Research, National Hospital Organization, Kure Medical Center/Chugoku Cancer Center, Kure, Japan (T.O.).

Funding: Grant-in-Aid for Research on Hepatitis and BSE from the Japanese Ministry of Health, Labor, and Welfare.

Address reprint requests to Takashi Onoe, MD, PhD, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. E-mail: tonoemd@gmail.com

0041-1345/14/$–see front matter
http://dx.doi.org/10.1016/j.transproceed.2013.12.027

[2,6]. Recent reports indicate that some gene polymorphisms may be related to NODM after solid organ transplantation [7]. These characteristics likely reflect inherited and acquired defects in insulin sensitivity and β-cell function that contribute to hyperglycemia.

NODM is thought to result from the combination development of insulin resistance and decreased insulin secretion. The use of transplantation immunosuppressive medications in addition to weight gain or steroid pulse therapy could aggravate pre-transplantation glucose intolerance [8]. Tacrolimus, one of the most frequently used calcineurin inhibitors (CNIs), seems to have the most pronounced diabetogenic effect. Kurzawski et al [7] showed that individuals taking tacrolimus had a significantly higher incidence of NODM than those not taking it. Compared with patients on cyclosporine treatment, those medicated with tacrolimus had a significantly higher incidence of NODM or impairment of glucose tolerance after 6 months of therapy (26.0% vs 33.4%, respectively) [9].

Some reports have shown that HCV infection causes a high rate of NODM in renal and liver transplant recipients [10,11]. The prevalence of pretransplant diabetes mellitus (DM) and glucose intolerance was apparently higher in recipients with HCV infection [12]. Soule et al [13] reported the HCV infection contributes to the development of DM in orthotopic liver transplant recipients.

However, most published data about NODM have come from single-center studies with relatively small sample sizes and from deceased-donor liver transplantation in Western populations. Harada et al [14] reported that higher body mass index (BMI), male sex, and older age were independent predictive factors for developing NODM after living-donor liver transplantation (LDLT) among Asian adult recipients, whereas the impact of HCV infection did not reach significance. In the present study, we aimed to investigate risk factors for development of NODM and to identify risk factors for progressive impaired glucose metabolism in recipients with pre-transplantation DM after LDLT at a single transplant center.

METHODS

A total of 188 adult patients underwent LDLT from June 1991 to December 2011 at Hiroshima University Hospital. Of the 188 patients, 16 recipients who died within 6 months after LDLT, 2 who required re-LDLT, and 4 who underwent deceased-donor liver transplantation were excluded from the study. Pre-transplantation DM was diagnosed in 32 recipients (19.3%). The procedures for donor evaluation, donor surgery, recipient surgery, and perioperative management followed in our hospital have been described elsewhere [15,16].

NODM was defined by the American Diabetes Association/World Health Organization criteria that were recommended in the 2003 International Consensus Guidelines to diagnose DM after transplantation [1]. Diagnostic criteria include the following: fasting blood glucose levels ≥126 mg/dL (7.0 mmol/L) on 2 separate occasions and/or a 2-hour postprandial blood glucose level ≥200 mg/dL (11.1 mmol/L) on 2 separate occasions. Alternatively, DM was defined as the requirement of glucose-lowering medications (insulin or oral hypoglycemic agents). Transient DM was defined as DM present in <2 follow-up post-transplantation intervals. Persistent DM was defined as DM present in ≥3 follow-up post-transplantation intervals with ≥1 interval beyond the 3rd post-transplantation month [10]. In our study, patients with postoperative progressive impairment of glucose metabolism were identified as those who needed to be medicated with new or higher doses of insulin and who later had diabetes-related renal failure or diabetic complications.

The basic immunosuppressive regimen after LDLT consisted of tacrolimus/cyclosporine and methylprednisolone, with doses gradually tapered off. In patients with HCV infection, the methylprednisolone dose was rapidly tapered off and administration was stopped within 1 month after LDLT, which would be beneficial for preventing enhanced viral replication [17].

The assessed recipient-related risk factors included age, sex, pre-transplantation BMI (kg/m^2), Model for End-Stage Liver Disease (MELD) score, and presence of HCV infection. HCV infection was defined in recipients with a positive anti-HCV test or an HCV-related diagnosis. Sustained virologic response (SVR) was defined as undetectable HCV RNA in the serum after the completion of HCV therapy. Baseline recipient factors according to the presence of postoperative impaired glucose metabolism or persistent and improved glucose metabolism were compared with the use of the chi-square test. Multivariate analysis was performed with the use of a logistic regression test. All P values were 2 tailed, and $P \leq .05$ was considered to be statistically significant. All analyses were performed with the use of the SPSS statistical software (IBM Japan, Tokyo, Japan).

RESULTS

The study population with which to identify NODM risk factors consisted of 51 female and 83 male recipients without pre-transplantation DM. As observed in Table 1, NODM was diagnosed in 8 (6.0%) of the 134 recipients. We analyzed recipient and donor risk factors for developing NODM in a univariate analysis. A total of 48 (35.8%) of the 134 patients were infected with HCV, whereas 86 (64.2%) of the 134 patients were not infected with HCV before LDLT. HCV infection and increased recipient age were risk factors for developing NODM on univariate analysis; recipient sex, recipient BMI, MELD score, donor age, donor sex, donor BMI, cold ischemia time, and operation time demonstrated no statistically significant differences. Older recipient age was considered to be the unique and important predictive risk factor for developing NODM on multivariate analysis (odds ratio, 9.191; 95% confidence interval, 1.083–76.478; $P = .042$). Table 2 shows that among the 166 patients, the HCV-positive recipients showed significantly higher proportional rate of pretransplant DM (19/67, 28.4%) than the HCV-negative recipients (13/99, 13.1%; $P < .05$, Fisher exact test). The outcomes of the patients with DM or non-DM were not significantly different regarding graft and patient survival (data not shown).

Next, a subgroup analysis was performed in the patients with pre-transplantation DM recipients with a special focus on the patients with post-transplantation progressive

Table 1. Risk Factors for Developing New-Onset Diabetes Mellitus

Variable	Overall (n = 134)	Non-NODM (n = 126)	NODM (n = 8)	Univariate	Multivariate
	n (%)	n (%)	n (%)	P Value	OR (95% CI) P Value
Recipient sex: male/female	83/51 (61.9%/38.1%)	77/49 (61.1%/38.9%)	6/2 (75%/25%)	.32	
Recipient age: ≥55 vs <55 y	62/72 (46.3%/53.7%)	55/71 (43.7%/56.3%)	7/1 (87.5%/12.5%)	.009	9.19 (1.08–76.48) .042
Recipient BMI (kg/m^2)	23.6 ± 3.9	23.8 ± 4.0	23.4 ± 2.8	.68	
MELD score	17.4 ± 8.5	17.0 ± 8.5	12.6 ± 6.6	.052	
Recipient HCV infection: yes/no	48/86 (35.8%/64.2%)	42/84 (33.3%/66.7%)	6/2 (75%/25%)	.01	.099
Donor sex: male/female	83/51 (61.9%/38.1%)	80/46 (63.5%/36.5%)	3/5 (37.5%/62.5%)	.14	
Donor age (y)	35.6 ± 12.2	35.8 ± 12.1	43.2 ± 13.7	.42	
Donor BMI (kg/m^2)	21.8 ± 2.6	22.0 ± 2.7	21.0 ± 2.0	.18	
Cold ischemia time (min)	98.4 ± 37.9	98.8 ± 38.0	103.3 ± 38.0	.73	
Operation time (min)	732 ± 138	737 ± 140	735 ± 84.9	.96	

Note: Unless otherwise indicated, data are presented as mean ± SD.
Abbreviations: BMI, body mass index; CI, confidence interval; HCV, hepatitis C virus; MELD, Model for End-Stage Liver Disease; NODM, new-onset diabetes mellitus; OR, odds ratio; SVR, sustained virologic response.

impairment of glucose metabolism (Table 3). We subdivided the patients into 2 groups: patients with post-transplantation impaired glucose metabolism and patients with post-transplantation persistent and improved glucose metabolism. Hypertriglyceridemia was the only independent risk factor for post-transplantation progressive glucose intolerance (odds ratio, 1.022; 95% confidence interval, 1.003–1.041; P = .020) on multivariate analysis; there were no differences in age, sex, high BMI, total cholesterol level, creatinine level, hemoglobin level, or MELD score. Donor factors such as age and sex were also analyzed in these 2 groups; there were no significant risk factors for post-operative progressive glucose disorder. All the patients with NODM at the time of diagnosis were treated with tacrolimus; 4 (50%) of 8 patients recovered from NODM by switching from tacrolimus to cyclosporine. Those 4 patients had transient DM. The 4 patients with persistent DM required insulin therapy, whereas 1 of the 4 patients required blood dialysis for diabetes-related chronic renal failure. However, the presence of NODM and post-transplantation progressive glucose intolerance did not have a significant effect on patient morbidity and mortality after LDLT (data not shown).

DISCUSSION

The incidence of NODM was previously reported to be 10.5%–50.4% [2,4]. The United Network for Organ Sharing database study reported an incidence of 15.7% in LDLT [2], whereas in the present study, only 8 patients (6.0%) developed NODM after LDLT, a lower incidence than in earlier reports. This variance in reported prevalence rates could be due to the differences in post-transplantation diabetes definitions. We previously reported the efficacy of carboxyfluorescein diacetate succinimidyl ester labeling–mixed lymphocyte reaction (CFSE-MLR) assay to monitor anti-donor alloreactivity for the accurate diagnosis of acute rejection after LDLT [17].

Table 2. Association Between Hepatitis C Virus (HCV) Infection and Pre-Transplantation Diabetes Mellitus

	HCV Positive	HCV Negative	Total
Pre-transplantation DM positive	19 (28.4%)*	13 (13.1%)	32
Pre-transplantation DM negative	48 (71.6%)	86 (86.9%)	134
Total	67	99	166

Note: The percentages in the parentheses represent the rates among the HCV-positive or HCV-negative population.
Abbreviation: DM, diabetes mellitus.
*P < .05 (Fisher exact test).

The first reason for the low incidence of NODM in our institution is that our CFSE-MLR assay plays an important role in avoiding CNI overdose and unnecessary corticosteroid pulse therapy. Corticosteroids induce a state of insulin resistance characterized by decreased binding of insulin to insulin receptors and decreased glucose utilization. The diabetogenic effects of corticosteroids seem to be dose related [18]. Some studies have reported no statistically significant difference in the overall incidence of NODM in steroid-free versus steroid-treated patients [19,20]. However, all studies showed trends toward less severe NODM among steroid-free patients based on a reduced need for insulin therapy; our results are consistent with these reports.

The second reason is that we could diagnose the patients with NODM and progressive impairment of glucose metabolism as early as we could and treat them with insulin therapy or by switching the CNI regimen, which led to better outcomes even in the patients who were developing NODM or progressive glucose disorder. The outcomes of these patients and those without DM among the patients with HCV infection were not significantly different (data not shown). It remains controversial whether switching a CNI improves glycemic control in recipients with NODM. The balance between the treatment of NODM and the prevention of acute or chronic rejection should be considered.

Well known risk factors for developing NODM, including older age, black race, obesity, HCV infection, family history of DM, and use of tacrolimus or corticosteroids, predict the development of NODM in transplant recipients [2,4,14]. Only older recipient age (≥55 years) was identified as an independent risk factor for NODM in the present study. It

Table 3. Comparison Between Post-Transplantation Impaired Glucose Metabolism and Persistent or Improved Glucose Metabolism Among the Pretransplant Recipients With Diabetes Mellitus

Variable	Post-Transplantation Progressive Impaired Glucose Metabolism (n = 13)	Perioperative Persistent or Improved Glucose Metabolism (n = 19)	Univariate	Multivariate
	n (%)	n (%)	P Value	OR (95% CI) P Value
Recipient sex: male/Female	9/4 (69.2%/30.8%)	12/7 (63.2%/36.8%)	.69	
Recipient age (y)	51.4 ± 9.8	52.8 ± 14.1	.54	
Recipient BMI (kg/m^2)	24.0 ± 4.0	22.6 ± 5.3	.29	
Obesity (BMI > 30 kg/m^2)	1 (7.7%)	4 (5.3%)	.40	
Recipient HCV infection: yes/no	11/2 (84.6%/15.4%)	8/11 (42.1%/57.9%)	.46	
Total cholesterol (mg/dL)	121.6 ± 54.7	132.3 ± 52.8	.37	
Triglycerides (mg/dL)	103.1 ± 111.1	80.8 ± 55.8	.017	1.022 (1.003–1.041) .020
Creatinine (nmol/L)	0.8 ± 0.4	1.1 ± 1.0	.13	
Hemoglobin (mg/dL)	10.4 ± 2.8	11.1 ± 2.0	.019	.417
MELD score: ≥25 vs <25	6/7 (46.2%/53.8%)	13/6 (68.4%/31.6%)	.94	
Donor sex: male/female	9/4 (69.2%/30.8%)	14/5 (73.7%/26.3%)	.22	
Donor age (y)	37.3 ± 12.2	34.8 ± 13.3	.22	
Donor BMI (kg/m^2)	22.1 ± 2.6	20.9 ± 4.6	.51	
Cold ischemia time (min)	93.8 ± 33.8	98.6 ± 41.1	.32	
Operation time (min)	722 ± 193	720 ± 129	.92	

Note: Unless otherwise indicated, data are presented as mean ± SD.
Abbreviations as in Table 1.

remains controversial whether age would be an independent predictive factor for NODM in deceased-donor liver transplantation. However, in LDLT recipients, older age could be an important factor for development of NODM, as seen in earlier studies [14].

Generally, patients infected with HCV have a high incidence of DM compared with patients who have other types of liver disease, as seen in our results presented in Table 2 [6]. The pathophysiologic mechanisms of HCV infection in the presence of hyperglycemia remain unclear. The hypothesis was that HCV infection could lead to DM by promoting or accentuating insulin resistance mediated by a post-receptor signaling defect and decreased hepatic glycogenesis [21]. This could be explained by the fact that 14 patients who achieved post-transplantation SVR did not develop NODM after antiviral treatment, although incidence of pre-transplantation HCV infection were not associated with the development of NODM on multivariate analysis. As such, HCV treatment might have a synergistic impact on glucose metabolism. Liver transplantation itself had a beneficial impact on the existing pre-transplantation peripheral insulin resistance and impaired glucose metabolism. As a matter of fact, we experienced 4 patients who underwent LDLT in whom preexisting DM completely recovered after liver transplantation. No patients with NODM or progressively impaired glucose metabolism recipients died during our study, but long-term outcomes have yet to be investigated.

Criterion standard methods to measure insulin resistance are both time-consuming and impractical. Different parameters of lipid metabolism are well correlated with insulin resistance when measured by accurate methods [22]. Of these, triglyceride levels and triglycerides–high-density lipoprotein cholesterol ratio showed the best correlation with insulin resistance [22]. Consistent with the earlier report, our data show that pre-transplantation hypertriglyceridemia was an independent risk factor for progressive glucose intolerance in pre-transplantation DM recipients.

In conclusion, older recipient age (≥55 years) was a significant risk factor for NODM. Pre-transplantation hypertriglyceridemia is the only independent risk factor for the development of progressive glucose metabolism in patients with pre-transplantation DM. However, in our study, perioperative impaired glucose metabolism had no negative impact on patient or graft survival.

ACKNOWLEDGMENTS

The authors thank Minoru Hattori for assisting with the statistical analysis.

REFERENCES

[1] Davidson J, Wilkinson A, Dantal J, et al. New-onset diabetes after transplantation: 2003 International Consensus Guidelines. Proceedings of an international expert panel meeting. Barcelona, Spain, 19 February 2003. Transplantation 2003;75:SS3–S24.

[2] Kuo HT, Sampaio MS, Ye X, et al. Risk factors for new-onset diabetes mellitus in adult liver transplant recipients, an analysis of the Organ Procurement and Transplant Network/United Network for Organ Sharing database. Transplantation 2010;89:1134–40.

[3] Pham PT, Pham PC, Lipshutz GS, et al. New onset diabetes mellitus after solid organ transplantation. Endocrinol Metab Clin North Am 2007;36:873–90. vii.

[4] Chen T, Jia H, Li J, et al. New onset diabetes mellitus after liver transplantation and hepatitis C virus infection: meta-analysis of clinical studies. Transpl Int 2009;22:408–15.

[5] Montori VM, Basu A, Erwin PJ, et al. Posttransplantation diabetes: a systematic review of the literature. Diabetes Care 2002;25:583–92.

[6] Bodziak KA, Hricik DE. New-onset diabetes mellitus after solid organ transplantation. Transpl Int 2009;22:519–30.

[7] Kurzawski M, Dziewanowski K, Lapczuk J, et al. Analysis of common type 2 diabetes mellitus genetic risk factors in new-onset diabetes after transplantation in kidney transplant patients medicated with tacrolimus. Eur J Clin Pharmacol 2012;68:1587–94.