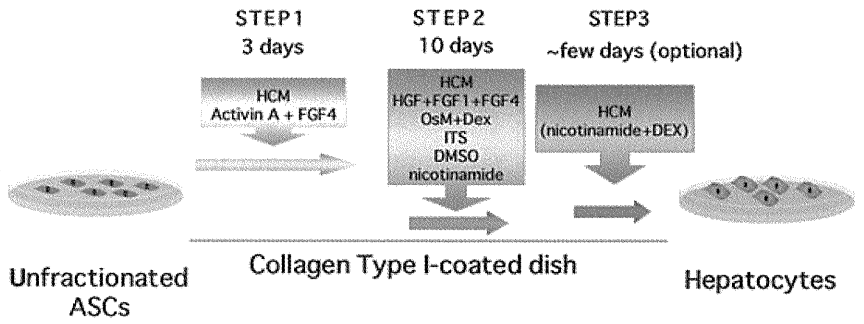

Figure 1 Improved and modified hepatogenic induction strategy. At present, approximately 2 weeks are required to induce hepatogenic characteristics in adipose-derived stem cells (ASC). Unfractionated ASC were plated on collagen type I-coated dishes and were treated with Activin A and FGF4 at step 1, followed by step 2, treatment with hepatocyte growth factor (HGF), fibroblast growth factor (FGF)1, FGF4, oncostatin M (OsM), dexamethasone, insulin-transferrin-selenium (ITS), dimethyl sulfoxide (DMSO), and nicotinamide. At this point, cells may be maintained a few days in hepatocyte culture medium (HCM) alone (or optionally supplemented with 10^{-8} mol/L dexamethasone and 0.05 mmol/L nicotinamide). MSC, mesenchymal stem cells.

lymphocyte antigen [HLA I]) and no MHC II (HLA II) expression, therefore reducing the risk of allogenic transplant rejection.[20–27]

Currently, attention is being given to adipose tissue (AT) as a source of MSC for regenerative medicine. From adipose tissue, a sufficient number of stem cells for a stem cell-based therapy may be obtained without invasiveness or damage to a patient's health. We have already demonstrated that human ASC have the ability to give rise to hepatocyte-like cells and that CD105 is a candidate mesenchymal stem cell marker.[19] However, this *in vitro* differentiation method is not applicable to a practical, clinical use, as more than 1 month is required to induce ASC into cells with hepatic functions.

In the present study, we evaluate the therapeutic potential of ASC-derived hepatocyte-like cells after transplantation into mice with liver injury. Clinical applications in the future would require a special approach, such as shortening as much as possible *ex vivo* manipulations, including cultivation and direct hepatic fate. Therefore, we improved and modified our hepatocyte differentiation strategy, based on the current knowledge on *in vivo* mouse fetal liver development. At present, a period as short as 13 days is required and that strategy is enriched by pretreatment with Activin A (PeproTech, EC, London, UK) and fibroblast growth factor (FGF)4 (PeproTech) (one of the factors secreted by septum transversum mesenchyme (STM) and cardiogenic mesoderm at the early stage of endoderm development *in vivo*). Additionally, we reorganized the content of the growth factor cocktail and enriched it with the addition of dimethyl sulfoxide (DMSO), nicotinamide and insulin-transferrin-selenium. Using the present protocol, we obtained functional hepatocyte-like cells in a much shorter period of time. Finally, we transplanted ASC-derived hepatocyte-like cells into immunodeficient mice with liver injury/non-severe acute liver injury. Our results showed a significant decrease of ammonia, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and uric acid (UA) in the blood plasma of mice after ASC-derived hepatocyte-like cell transplantation. The results show a very important step towards future establishment of an alternative and successful therapy for liver disease.

Methods

Isolation and culturing of ASC

Adipose-derived stem cells were derived from abdominal subcutaneous adipose tissue, which was obtained from two female gastric cancer patients (Donor #1 [36 years old] and Donor #2 [45 years old]), undergoing gastrectomy at the International Medical Center of Japan, Tokyo. The hospital's committee of ethics approved this study, and informed consent was obtained from both patients. Adipose tissue was processed as previously described.[19] For *in vitro* differentiation, the cells (ASC062801, ASC012202, ASC0025) obtained from DS Pharma Biomedical Co., Osaka, Japan) were also analyzed.

Hepatic differentiation

At passage five to 10, the cells were plated on collagen type I-coated dishes at a concentration of $3.0–4.0 \times 10^4$ cells/cm^2 (Fig. 1). When the cells reached confluency, hepatogenic induction was carried out over a period of 2 weeks. First, the cells were treated for 3 days with DMEM (GibcoBRL, Tokyo, Japan) (serum free) supplemented with 20 ng/mL Activin A and 20 ng/mL FGF4 (PeproTech EC, London, UK). Afterwards, the cells were cultured for 10 days in a hepatocyte culture medium (HCM), containing 5 μg/mL transferrin, 10^{-6} mol/L hydrocortisone-21-hemisuccinate, 0.5 mg/mL bovine serum albumin, 2 mmol/L ascorbic acid, 20 ng/mL epidermal growth factor, 5 μg/mL insulin, 50 μg/mL gentamicin (Cambrex Corp., Walkersville, MD, USA) and supplemented with 150 ng/mL hepatocyte growth factor (HGF), 100 ng/mL FGF1, 25 ng/mL FGF4, 30 ng/mL oncostatin M (OsM; PeproTech), (2×10^{-5} mol/L) dexamethasone (Dex; Sigma, Tokyo, Japan), 1 × insulin-transferrin-selenium (ITS; Gibco), 0.05 mmol/L nicotinamide (Sigma), and 0.1% DMSO (Sigma). For the next few days, the cells were maintained with HCM alone. For *in vivo* transplantation, hepatocyte-like cells from two donors (#1 and #2) were harvested by treatment with a 0.05% collagenase/1000 U/mL dispase solution for 3–5 min, dissolved in

PBS (–) and injected intravenously into mice with liver injury caused by CCl_4 injection.

Quantitative real-time PCR

In order to confirm the regulation of the hepatocyte-specific genes in ASC-derived hepatocytes, we performed real-time polymerase chain reaction (PCR) for albumin (ALB) and tryptophan 2,3-dioxygenase (TDO2), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference gene. After retro-transcription, cDNA was subjected to real-time PCR by using Platinum Quantitative PCR Super Mix-UDG (Invitrogen, Tokyo, Japan) and specific primers for ALB (NM_000477): F:GTCACCAAATG CTGCACAGA, R:ACGAGCTCAACAAGTGCAGT for TDO2 (NM_005651): F:GTGTGCATGGTGCACAGAAT, R:GGGTT CATCTTCGGTATCCA, for FOXA2 (NM_021784): F:GGGA GCGGTGAAGATGGAAG, R:TGCCAGCGCCCACGTA and for GAPDH (NG_007073): F:GAAGGTGAAGGTCGGAGT, and R:GAAGATGGTGATGGGATTTC, based on the human genome database. The PCR conditions were as follows: denaturation at 95°C for 30 s, annealing at 56°C or 60°C for 30 s, and extension at 72°C for 30 s for up to 45 cycles. Real-time PCR was carried out by using the Applied Biosystems (Tokyo, Japan) PRISM 7700 Sequence Detection System.

Immunofluorescence

Cells were fixed in 4% formaldehyde for 10 min, followed by incubation with Protein Block (DakoCytomation, Carpinteria, CA, USA) for 30 min. ASC-derived hepatocytes were analyzed by immunohistochemistry using monoclonal anti-human specific albumin ALB (clone HAS-11, 1:250; Sigma) antibody overnight at 4°C. The Alexa Fluor 488 (green, 1:1000)-conjugated secondary antibody (Invitrogen, Tokyo, Japan) was applied for 30 min. Nuclei staining was performed using 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA).

Albumin production

Albumin production was evaluated by enzyme linked immunosorbent assay (ELISA, E80-129; Bethyl Laboratories, Montgomery, TX, USA). The antibody is human specific and does not cross-react with mouse, rat, bovine, goat, and pig albumin. Briefly, the supernatant during hepatogenic induction was collected every 3 days at days 3, 6 and 9, and ELISA assay was performed. Data are reported as the mean ± SD and were analyzed by Student's *t*-test, $n = 3$ ($^*P < 0.05$).

ASC-derived hepatocyte transplantation into mice with CCl_4-induced injury

Animal studies were carried out in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute. Six-week-old female BALB/c nude mice (CLEA Japan Inc., Tokyo, Japan) were used. An acute liver failure model was produced by giving one dose of CCl_4. At day 0, mice underwent i.p. injection of 100 μL/20 g bodyweight of olive oil containing 10 μL CCl_4. At day 1, mice underwent transplantation of ASC-derived hepatocyte-like cells (Donor #1 ($n = 4$), or Donor #2 ($n = 4$) at a concentration of 1.5×10^6 cells per mouse (0.2 mL cell suspension was injected through the tail vein). As a control, non-transplanted CCl_4-treated mice ($n = 3$) and non-transplanted CCl_4-non-treated (olive oil) ($n = 3$) mice were used. Twenty-four hours after transplantation, blood serum was collected and evaluated for biochemical parameters, such as AST, ALT, UA and ammonia concentration levels.

Assessment of liver functions

Blood samples were obtained from each mouse, centrifuged for 20 min at 400 *g* and serum was collected. Serum samples were tested for ammonia concentration level by using the Ammonia Test-Wako (Wako Pure Chemicals, Tokyo, Japan). The concentration of markers of liver injury such as ALT, AST and UA was analyzed by using a FUJIFILM DRI-CHEM 3500 machine and FUJI DRI-CHEM Slides for ALT/ALT-PIII, AST/AST-PIII, and UA-PIII, respectively (Fujifilm Co., Tokyo, Japan).

Statistical analysis

The results are given as the mean ± SD. Statistical analyses were conducted using either the variance with the Bonferroni correction for multiple comparisons or the Student's *t*-test. The statistical analysis of quantitative relative expression was evaluated by using the Pair Wise Fixed Reallocation Randomization Test©, Relative Expression Software Tool-XL = REST-XL© (http://www.gene-quantification.info/). A P value < 0.05 was considered significant.

Results

Hepatic fate specification of ASC

A direct fate hepatic specification (Fig. 1) was performed within 13 days. After that, ASC-derived hepatocyte-like cells could be maintained for a few days in HCM alone (optionally supplemented with Dex 10^{-8} mol/L and nicotinamide 0.05 mmol/L). After 3 days of pretreatment with FGF4 and Activin A, ASC expressed FOXA2 (Fig. 2a), the expression of which was decreased at day 6 of the induction system (3 days of pretreatment with FGF4 and Activin A, followed by 3 days of treatment with a cocktail containing HGF, FGF1, FGF4, OsM, Dex, ITS, DMSO and nicotinamide) (Fig. 2a). FOXA2, so-called hepatocyte nuclear factor 3β (HNF-3β) is an essential transcription factor for endoderm specification as well as hepatogenic fate. Similarly, ALB (hepatocyte-specific protein) and TDO2 (hepatocyte-specific enzyme, expressed by mature hepatocytes) were also detected by quantitative PCR at day 3 and their expression increased at day 6 of the induction system (Fig. 2a). The representative morphology of the ASC-derived hepatocyte-like cells of either a cancer patient's ASC or from the commercialized cells at the 13th day of induction is shown in Figure 2b. Importantly, 24 h of incubation with our new cocktail (Step II) alone is enough to dramatically influence the morphology of ASC (Donor #2) from fibroblast to epithelial (Fig. 3a). The pretreatment with Activin A and FGF4, however, is very important, because it induces the endodermal fate and alters further morphological changes and maturation of hepatocyte-like cells. As shown

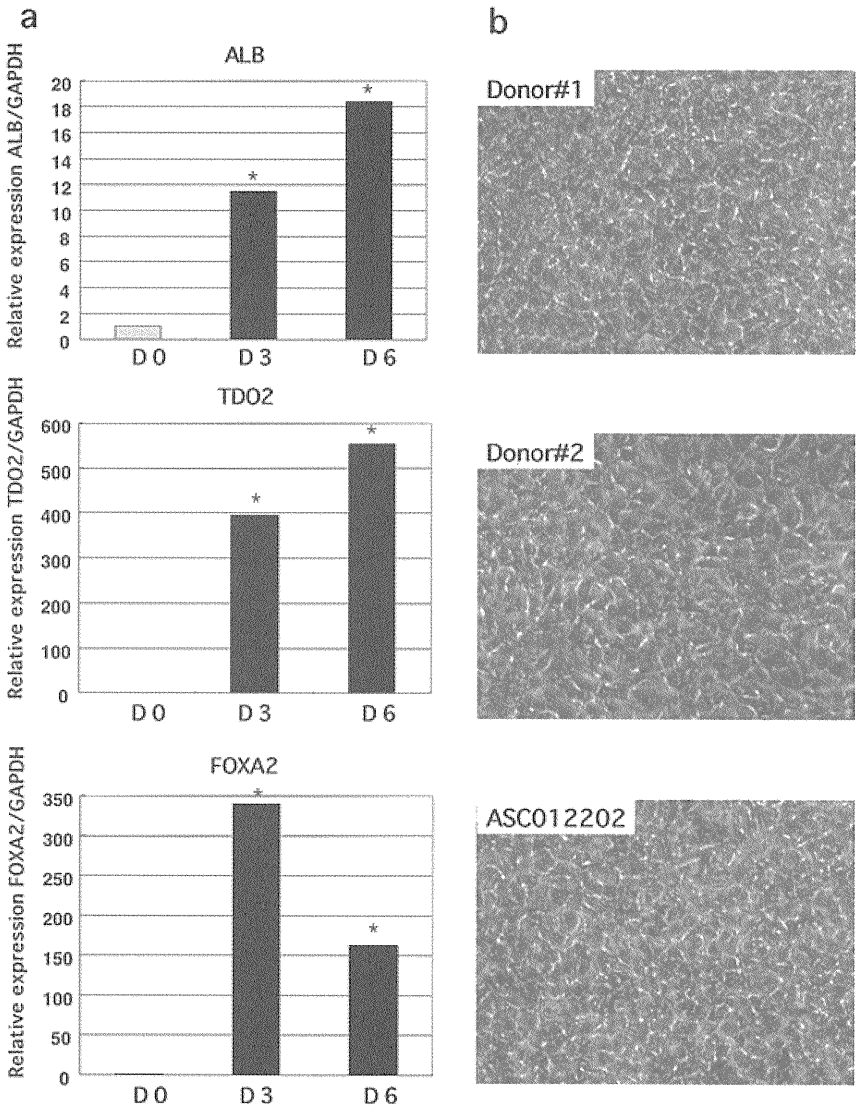

Figure 2 (a) Expression of albumin (ALB), tryptophan 2,3-dioxygenase (TDO2) and FOXA2 at day 3 (D3) (pretreatment with fibroblast growth factor [FGF]4 and Activin A) and day 6 (D6) (3 days of pretreatment with FGF4 and Activin A, and 3 days of treatment with hepatocyte growth factor [HGF], FGF1, FGF4, oncostatin M [OsM], dexamethasone [Dex], insulin-transferrin-selenium [ITS], dimethyl sulfoxide [DMSO], and nicotinamide) (■). Undifferentiated adipose-derived stem cells (ASC) (D0) (▨). Data were analyzed by the Pair Wise Fixed Reallocation Randomization Test©, $n = 3$. $^*P < 0.05$). (b) Morphological features of ASC-derived hepatocyte-like cells of ASC derived from Donor #1, Donor #2, and commercially available ASC012202.

in Figure 3(a), changes in the morphology of ASC-derived hepatocyte-like cells (Donor #2) at days 0, 4, 9 and 16 of hepatogenic induction indicate hepatocyte maturation. At day 13, ASC-derived hepatocyte-like cells expressed albumin (Fig. 3b), which was detected by immunostaining, using anti-human specific antibody. Undifferentiated ASC, however, did not express albumin (data not shown). We also checked the functionality of ASC-derived hepatocyte-like cells. Figure 3(c) represents the albumin production at days 3, 6 and 9 of the induction process. ASC-derived hepatocyte-like cells also revealed an ability to uptake low-density lipoprotein (LDL) and store glycogen (Fig. 4).

Transplantation of ASC-derived hepatocyte-like cells into mice with liver injury

To address whether ASC reveal therapeutic abilities to regenerate an injured liver, we transplanted ASC-derived hepatocyte-like cells of Donors #1 and #2 into nude mice with acute liver failure. CCl_4 injury generated oxidative stress and hepatocyte necrosis. Twenty-four hours after CCl_4 injection, mice revealed serious liver injury. Biochemical parameters such as ALT, AST, UA and ammonia were increased in mice with CCl_4 injury compared with non-injured mice (Fig. 5). We transplanted 1.5×10^6 cells of ASC-derived hepatocyte-like cells into a CCl_4-injured mouse. After transplantation, ALT and AST were significantly decreased to a value more than 50% lower than in non-transplanted and injured mice (Fig. 5). Likewise, ammonia concentration was significantly decreased after ASC-derived hepatocyte-like cell transplantation. UA, a marker of oxidative stress, was significantly decreased up to a normal level after transplantation of ASC-derived hepatocyte-like cells (Fig. 5). Hematoxylin-eosin staining revealed that the level of injury was the same in the injured, non-transplanted mice (Fig. 6b,e) as well as in the injured transplanted mice (Fig. 6c,f), in contrast to the non-injured non-transplanted mice (Fig. 6a,d). Significant morphological changes between those mice, however, were detected in the hepatocytes of the non-necrotic area. The

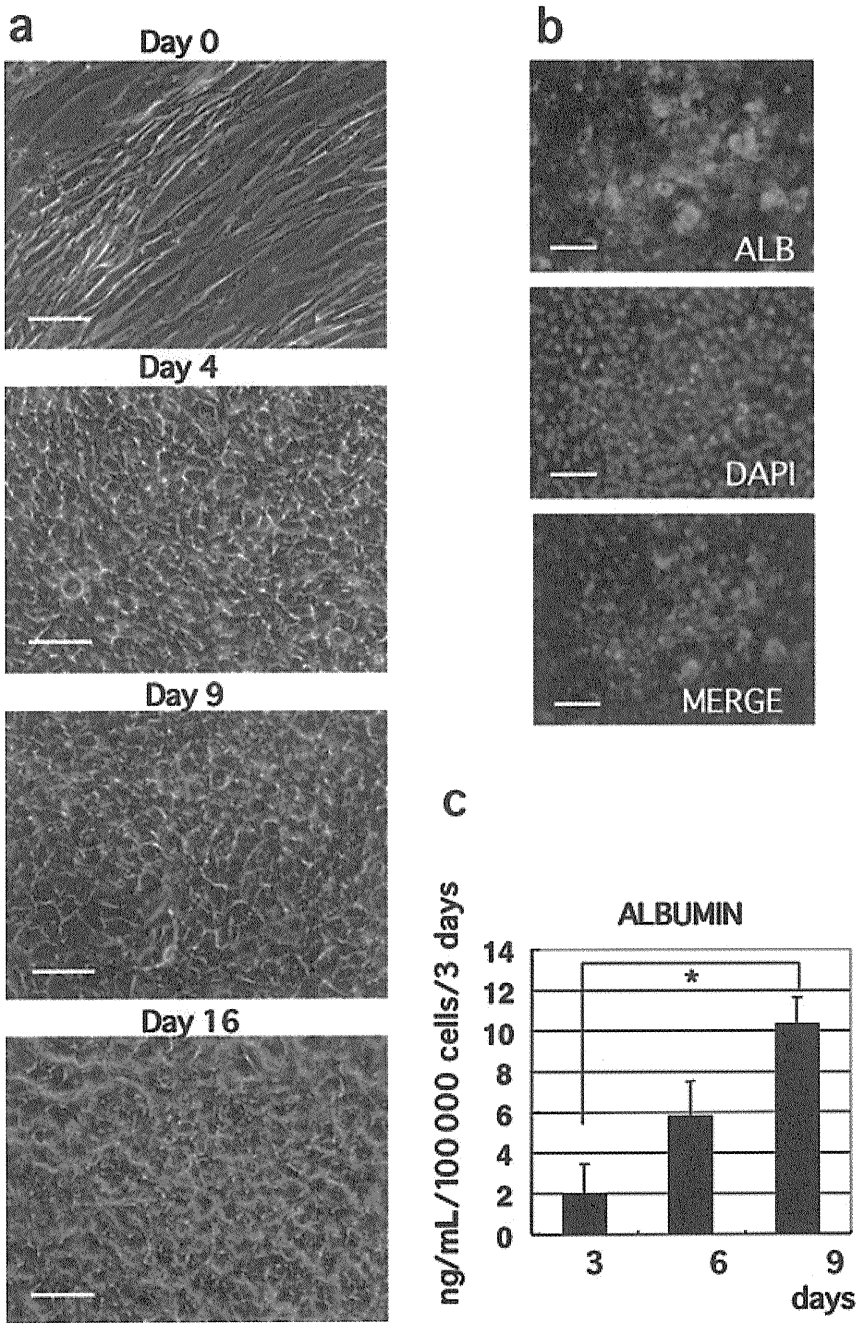

Figure 3 (a) Morphology of adipose-derived stem cells (ASC) (Donor #2) at days 0, 4, 9 and 16 during the hepatogenic induction process. (b) Albumin immunostaining analyses of ASC-derived hepatocyte-like cells at day 13 of induction. (c) Albumin production by ASC-derived hepatocyte-like cells at days 3, 6 and 9 of induction. Data are reported as the mean ± SD and were analyzed by Student's *t*-test, $n = 3$. $^*P < 0.05$). ALB, albumin; DAPI, 4,6-diamidino-2-phenylindole. Bar, 50 μm.

livers of injured, transplanted mice revealed less vacuolar degeneration caused by dilatation of mitochondria and rough endoplasmic reticulum. These observations reflect the data of the decrease of ALT and AST levels in injured transplanted mice. Therefore, transplantation of ASC-derived hepatocyte-like cells provided protection against CCl_4-induced hepatic injury. The above results indicate that ASC-derived hepatocyte-like cells generated within 13 days reveal hepatocyte-specific markers and functions *in vitro*, and improve liver function *in vivo*.

Discussion

Transplantation of hepatocytes generated from stem cells might become an easier, more efficient, and safer way than whole organ transplantation to cure patients suffering from liver disease. ASC can be very easily obtained with minimal invasiveness from a patient's own adipose tissue. Such a possibility sidesteps the obstacles regarding the risk of rejection, ethical issues, and availability of stem cells. We have already demonstrated mouse

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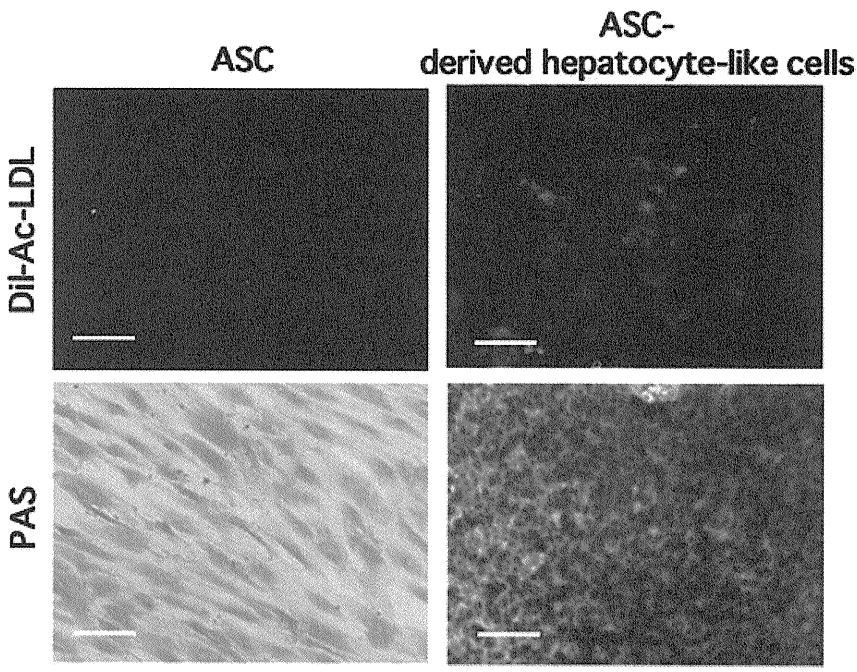

Figure 4 Low-density lipoprotein (LDL) uptake ability and glycogen storage ability (PAS) of adipose-derived stem cells (ASC)-derived hepatocyte-like cells at day 13 of induction. Dil-Ac-LDL, 1,1′-dioctade-cyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (Dil)-labeled acetylated LDL. Bar, 50 μm.

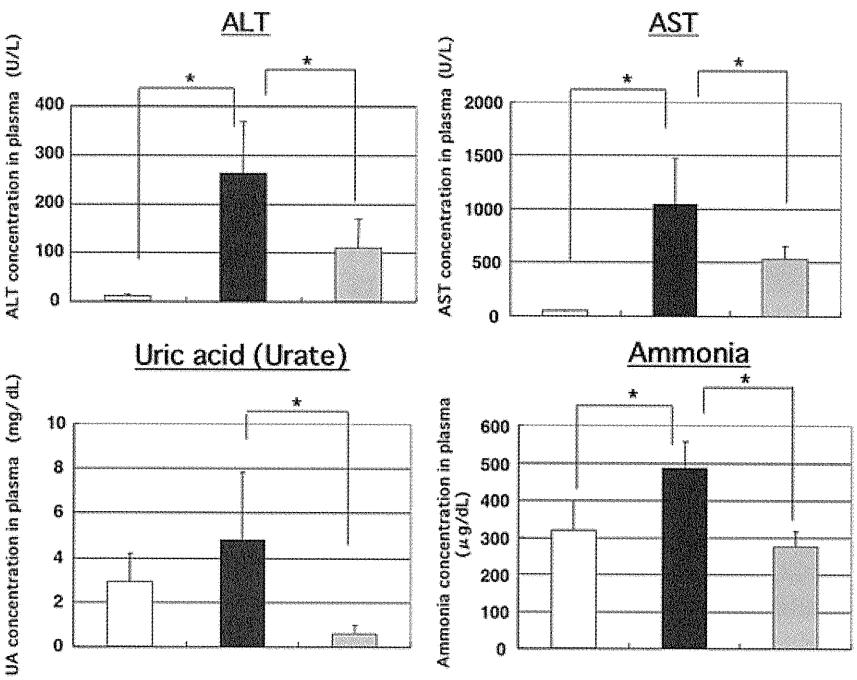

Figure 5 Biochemical analysis. Concentration of ammonia, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and UA (uric acid/urate) in blood serum of killed mice. □, non-injured, non-transplanted mice; ■, injured and non-transplanted mice; ▨, injured and transplanted with adipose-derived stem cells (ASC)-derived hepatocyte-like cells (combined data of Donors #1 and #2). Data are presented as the mean ± SD and were analyzed by the Bonferroni correction $n = 3$. (*$P < 0.05$).

embryonic stem cell[28–30] and human adult ASC[19] hepatogenic differentiation.

In the present study, we presented induction within a very short time of human ASC into hepatocyte-like cells. Thirteen days is sufficient to generate *in vitro* cells, which reveal hepatocyte-specific morphology, marker profile, and functionality. This is first time for such a short hepatogenic differentiation protocol to be presented. At the beginning we treated the cells with Activin A together with FGF4, which are important factors at early stages of endoderm formation in mouse liver development. Afterwards we used a number of factors essential for hepatogenic specification and hepatocyte morphology maintenance. We compared the hepatocyte-like cells obtained by a new rapid protocol with the hepatocyte-like cells of an original protocol,[19] and have found that they reveal all the analyzed functions, albeit much earlier. We observed that 24 h of *in vitro* cocktail treatment (HGF, FGF1,

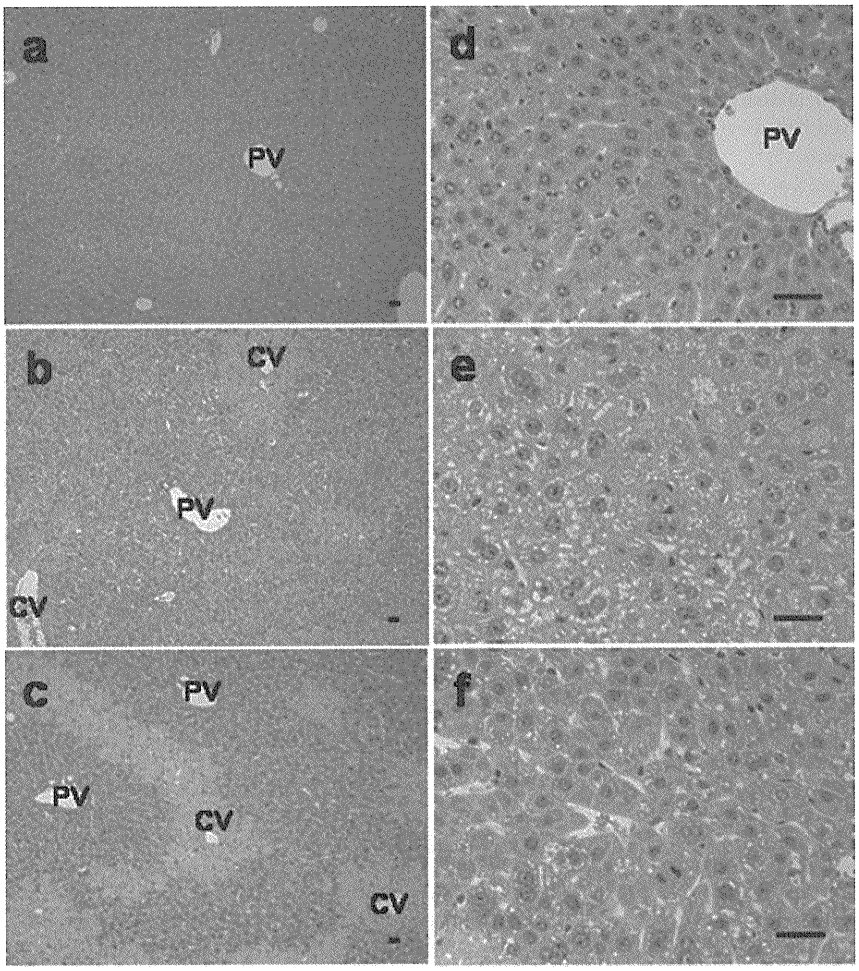

Figure 6 Hematoxylin-eosin staining of liver sections from (a,d) non-injured non-transplanted, (administered with olive oil and phosphate-buffered saline [PBS] [–]) mice ($n = 3$); (b,e) injured non-transplanted (administered with CCl_4 and PBS [–]) mice ($n = 3$); (c,f) injured transplanted (administered with CCl_4, 1 day after 1.5×10^6 ASC-derived hepatocyte-like cells transplantation) mice ($n = 4$). Panels a–c lower magnification 100 ×, panels d–f higher magnification 400 ×. CV, central vein; PV, portal vein. Scale bars represent 50 μm.

FGF4, OsM, Dex, ITS, nicotinamide, and DMSO) induces a dramatic change in morphology followed by little production of albumin at day 6 and a significant increase in the albumin level at day 9. However, using a previous protocol, albumin production was detected at days 30–50.

Prior to *in vivo* transplantation, it is important to induce hepatic fate within a short period of time and transplant the cells as fast as possible back into the patient with liver disease. Such a short period of time does not require large quantities of growth factors and may save much on expenses. Additionally, it will serve as hope and a great chance for a patient's total recovery. Significant morphological changes and albumin production as early as within 9 days suggest that it may be possible to even shorten the hepatic fate prior to transplantation. In the context of future clinical usage, a short period of stimulation to induce hepatic fate may be sufficient, because cells after transplantation may undergo further maturation in a regeneration environment.

Transplantation of *in vitro*-generated hepatocyte-like cells into CCl_4-injured nude mice resulted in the improvement of liver function *in vivo*. Interestingly, *in vivo* liver functions illustrated by the concentrations of ALT, AST, UA and ammonia were significantly decreased after ASC-derived hepatocyte-like cell transplantation (Fig. 5). The functional benefits of ASC-derived hepatocyte-like cell transplantation may be because of the functional support of the transplanted cells. It is still not clear by which mechanisms the transplanted cells improve the functioning of the liver. Fusion with host hepatocytes is not excluded. Likewise, the support and activation of endogenous progenitors are possible. Further studies examining the *in vivo* mechanism of homing, engraftment, and liver regeneration need to be conducted. It has been reported that in recipient liver, partial portal embolization, not partial portal ligation, improves engraftment of transplanted hepatocytes in a monkey primate preclinical model.[31] This provides new possibilities and strategies for future cell transplantation. It is essential to exclude any post-transplantation complications prior to any clinical trials. A long-term course experiment as well as safety issues should be carefully evaluated. Interestingly, in another study,[32] we observed that parameters such as ALT, AST, UA and ammonia were also decreased after undifferentiated ASC transplantation and we postulate that undifferentiated ASC per se compose a very attractive tool for the establishment of successful therapy for the liver.[32] We also speculate that the therapeutic potential of ASC may be due to the trophic activity of ASC.[32] These findings require additional studies with respect to safety issues post-

transplantation; however, they give great promise for future clinical applications.

Short-term hepatogenic induction methods may also have great usage in drug metabolism studies and toxicological analyses. In fact, we have already observed that ASC-derived hepatocyte-like cells reveal cytochrome activities (data not shown).

In conclusion, our study revealed that ASC have a special affinity towards hepatocyte differentiation *in vitro* and hepatocyte regeneration *in vivo*. Thus, ASC may be a superior choice for the establishment of therapy for an injured liver.

Acknowledgments

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研究成果の刊行に関する一覧表

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Ⅱ. 基礎的メカニズムの解明
3. NASHと慢性炎症

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[Summary]

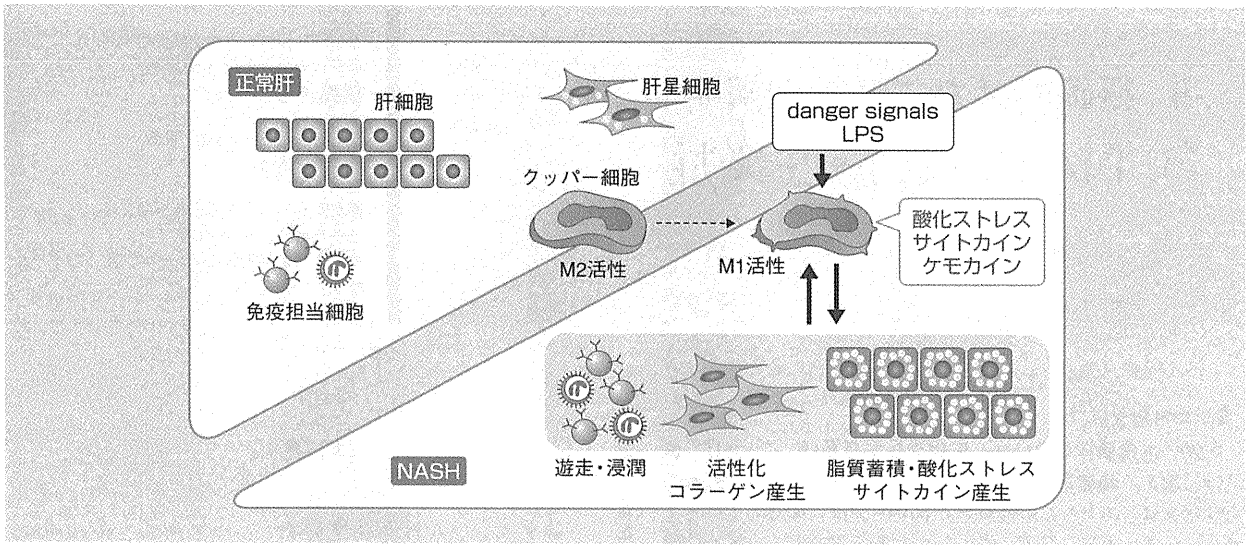
肥満の進展過程において，肝臓では脂質蓄積・インスリン抵抗性に加え，組織常在性マクロファージであるクッパー細胞の活性化をはじめとした慢性炎症がNASH発症に関与していると考えられる．近年，肥満の脂肪組織や動脈硬化症など多くの肥満関連疾患の基盤病態として慢性炎症が注目され，病原体センサーTLR4と内因性リガンドの相互作用により誘導される「自然炎症」の病態生理的意義も明らかにされつつある．NASHは適切な動物モデルが存在しないため，NASHの進展とTLR4の関連は十分に検討されていない．今後，NASHにおける病態形成と「自然炎症」の関与が明らかになり，NASHの自然史が解明されることが期待される．

Key Words:

クッパー細胞□TLR4□自然炎症□飽和脂肪酸

はじめに

非アルコール性脂肪性肝炎（nonalcoholic steatohepatitis；NASH）は非アルコール性脂肪性肝疾患（nonalcoholic fatty liver disease；NAFLD）の中で，ときに肝硬変・肝癌に進展する重症型と考えられる．その発症機序としてTwo-hit theoryが提唱されているが，その詳細な発症メカニズムにはいまだ不明な部分が多い[1]．肝臓は糖脂質代謝において重要な臓器であるとともに，自然免疫系においても最前線として機能する臓器であり，NASHの病態には自然免疫が深くかかわっていると考えられる．近年，肥満，糖尿病や動脈硬化性疾患においてマクロファージの役割に対する理解が深まり，生活習慣病やメタボリックシンドロームに共通する基盤病態として「慢性炎症」が注目されている[2]．慢性炎症では，比較的短期間に炎症反応の活性化と退縮を生じる急性炎症と異なり，長期にわたるストレス応答のために実質細胞と間質細胞の相互作用が遷延化し，適応の破綻により不可逆的な「組織リモデリング」を生じて臓器の機能障害をもたらす．NASHは肝臓に脂質が蓄積し，肝細胞障害やクッパー細胞を含む免疫担当細胞の活性化により，肝星細胞が活性化して細胞外基質を過剰に産生し，線維化という組織リモデリングに至った状態と考えられる（図❶）．本稿では，組織常在性マクロファージであるクッパー細胞に焦点を当て，NASHの病態への関与と，



図❶ クッパー細胞の活性化と細胞間相互作用

正常肝は糖脂質代謝と自然免疫系を司る組織として，種々の細胞が恒常性維持に働いている．一方，肥満に伴って細胞障害によって放出されるdanger signalやLPSなどに曝されると，クッパー細胞は活性化し，さらに多くの酸化ストレス，サイトカイン，ケモカインなどを産生する．さまざまな細胞同士の相互作用の結果，炎症の遷延化・線維化といった組織リモデリングにつながる

病原体センサーと内因性リガンドの相互作用により誘導される「自然炎症」という新たな概念について述べる．

クッパー細胞と肝臓における炎症性変化

クッパー細胞は全身の組織常在性マクロファージの80~90%，肝非実質細胞の約10%を占める[3]．肝臓に流入する血流の約80%は門脈血であり，消化管由来の病原微生物，環境毒素，栄養素などが直接流入する．クッパー細胞は類洞に広く分布するため，生体に有害な物質を認識・除去することで，生体の恒常性維持に働いている[3]．具体的には，病原体やアポトーシス細胞，死細胞の残骸などの貪食や，活性酸素の産生による病原体の殺滅，ケモカイン産生による好中球や単球などの遊走を促す．また，抗原提示作用も有し，獲得免疫にも関与する．これらのクッパー細胞の機能は炎症応答を適正に調節するために厳格にコントロールされているが，慢性的な組織障害によってクッパー細胞の機能調節に破綻が起きると，さまざまな側面からNAFLDの病態形成に影響を与えることが予想される．

ガドリニウムやクロドロネートリポソーム法によってクッパー細胞を特異的に消去すると，四塩化炭素による肝障害や虚血再灌流障害，メチオニン・コリン欠乏食負荷における肝障害が軽減する[4,5]．これらの結果は，クッパー細胞がさまざまな肝障害モデルにおいて炎症と組織障害を増悪することを示している．一方で，四塩化炭素障害後の回復期にはクッパー細胞が組織修復を促進することから，障害の時期によって異なる役割を担っていると考えられる[5]．NASHのように慢性的なストレスによって発症する病態において，病態の進展過程で異なる役割をもつのかどうか，現在のところ明らかではない．また，上述の方法を用いて，高脂肪食あるいは高脂肪・高ショ糖食下にクッパー細胞を

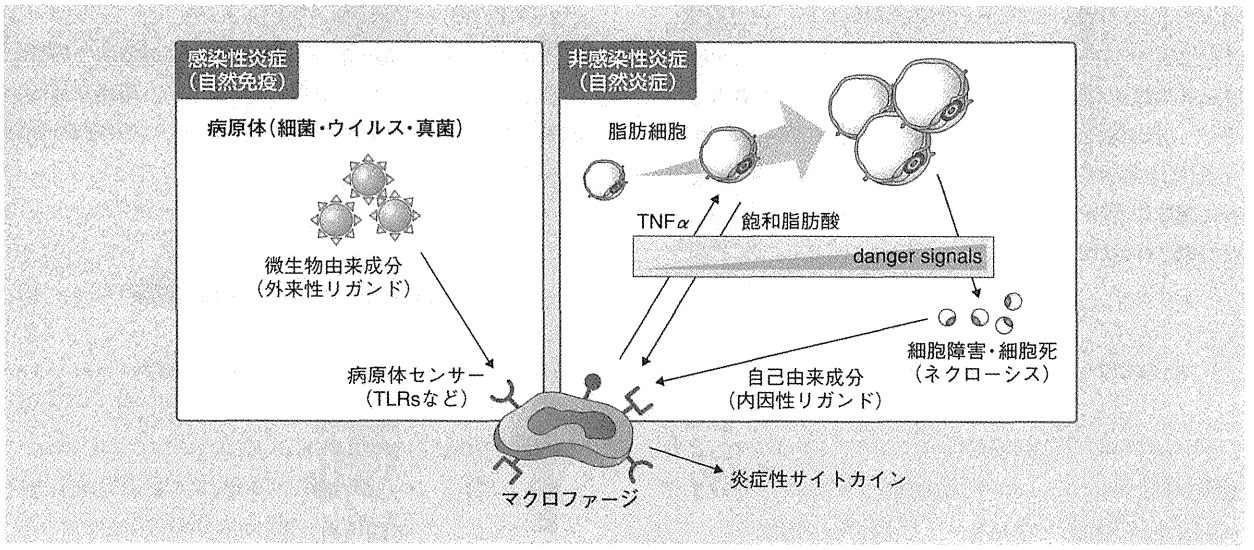
消去すると，肝臓の中性脂肪含量が減少し，インスリン感受性が改善することが報告されている[6,7]．また，クッパー細胞と肝細胞の共培養系においてクッパー細胞を活性化させると，肝細胞における脂質合成系遺伝子の発現が増加し，β酸化が抑制されるということから，クッパー細胞が糖脂質代謝に影響を与え，脂肪肝そのものを促進していることが示唆される[7]．

マクロファージの活性化状態とNAFLD

マクロファージには炎症性サイトカインを産生する活性型のM1マクロファージと抗炎症性サイトカインを産生する非活性型のM2マクロファージがあり，病態に応じてマクロファージの性質に変化があると考えられている．例えば，非肥満の脂肪組織では非活性型のM2マクロファージが中心であるが，肥満に伴い増加する活性型のM1マクロファージは多くの炎症性サイトカインを分泌して脂肪組織の炎症性変化を促進するという[8,9]．このM1/M2極性に影響を与えるものとして，核内受容体であるperoxisome proliferators-activated receptor γ（PPARγ）やperoxisome proliferators-activated receptor δ（PPARδ）の関与が示唆されている[10,11]．PPARγあるいはPPARδを欠損するとマクロファージはM1側に傾き，高脂肪食負荷による肥満が誘導されやすく，インスリン抵抗性や脂肪肝が増悪する．特に，PPARδはクッパー細胞のM2極性に必要であるといわれている[11]．NAFLDの肝臓ではマクロファージにおけるM1サイトカイン産生増加が知られるが[7]，NASHにおいて常在性マクロファージの活性化と，浸潤マクロファージがそれぞれどのような影響を与えているのかは不明である．最近，脂肪肝では骨髄由来のC-C chemokine receptor 2（CCR2）陽性細胞が増加し，これらの細胞群はクッパー細胞に比較してM1サイトカインの産生が多く，肝中性脂肪含量の増加に関与していることが示された[12]．臨床的には，肥満や2型糖尿病の患者では循環血液中の単球の極性がM1様に変化していること，インスリン抵抗性改善薬であるチアゾリジン誘導体が単球の極性をM2様に変化させることが報告されており，循環血液中の単球の活性制御がメタボリックシンドロームの病態に関連する可能性が示唆されている[13,14]．

病原体センサーと自然炎症

Toll様受容体（toll-like receptors；TLRs）はパターン認識受容体ファミリーに属し，自然免疫系において病原体の認識と炎症性シグナル応答に重要であることが知られている[15]．特に，クッパー細胞に発現するTLR4は病原体センサーとしてグラム陰性菌の構成成分であるリポポリサッカライド（lipopolysaccharide；LPS）を認識し，細菌感染の免疫応答に中心的役割を果たす[15]．一方で，肥満の脂肪組織や動脈硬化症など非感染性の慢性炎症にTLR4が関与することが報告されている[16-18]．このような病態においては，自己の細胞から分泌される代謝産物，死細胞や細胞外基質より放出される因子（danger signal）が内因性リガンドとして作用していると考えられ，内因性リガンドに誘導される応答を「自然炎症」と捉える概念が提唱されている（図❷）．実際われわれは，脂肪細胞から放出される飽和脂肪酸がTLR4の内因性リガンドとして作用し，肥満脂肪組織におけるマクロファージとの相互作用や動脈硬化の初期病態とされる血管内皮への単球接着に関与することを報告した[19,20]．肝臓の線維化モデルとして汎用されるメチオニン・コリン欠乏食負荷モデルにおいても，TLR4を欠損すると肝脂質蓄積，炎症，線維化が改善する[21]．肥満では消化管において細菌の過増殖や消化管の透過性に変化が生じ，血中のLPS濃度が増加するといわれる[22]．消化管から流入したLPSは門脈を介して肝臓に到達するため，クッパー細胞はLPSの曝露を受け，これがNASH進展過程における2nd hitのひとつとして捉えられている[1]．一方で，NAFLD/NASHでは食物由来あるいは内臓脂肪での脂肪



図❷ 肥満の脂肪組織における自然炎症

マクロファージの細胞表面上にはTLRsなどの病原体センサーを発現する．従来，病原体センサーは宿主には存在しない微生物由来成分をリガンドとすると考えられてきた(外来性リガンド)．しかしながら，細胞障害や細胞死(ネクローシス)など細胞が危機的な状況に陥ると，普段は放出されることのない自己由来成分(内因性リガンド)が「danger signal」として放出され，病原体センサーを介して慢性炎症を誘導・増悪する

分解に由来する脂肪酸の流入も増加していると考えられ，脂質を含むさまざまな因子が病原体センサーのリガンドとして作用している可能性がある．しかし，現在のところ，飽和脂肪酸以外にメタボリックシンドロームや動脈硬化の発症に関与するTLR4の内因性リガンドは不明であり，今後の重要な検討課題である．

おわりに

NASHの病態において，クッパー細胞をはじめとした自然免疫系の活性化と，肝細胞や肝星細胞など多彩な細胞との相互作用が深く関与している．そのメカニズムのひとつとして，クッパー細胞の病原体センサーTLRsとdanger signalの相互作用により誘導される「自然炎症」が経時的に増大していくと考えられる．また，TLRsはクッパー細胞だけでなく，肝星細胞にも発現しており，特に，胆管結紮モデルでは肝星細胞のTLR4が重要であることが報告されている[23]．NASHの臨床像を反映する適切な動物モデルが存在しないため，NASHの病態におけるTLRsの関与は十分検討されていない．今後，TLRsをはじめとした病原体センサーが，どのような細胞で何をリガンドとしてNASHの病態形成に関与しているかが明らかとなり，NASHの自然史が解明されることが期待される．

■文 献

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