Novel Management of Acute or Secondary Biliary Liver Conditions Using Hepatically Differentiated Human Dental Pulp Cells

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The current definitive treatment for acute or chronic liver condition, that is, cirrhosis, is liver transplantation from a limited number of donors, which might cause complications after donation. Hence, bone marrow stem cell transplantation has been developed, but the risk of carcinogenesis remains. We have recently developed a protocol for hepatic differentiation of CD117⁺ stem cells from human exfoliated deciduous teeth (SHED). In the present study, we examine whether SHED hepatically differentiated (hd) *in vitro* could be used to treat acute liver injury (ALI) and secondary biliary cirrhosis. The CD117⁺ cell fraction was magnetically separated from SHED and then differentiated into hepatocyte-like cells *in vitro*. The cells were transplanted into rats with either ALI or induced secondary biliary cirrhosis. Engraftment of human liver cells was determined immunohistochemically and by *in situ* hybridization. Recovery of liver function was examined by means of histochemical and serological tests. Livers of transplanted animals were strongly positive for human immunohistochemical factors, and *in situ* hybridization confirmed engraftment of human hepatocytes. The tests for recovery of liver function confirmed the presence of human hepatic markers in the animals' blood serum and lack of fibrosis and functional integration of transplanted human cells into livers. No evidence of malignancy was found. We show that *in vitro* hdSHED engraft morphologically and functionally into the livers of rats having acute injury or secondary biliary cirrhosis. SHED are readily accessible adult stem cells, capable of proliferating in large numbers before differentiating *in vitro*. This makes SHED an appropriate and safe stem cell source for regenerative medicine.

Introduction

LIVER TRANSPLANTATION is hampered by difficulties in finding donors and in avoiding health concerns after donation.^{1,2} Stem cell therapy may offer an alternative method for transplantation, raising great hopes for this treatment. However, transplantation of undifferentiated stem cells increases the incidence of cancers or teratoma.^{3,4} Even without malignancy, transplanted stem cell lines will lose their ability to repopulate tissues if they have not yet differentiated enough.⁵ The objective of this study was to determine whether hepatocyte-like CD117⁺ stem cells from human exfoliated deciduous tooth pulp differentiated *in vitro* (SHED) will allow recovery from secondary biliary cirrhosis or acute liver injury without severe complica-

tions.^{1,6,7} Transplantation of stem cells differentiated *in vitro* can be expected to be safer and more efficient compared with undifferentiated cells.

To produce enough of well-differentiated cells is one of the biggest challenges in human transplantation. We have not yet managed to proliferate a large number of stem cells *in vitro* while still keeping their stem cell properties. Moreover, autodifferentiation starts during very early passages^{8–10} and interferes with this procedure. There is no effective protocol yet, even at the *in vitro* research level.¹¹ Lysy *et al.* mention that while it is possible to use stem cell-derived hepatocyte-like cells for clinical purposes, major difficulties remain; there is a continuing lack of liver function, and no evidence of adequate liver regeneration has yet been reported.¹² Furthermore, expression of the acquired

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hepatocyte phenotype is severely impaired, with very little or no engraftment of transplanted bone marrow-derived stem cells into liver tissue.¹³ A high enough generation level using both human stem cell-derived hepatocyte-like cells and successful animal models has not yet been established for liver regenerative medicine.

We have previously reported that adult dental pulp stem cells or SHED may be an excellent cell source for the purposes mentioned earlier.^{10,14,15} The cells have been shown to differentiate into hepatocyte-like cells displaying excellent hepatic functions with complete purity of the hepatically differentiated SHED (hdSHED). Furthermore, the protocol employs a serum-free medium, which reduces future problems after differentiation.¹⁶ We managed to induce hepatic differentiation in CD117⁺ SHED cultures *in vitro*, at around 50% of the population of the primary culture of SHED.^{10,14} The CD117 fractionating protocol allows cells to keep their stem cell properties after 50 passages, meaning that we can produce enough *in vitro* differentiated cells for human transplantation. All of the CD117⁺ cells expressed confirmed hepatic markers after differentiation, including α -fetoprotein (AFP), albumin, carbamoyl phosphate synthetase-1 (CPS-1), and insulin-like growth factor-I (IGF-I). Moreover, it has been suggested that SHED retain more of the capabilities of stem cells than cells from other sources.^{15,17} Hepatic differentiated SHED stem cells may be able to restore liver function after resection of the liver or secondary biliary cirrhosis. We establish a human-specific hepatocyte source suitable for human transplantation.

Materials and Methods

Isolation of primary cell cultures

All protocols for the present study were approved by the Research Ethics Committee of Nippon Dental University. The pulp of extracted deciduous teeth was accessed through the resorbed tooth root canal and extracted by sterile barbed broach. The pulp was then digested for 1 h at 37°C in 4 mg/mL collagenase type I (Wako Pure Chemical Industries Ltd., Osaka, Japan) and dispase (Invitrogen, Grand Island, NY). All cell suspensions were seeded into 25-cm² flasks (TPP, Trasadingen, Switzerland) and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Eugene, OR) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT), 100 U/mL penicillin, 100 μ g/mL streptomycin, and 0.25 μ g/mL amphotericin (Invitrogen, Carlsbad, CA). Cell cultures were grown until they reached 85–90% confluency and then passaged into new flasks (10⁵ cells/cm²).

Magnetic separation of CD117⁺ cells and cell culture

The cells were grown in DMEM supplemented with 10% FBS for three passages. Up to 10⁶ SHED were collected by trypsinization and subjected to the Manual MACS[®] Cell Separation protocol (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).^{14,15} In brief, the cells were incubated with mouse anti-human CD117 IgG conjugated with magnetic microbeads (Miltenyi Biotec GmbH), then the cell suspension was loaded into a column placed in the magnetic field of a mini-MACS Separator (Miltenyi Biotec, Inc., Auburn, CA). The magnetically labeled CD117⁺ cell fraction was retained in the column. After removing the column

from the magnetic field, the cells were flushed out with 2 mL DMEM into a new culture flask.

hdSHED in serum-free medium

CD117⁺ hdSHED were cultured in the serum-free DMEM, as reported previously,^{10,14} and then induced to undergo *in vitro* hepatic differentiation. In short, 20 ng/mL of recombinant human hepatocyte growth factor (HGF; R&D Systems, Inc., Minneapolis, MN) was added for 5 days; later, a mixture of 10 ng/mL Oncostatin M (R&D Systems, Inc.) and 10 nM dexamethasone (Wako Pure Chemical Industries Ltd.) was added to the HGF-supplemented medium for another 15 days. All types of media were changed every third day.^{10,14,15}

Animal liver injury models

Acute liver injury. All animal studies were approved by the animal study committee, Nippon Dental University. Appropriate measures were taken to minimize pain or discomfort. Each rat was kept under observation in an individually ventilated cage system during the experiments. All surgeries were performed under sodium pentobarbital anesthesia, and every effort was made to minimize suffering. Twelve male, 10-week-old nude rats (F344/NJc1-rnu/rnu; Nihon Clea Co. Ltd., Tokyo, Japan) were anesthetized with Somnopentyl[®] (6–10 mg/100 g bodyweight of pentobarbital sodium; Kyoritsu Seiyaku Corporation, Tokyo, Japan) and subjected to 80–90% surgical resection of the liver. hdSHED were re-suspended in Hanks' Balanced Salt Solution (Gibco[®], Grand Island, NY) at a density of 2×10^6 cells/100 μ L. Immediately after resection, hdSHED (2×10^6 /100 μ L HBSS) were implanted into the spleens of six of the animals. Another six animals were injected with 100 μ L HBSS after liver resection and were left as controls or sham-operated animals. One animal from the control group died within 24 h after resection because of liver insufficiency, so there were only five control animals left. All animals were euthanized 20 days after transplantation by intraperitoneal injection of an overdose of the pentobarbital sodium mentioned earlier (100 mg/kg), and the livers, spleens, and whole blood were collected.

Secondary biliary cirrhosis. Eighteen male 9-week-old F344/NJc1-rnu/rnu nude rats (Nihon Clea Co. Ltd.) were divided into three groups. Twelve animals were subjected to ligation of the main bile duct. The common bile duct was ligated close to the beginning of the intrapancreatic portion with polypropylene sutures (4-0; Ethicon, Inc., San Angelo, TX), using microdissection forceps. hdSHED (2×10^6 /100 μ L HBSS) were implanted into the spleens of six of the animals (cirrhosis transplanted group). Another six rats were not transplanted and were used as positive controls (cirrhosis nontransplanted group). Six rats were not subjected to ligation or transplantation and were used as negative controls (control group). Sixty days later, the rats were euthanized, and the livers, spleens, and whole blood were collected.

Morphological determination

The internal organs were fixed in 4% buffered formaldehyde, dehydrated, and embedded in paraffin. Sections were stained with hematoxylin–eosin (HE) or Masson's trichrome stains. For fluorescent staining, the sections were digested

with 0.5% Trypsin (Invitrogen, Carlsbad, CA) in phosphate-buffered saline (PBS), blocked with 1% bovine serum albumin (Invitrogen, Carlsbad, CA), and incubated at 4°C overnight with the following primary antibodies at 1:200 dilution in PBS—primary mouse anti-human antibodies: anti-serum albumin, anti-AFP, anti-HGFR/c-MET (R&D Systems, Inc.), anti-IGF-I (Raybiotech, Norcross, GA), anti-prothrombin, anti-CPS-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and anti-human mitochondria (LifeSpan Bioscience, Inc., Seattle, WA). The Alexa fluor[®]568-conjugated donkey anti-mouse antibody (Invitrogen, Carlsbad, CA) was used as the secondary antibody at 1:500 dilution. After washing, the slides were mounted with the Vectashield mounting medium with DAPI (Vector Laboratories, Inc., Burlingame, CA). The resulting fluorescent signal was detected under a confocal scanning laser fluorescence microscope (Leica DMRE; Leica Microsystems GmbH, Wetzlar, Germany).

For mitochondrial staining, after rehydrating and blocking with 1% BSA, the sections were incubated with the anti-human mitochondria antibody, produced in mouse, for 1 h at room temperature. Subsequently, slides were washed twice in 0.1 M PBS, incubated with N-Histofine Simple Stain Max PO (Nichirei Corp., Tokyo, Japan) at room temperature for 30 min, washed again, and incubated with the Metal Enhanced DAB Substrate Kit (Thermo Scientific, Rockford, IL) for 15 min until a dark brown reaction product could be seen. Slides were then mounted with the Entellan new mounting medium (Merck K GaA, Darmstadt, Germany), and images were obtained using an Olympus IX71 inverted microscope (Olympus Corp., Tokyo, Japan).

In situ hybridization

In situ hybridization was carried out by Ourgenic Co., Ltd. (Narutoshi, Japan) using their protocol. Briefly, a 520 bp cDNA encoding positions 361–880 of human Albumin CDS (1827bp) gene was synthesized using T3 (sense: GGAT CCAATTAACCTCACTAAAGGG) and T7 (antisense: CCCTATAGTGAGTCGTATTAGGATCC) promoters. Digoxigenin-labeled RNA probes were synthesized with a Dig RNA labeling mix (Roche Diagnostics Ltd., Burgess Hill, United Kingdom). The antisense probe was first produced with T7 RNA polymerase (Invitrogen, Carlsbad, CA) and then transcribed using T3 RNA polymerase (Stratagene, La Jolla, CA) to produce the sense probe. The fixed sections were digested in 5 µg/mL proteinase K (Roche Diagnostics Ltd.) for 20 min at room temperature and then prehybridized for 60 min at 54°C, after which 1 µg/mL labeled probe in the hybridization buffer was applied to the sections. Hybridization of the probe with the native mRNA was allowed for 24 h at 54°C.

The signal was detected by a chromogen nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) solution (Roche Diagnostics Ltd.). At optimal color development, the slides were counterstained with neutral red and visualized by light microscope.

Serological tests

The serum of the acute liver injury experiment animals was tested by the following methods: direct immunoradiometric assay was used for detecting human IGF-I; nephelometry followed by the Bromocresol Green assay was employed for detecting human albumin in rat serum; che-

miluminescence enzyme immunoassay (Lumipulse; Fujirebio, Tokyo, Japan) was used for detecting human AFP.

White blood cells (WBC) of the animals involved in the chronic liver condition model were counted. Serological tests of that animal group also included the total bilirubin (T.Bil.) measurement by a colorimetric assay (Total Bilirubin V; Wako Pure Chemical Industries Ltd.), using vanadate as the chemical oxidizing reagent. Blood urea nitrogen (BUN) concentrations were also tested using a commercially available kit (BUN-test; Wako Pure Chemical Industries Ltd.). Hepaplastin was detected using the Owner method. All standards and samples were analyzed in triplicate.

Statistics

Statistical analysis was performed by Bonferroni's multiple comparison procedure, using Windows SPSS Ver. 16 (SPSS, Inc., Chicago, IL).

Results

Acute liver injury

After the resection and transplantation, all rats in the transplanted group survived. The livers of the animals had

FIG. 1. After hepatically differentiated CD117⁺ stem cells from human exfoliated deciduous teeth had been transplanted into rats with acute injury, human mitochondria and human hepatic markers, α -fetoprotein (AFP), albumin, insulin-like growth factor-I (IGF-I), carbamoyl phosphate synthetase-1 (CPS-1), and c-MET, were scattered all over the regenerated livers of the transplanted animals. Cells were often grouped as if forming an island. Red indicates that the target protein reacted with the antibody in each case. In the control groups, we could not discover any human hepatic cells using the antibodies mentioned above (data are not shown). Blue shows nuclei. Magnification 400 $\times$. Color images available online at www.liebertpub.com/tea

fully regenerated 20 days after the operation. The surface of all the livers was uniform and flat. Histological staining of the rat livers revealed groups of cells expressing human markers distributed all over the regenerated organs of the transplanted animals (Fig. 1). Engrafted human hepatic cells were found in the entire liver. The cells were often grouped close to the newly formed blood vessels, but small groups and single cells were also detected in the parenchymal part of the livers. The presence of cells expressing the most important human hepatic marker, human albumin, was confirmed by *in situ* hybridization (Fig. 2). Moreover, human-specific hepatic markers were found in the rat serum as follows: albumin—28.1 mg/dL (± 1.75 mg/dL); IGF-I—30.26 ng/mL (± 1.18 ng/mL); and AFP—3.49 ng/mL (± 0.22 ng/mL). We confirmed that hdSHED had become incorporated into structural components of the liver after acute injury. In the control groups, we could not find any human hepatic cells using the antibodies mentioned earlier. The above results led us to the bile duct ligation experiments described below.

Secondary biliary cirrhosis

The livers of the animals were normal sized. Yellowish collections of bile were visible on the surface of all the livers of the cirrhosis nontransplanted group. Despite marginal bile duct proliferation and drainage of the bile, found in both the cirrhosis transplanted and the cirrhosis non-

transplanted groups, our results show that the transplantation group has significantly fewer markers for developing cirrhosis. Blood examinations in the cirrhosis nontransplanted group showed an increase in WBC, T.Bil. and BUN and a decrease in hepaplastin and rat albumin ($p < 0.001$) compared to the control group (Fig. 3). All markers in the cirrhosis transplanted group were significantly different from those in the cirrhosis nontransplanted group ($p < 0.001$). Although rat albumin was not recovered, human albumin appeared to compensate for the loss of rat albumin (Fig. 4). In the serum of the cirrhosis transplanted group, human-specific AFP (33 ± 6.0 ng/mL) was also found to be positive for the same reason, while these markers were not found in the control and cirrhosis nontransplanted groups. HE staining in the cirrhosis nontransplanted group showed typical aspects of liver cirrhosis, in that fibrous tissues were markedly increased, whereas it is very difficult to find morphological properties of cirrhosis in the cirrhosis transplanted group (Fig. 5). Immunological staining showed no cells positive for the human-specific hepatic marker antibodies AFP, albumin, CPS-1, IGF-I, and prothrombin in the nontransplanted group, while groups of cells that were distributed with high density in the entire livers of the cirrhosis transplanted group were strongly positive for these human-specific liver markers, as shown in Figure 6. Since hdSHED were originally transplanted into the spleen, cells expressing human-specific markers were found in the spleens of the transplanted animals (Fig. 7).

FIG. 2. The organization of the cells expressing human albumin as displayed by *in situ* hybridization. The antisense probe was transferred to the sense probe by T7 RNA polymerase, and then the production was confirmed by agarose gel electrophoresis. Magnification 200 $\times$. Color images available online at www.liebertpub.com/tea

FIG. 3. The serological tests in the nontransplanted group showed an increase of white blood cells (WBC) ($18,600 \pm 1970$ cells/ μ L– 6267 ± 728 cells/ μ L, $p < 0.001$), total bilirubin (T.Bil.) (0.7 ± 0.3 mg/dL– 7.7 ± 0.3 mg/dL, $p < 0.001$) and blood urea nitrogen (BUN) (13.7 ± 2.7 mg/dL– 60.5 ± 14.1 mg/dL, $p < 0.001$), and a decrease in hepaplastin ($100\% \pm 15.5\%$ – $25\% \pm 5.5\%$, $p < 0.001$) compared to the control group. In the transplanted group, WBC, T.Bil., BUN, and hepaplastin levels were 7150 ± 442 cells/ μ L, 1.7 ± 0.4 mg/dL, 21.3 ± 1.4 mg/dL, and $100\% \pm 9.0\%$, respectively. * $p < 0.001$.

Discussion

For severe liver failure, as in fulminant hepatitis or cirrhosis, hepatic transplantation might be the only life-saving definitive therapy.^{18–20} Transplantation has limited application because there are few donors and because of health concerns after removing part of the donors' livers. Hence, stem cell transplantation is expected to be a novel

treatment for repopulating a liver damaged by various conditions.^{21,22}

In our study, we subjected two groups of nude rats to acute injury or secondary biliary cirrhosis and assessed whether engrafted hdSHED could promote the healing process in the livers by intrasplenic transplantation instead of by introducing the cells into the portal vascular system.²³ Two lines of defense against liver condition were defined—regeneration by hepatocytes and regeneration by liver progenitor cells, which contribute differently to the regeneration of liver.²⁴ There is evidence that liver progenitor cells are activated to a large degree after liver injury.²⁵ To repopulate liver progenitor cells and to help the organ overcome severe liver conditions, various stem cell sources have been proposed.²⁶ Despite the increasing amount of data in the literature showing that bone marrow stem cells engraft into the recipient's liver by means of cell fusion with²⁷ hepatocytes or by transdifferentiation²⁸ and also reduce liver fibrosis,²⁹ the mechanism of repopulation of the damaged liver remains a highly disputable topic. Moreover, Kaibori *et al.*²⁸ speculated that transplanted adult stem cells may produce hepatopoietic factors in the early period after acute or chronic trauma has occurred, which would stimulate the regeneration of the injured liver. However, it has not been yet determined whether such speculation is consistent with the data on *in vivo* liver regeneration.

After resection of the liver, the hepatocytes immediately start regeneration of the damaged organ. The existence of CD117⁺ cells in the liver and an increase in the levels of kit ligand in the healing liver have been reported.^{30,31} We have previously reported the pluripotent differentiation potential

FIG. 4. Albumin content in animal serum (g/dL). In the serum of the transplanted group, human albumin appeared at 3.0 ± 0.3 g/dL and rat albumin levels were 2.3 ± 0.2 g/dL. Rat albumin levels in the nontransplanted group were significantly lower (2.2 ± 0.2 g/dL) than in the control group (4.2 ± 0.16 g/dL).

FIG. 5. Hematoxylin–eosin (HE) staining clearly shows that secondary biliary cirrhosis occurred in the cirrhosis non-transplanted group. Fibrous tissues are markedly increased. However, it is difficult to find any indication of cirrhosis in the cirrhosis transplanted group. Cirrhosis non-transplanted and transplanted: (A) HE stain. Magnification 200 $\times$. (B) Masson's trichrome stain. Magnification 200 $\times$. Cirrhosis non-transplanted: (C) Masson's trichrome stain. Magnification 50 $\times$.

of CD117⁺ SHED.³² The importance of CD117 in the hepatocyte repopulation of injured liver prompted us to use CD117⁺ SHED for our research. Blood examinations in this study show that the transplanted hdSHED produced human-specific hepatic markers in acute injury. All human hepatic markers assessed were positive in the livers of the cirrhosis

transplanted group. Successful engraftment of rat liver tissue with acute injury by means of human tissue was confirmed by the *in situ* hybridization of human albumin. In this study, we have further demonstrated that the original liver matrix represented a model of structure scaffold for hepatic cell transplantation.³³ The production of liver-specific markers

FIG. 6. Immunofluorescent histological staining in the transplanted group shows cells positive for human-specific liver markers scattered with high density in the entire liver, as shown in the figures. Red indicates that each target protein reacted with the antibody. In the control and nontransplanted groups, we could not discover any human hepatic cells using the antibodies mentioned above (data are not shown). Blue shows nuclei. Magnification 400 $\times$ except prothrombin 500 $\times$. Color images available online at www.liebertpub.com/tea

FIG. 7. After transplantation, cells expressing human hepatic markers, AFP, albumin, IGF-I, and CPS-1, were found in the spleens of the transplanted animals. *Red* indicates that the target protein reacted with the antibody in each case. *Blue* shows nuclei stained with DAPI. Magnification 400 $\times$. Color images available online at www.liebertpub.com/tea

by the transplanted hdSHED may be the key factor that helps the injured organ to overcome the stress and to sustain the levels of these markers in the blood, enabling the animals to survive the acute phase of hepatic injury.

Bile has a toxic effect on hepatocytes if not evacuated through the bile duct. In the animals subjected to common bile duct ligation, we found proliferation of small bile duct vessels, connecting the liver with the duodenum. All these animals were losing weight and appeared obviously sick, displaying much less activity (data are not shown). After the animals were euthanized, we found elevated liver cirrhotic markers in the cirrhosis nontransplanted group, while in the cirrhosis transplanted group, the cirrhotic markers were within the norm. Rat albumin stayed low, while human albumin, which may function as an alternative to rat albumin was produced, compensating for the shortage of this protein. Production of other hepatopoietic factors may also stimulate the faster proliferation of rat hepatocytes and the small bile ducts and so prevent the toxic effect of the bile on the transplanted livers; hence, it is very difficult to find fibrosis in the cirrhosis transplanted group of animals.

Morphological studies proved the engraftment and integration of transplanted hdSHED into rat livers. The same results were also observed in the acute liver injury transplanted group. Since hdSHED were originally transplanted into the spleen, the presence of hdSHED in the spleen may also account for the normal levels of the markers found in the serum of the transplanted rats.

We employed indirect transplantation into the liver through the spleen to avoid ectopic liver formation. Our preliminary study employing direct transplantation into the liver caused ectopic liver formation in the lung. The data here show that our hdSHED are well differentiated and do not require niches for differentiation after transplantation. Since a protocol using the portal vein would be employed for large animals, including human, ectopic liver formation in the spleen would not happen; hence, our use of transplantation to the spleen. Moreover, the ectopic liver for-

mation described above also means that our *in vitro* differentiated hdSHED have a much lower possibility of producing teratomata or other neoplasms. This project has demonstrated a much higher potential for adaptation to clinical application than any other previously published protocols.^{34,35} The use of CD117⁺ SHED and *in vitro* hepatic differentiation of 100% of the cells in the serum-free medium can dramatically reduce the risk of severe complications after implantation.^{36–38} We have also demonstrated previously that the differentiation level in CD117⁺ SHED is promoted more than in bone marrow stem cells, and that hydrogen sulfide increases not only hepatic differentiation but also pancreatic differentiation.^{15,39} Hepatically differentiated SHED were proven to engraft morphologically and functionally into the acutely injured or secondary biliary cirrhosis liver, whereas the *in vivo* process of liver regeneration is still obscure. Further study of hdSHED using hydrogen sulfide may reveal the process, including the hepatopoietic factors mentioned earlier.

SHED are a suitable, convenient, and safe source of stem cells of nonembryonic origin for hepatic cell therapy. Moreover, *in vitro* differentiation of adult stem cells and subsequent transplantation may prove to be an efficient and safer method for developing cell therapy protocols.

Disclosure Statement

No competing financial interests exist.

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