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APPLICATION OF COLLAGEN SCAFFOLDS FOR HEPATIC STEM-LIKE CELL TRANSPLANTATION

Yasuharu Ueno[1)], Kunihiko Terada[1)], Hirokazu Nagai[2)], Go Watanabe[3)], Kiyoshi Ishikawa[1)], Kiwamu Yoshikawa[1)], Yasuhiro Hirayama[1)], Erika Endo[1)], Takashi Kameda[1)], Yang Liu[1)] and Toshihiro Sugiyama[1)]

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[1)]*Division of Molecular Cell Biology, Department of Anatomy and Biochemistry,* [2)]*Division of Dentistry and Oral Surgery, and* [3)]*Division of Gastroenterological Surgery, Department of Surgery, Akita University School of Medicine, Akita 010-8543, Japan*

Abstract: Tissue engineering using somatic stem cells is a new and exciting technique which has the potential to facilitate tissue reconstruction. Recent studies on tissue engineering have indicated advantages in using collagen matrices to restitute the structure of an organ. Here we have demonstrated the efficacy of transplantation of hepatic stem-like (HSL) cells, a previously established liver epithelial cell line with potential for differentiation, using type I collagen sponge. To this end, HSL cells were cultured and transplanted into Nagase's analbuminemic rat with spongy type I collagen matrices. Consequently, HSL was able to express both albumin and TAT after the culture in collagen sponge. In addition, the levels of serum albumin in recipient rats were found to increase up to 1.5 fold relative to controls after transplantation. These findings suggest that HSL cells are able to differentiate into functional hepatocytes, and that biodegradable collagen sponge may enhance hepatic differentiation of HSL cells by providing an appropriate microenvironment.

Key words: Somatic stem cell, Collagen, Differentiation, Scaffold, Cell transplantation

Introduction

Liver has been believed to contain somatic stem cells that have the potential to differentiate into both hepatocytes and biliary epithelial cells[1-3]. They are thought to constitute a reserve compartment that is activated in situations of severe liver injury in which hepatocytes cannot mount an appropriate proliferative response[1,2]. So far, several cloned lines of nonparenchymal epithelial cells derived from healthy livers of adult rats have been established. These liver epithelial cells are thought to be putative hepatic stem cells because of their capabilities to proliferate and differentiate into either hepatocytes or biliary epithelial cells[4-6]. Recently, we have demonstrated that HSL cells, a cloned liver epithelial cell line, have potential to differentiate into albumin-positive cells when cocultured with hepatic stellate cells[7], known as important cellular sources of extracellular matrices (ECMs) including types I, III, and IV collagens, and various growth factors[8,9]. Our study revealed that the maturation of HSL cells was accelerated by direct contact with stellate cells, indicating that the cell-matrix interaction was significant for the maturation of HSL cells.

The ECMs are involved in various biologic processes including the adhesion, migration, and

differentiation of cells via cell-matrix interaction[10,11]. In liver, the ECMs produced by stellate cells regulate the proliferation and differentiation of hepatocytes[12]. Especially, of all collagens in liver, type I collagen is very abundant and distributes widely around the vascular spaces and Glisson's capsule, and within the space of Disse in contact with hepatocytes and sinusoidal endothelial cells, providing an appropriate microenvironment for hepatic differentiation[9,10,13]. Several *in vitro* studies reported that primary hepatocytes cultured in collagen gels maintained their function long term[14,15]. Notably, collagen sponge scaffolds obtained by freeze-drying and crosslinking with UV treatment have improved mechanical properties including rigidity against mechanical stress and a reduced biodegradation rate compared with collagen gel[16], thereby they were thought to facilitate cell transplantation.

In this study, we cultured and transplanted HSL cells with spongy type I collagen scaffolds to investigate their differentiation potential and to evaluate the efficacy of transplantation for possible therapeutic applications.

Materials and Methods

Animals

Male Nagase analbuminemic (NA) rats, a mutant strain derived from SD rats, at 4 weeks of age were obtained from Japan SLC (Hamamatsu, Japan). Studies involving the use of animals were carried out in accordance with the animal guidelines of Akita University School of Medicine.

Cell culture and collagens

HSL cells, the liver epithelial cell line, were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St Louis, USA) supplemented with 10% fetal calf serum (FCS) at 37°C in a 5% CO_2 atmosphere[7]. Insoluble type-I collagen sponges with pores of approximately 100 μm were prepared as described[17]. The sponges ($2\times2\times0.5$ cm^3) were immersed in DMEM supplemented with 10% FCS, then 1×10^7 cells were inoculated into each sponge and cultured with DMEM supplemented with 10% FCS.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the cells using ISOGEN (Nippon Gene, Japan) according to manufacturer's instructions. RT-PCR was carried out using the SUPERSCRIPT One-Step RT-PCR System (Invitrogen, USA). The mixture for each reaction contained 1 μg total RNA, 0.2 μM forward and reverse primers, reverse transcriptase, and Taq DNA polymerase. The resulting complementary DNA was amplified using TaKaRa PCR Thermal Cycler TP-3000 (TaKaRa, Japan) with the following sets of primers. For albumin (302 base pairs) : forward, 5′-ATTCACACTCTCTTCGGAGAC-3′ ; reverse, 5′-GGTCAGAACCTCATTGTATTT-3′ ; for tyrosine amino-transferase (TAT) (402 base pairs) : forward, 5′-ATGAGATTCGAGACGGG-CTG-3′ ; reverse, 5′-GACGGGTGAGGGCTTATT-TG-3′ ; for β-actin (191 base pairs) : forward, 5′-ACCACCATGTACCCAGGCAT-3′ ; reverse, 5′-CCGGACTCATCGTACTCCTG-3′. For cDNA synthesis, the mixtures were incubated at 50°C for 30 min. Subsequently, PCR was performed with 40 cycles at 94°C for 15 s, 49°C for 30 s, and 72°C for 30 s for albumin, 40 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 45 s for TAT, and 35 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s for β-actin. The PCR products were subjected to 1.5% agarose gel electrophoresis and visualized by staining with ethidium bromide.

Cell transplantation

HSL cells (1×10^7) were inoculated into the collagen sponges and cultured for 3 days. After being rinsed with PBS, the collagen sponges containing HSL cells were placed on the greater omentum of NA rats. Sham operations omitting the cells were also carried out. All recipients used in this study underwent a two-thirds partial hepatectomy (PH) immediately before transplantation. FK506 (Fuji-

sawa Pharmaceutical, Japan) was injected daily at a dosage of 1 mg per kg body weight after the transplantation.

Immunohistochemistry

The collagen sponge with HSL cells was embedded in OCT compound, and 8 μm frozen sections were prepared by cryostat (Leica, Germany). For immunoperoxidase staining, the specimens were fixed in 4% paraformaldehyde in PBS for 10 min. After rinsing with PBS, samples were treated for 10 min with 3% H_2O_2 in methanol to inactivate endogenous peroxidase, and then blocked with 10% skim milk dissolved in PBS containing 0.1% Tween-20 (PBS-T) for 30 min. After rinsing with PBS-T, samples were incubated with rabbit anti-rat albumin polyclonal antibody (Cappel, USA) diluted to 1 : 200 for 60 min. After rinsing with PBS, the samples were incubated for 30 min with HRP conjugated goat anti-rabbit IgG (DAKO) diluted to 1 : 200. Peroxidase activity was developed with 1 mM 3, 3′-diaminobenzidine (DAB)/50 mM Tris-HCl, pH 7.6/0.03% H_2O_2, and then samples were counterstained with hematoxylin. Samples were observed with a light microscope (NIKON, Japan). All staining procedures were performed at room temperature.

Western blot analysis of serum albumin

Blood samples were collected from the jugular veins of recipient rats at various intervals. Sera were stored at −80°C until use. The samples were resolved by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, USA). Immunodetection was performed using an ECL Western Blotting Detection Kit (Amersham, England) as manufacture's manual. The primary antibodies were rabbit anti-rat albumin polyclonal antibodies (SRL, Japan) diluted at 1 : 1500. For secondary antibodies, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibodies (Cappel) were diluted 1 : 1500. All Antibodies were diluted in PBS containing 2% skim milk and 0.2% Tween-20. To evaluate increments in serum albumin, the densities of bands corresponding to albumin were numerated using NIH image software.

Results

Differentiation of HSL cells in collagen sponge

We cultured HSL cells in type I collagen sponge and examined their changes in gene expression, since a three-dimensional culture using collagen matrices are known to induce cell differentiation. We inoculated insoluble collagen sponges, a useful scaffold for cell transplantation, with HSL cells and examined the effect on differentiation. HSL cells adhered to the collagen fibers after 2 days of culture (Fig. 1).

To investigate the effect of collagen sponge on HSL cells, we performed RT-PCR analysis to detect hepatocytic markers by RT-PCR. The expression of albumin and TAT were extremely low in HSL cells cultured in monolayer (Fig. 2). On the other hand, HSL cells cultured in collagen sponge express albumin and TAT, indicating that the collagen sponge stimulated HSL cells to mature hepatocyte.

Transplantation of HSL cells

In this study we have used NA rats, a mutant

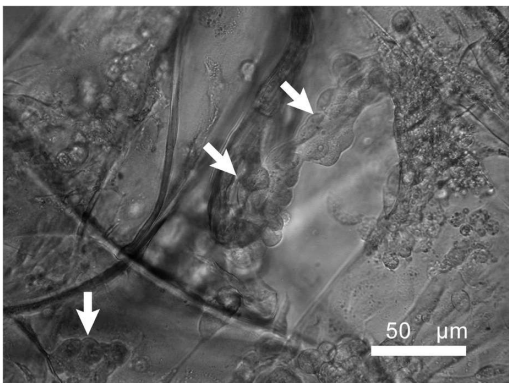

Fig. 1 HSL cells cultured in collagen sponge. Phase-contrast micrograph of HSL cells cultured in a collagen sponge with approximately 100 μm of pores for 7 days, showing the attachment of HSL cells (arrow) along collagen fibers. Scale bars, 50 μm.

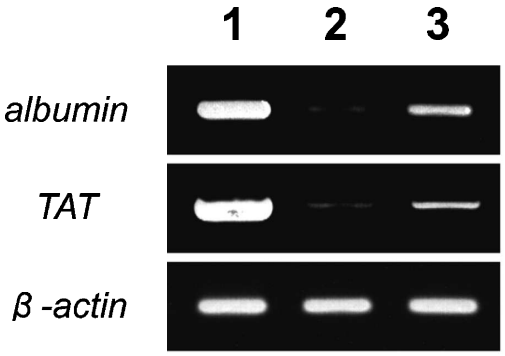

Fig. 2 Effect of collagen sponge on the expression of liver-specific genes in HSL cells.
Total RNA prepared from samples was amplified to detect albumin m-RNA, TAT m-RNA, and β-actin m-RNA. Template total RNA used for reactions were prepared from normal rat liver (lane 1), HSL cells cultured in the monolayer for two weeks (lane 2), HSL cells cultured in the collagen sponge for two weeks (lane 3).

strain derived from SD rats, as host animals for transplantation of HSL cells. The NA rat has a deletion of 7 base pairs at the 5′ splice site of intron H in the albumin gene, leading to a remarkable reduction in albumin mRNA synthesis to 1/4,000 relative to normal rats and a subsequent decrease in serum albumin levels[20-22]. This enables one to evaluate the production of albumin derived from the transplanted HSL cells. In the present study, we transplanted the collagen sponge adhered HSL cells, and investigated the engraftment of transplanted cells.

After the transplantation, we extirpated a collagen sponge from a recipient six weeks after transplantation and prepared frozen sections for immunohistochemical staining. We found albumin-positive cells in the transplanted sponge (data not shown), showing the existence of HSL cells in the collagen sponge.

Detection of serum albumin in NA rats after transplantation

To examine whether transplanted HSL cells function as hepatocytes, we investigated the changes in the level of serum albumin in NA rats. As shown

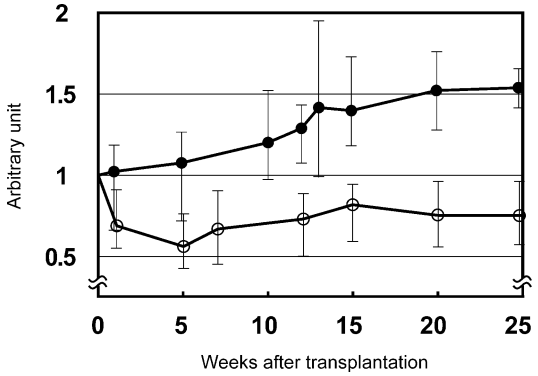

Fig. 3. Albumin secretion in NA rats after HSL cell transplantation.
The levels of serum albumin in NA rats after transplantation of HSL cell (1×10^7 cells) with collagen sponge (●) and control (○) were shown at each period after transplantation. Albumin concentrations were quantitated by measuring the chemiluminescent of the Western blots, and then computed the folds of elevation comparing with the level of before transplantation, expressed as "arbitrary unit". Each point is an average of values obtained from rats examined. Data are expressed as means±S.D.

Table 1 Summary of changes in serum albumin levels in NA rats after HSL cell transplantation with collagen scaffolds.

	Increased*	Unchanged
Collagen sponge ($n=10$)	6	4
Sham operation ($n=5$)	0	5

Transplantations of HSL cells into NA rats were performed with collagen sponges ($n=10$).
*Increased, represents the number of NA rats that exhibited more than a 1.2 fold elevation in serum albumin at 25 weeks of transplantation relative to before the transplantation.

in Fig. 3, the levels of serum albumin increased gradually up to 25 weeks after transplantation, implying that HSL cells can stably produce albumin *in vivo*. On the other hand, no elevation was observed in the control group. The transplantation with spongy collagen resulted in a 1.5-fold increase in serum albumin. These phenomena were observed in six of 10 rats (60%) transplanted with spongy collagen, as summarized in Table 1, indicating that

HSL cells can stably produce albumin *in vivo*.

Discussion

Many studies have indicated that the proliferation and differentiation of stem cells are regulated by ECM components and growth factors provided by the niches[2,13,21,22]. The ECMs are known to associate with growth factors and release them at specific times for interaction with their receptors[9,23,24]. In addition, it was reported that ECM molecules contained specific motifs of amino acids that allowed them to bind to cell surface receptors directly[10−13,24]. Of ECMs, type I collagen, the most abundant in liver, is known to assist in the maintenance of attachment-dependent cell types in culture by binding to integrin-binding domains on cells through its RGDT motif[24]. Three-dimensional culture systems using biodegradable collagen gels are known to maintain the function of cultured epithelial cells. Dunn *et al*. showed that primary hepatocytes maintained their functions for a long time and that dedifferentiated hepatocytes recovered their functions in a collagen sandwich culture[14]. Lazaro *et al*. achieved the differentiation of oval cells into mature hepatocytes using a three-dimensional culture consisting of collagen gels and a fibroblast feeder layer[21]. These observations suggest that biodegradable collagen matrices regulate the differentiation of hepatic cells by providing an appropriate microenvironment. In this study, we cultured HSL cells in type I collagen sponge. Consequently, HSL cells expressed liver-specific markers, albumin and TAT, implying that this culture system using collagen sponge was providing an appropriate microenvironment for hepatocytic differentiation of HSL cells.

Recent studies on tissue engineering have indicated advantages in using collagen as a scaffold to restitute the structure of an organ, which may facilitate cellular function after transplantation[25]. Because collagen polymers make excellent scaffolds due to their biocompatibility and capability for molding, they are widely used in many fields of tissue engineering. Recently, Takeshita *et al*. have shown the advantage of spongy collagen for construction of liver-like tissue[26]. In the present study, we tried to evaluate the efficacy of procedures using spongy collagen for cell transplantation. Consequently, we observed increased levels of serum albumin after the transplantation, indicating that the transplantation using the collagen scaffolds achieved the manifestation of hepatic function by HSL cells *in vivo* to some extent. This implies that the hepatic microenvironment is important to the function of transplanted cells.

As the standard level of serum albumin in five-week-old NA rats is 5 μg/ml[27], the 1.5-fold elevation in serum albumin after transplantation corresponds to 7.5 μg/ml. Since the standard level of serum albumin in five week-old SD rats is 22 mg/ml, the recovery of albumin production in transplanted NA rats is only 0.034% of that in SD rats. This result may come from a deficient number of transplanted cells, insufficient engraftment of cells and inadequate maturation of transplanted cells. To obtain adequate hepatic function, further improvements to transplantation procedures will be needed.

In conclusion, our study suggests that HSL cells have the potential to differentiate into functional hepatocytes and that the transplantation in combination with the use of hepatic stem cells and collagen matrix is a possible new approach to liver regeneration therapy.

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References

1) Grisham, J.W. and Thorgeirsson, S.S. (1997) Liver stem cells. In Potten, C.S. (ed.) *Stem Cells*. Academic Press, London, pp. 233-282.
2) Thorgeirsson, S.S. (1996) Hepatic stem cells in liver regeneration. *FASEB J*., **10**, 1249-1256.
3) Marceau, N. (1994) Epithelial cell lineages in developing, restoring, and transforming liver: evidence for the existence of a 'differentiation window'. *Gut*, **35**, 294-296.
4) Coleman, W.B., McCullough, K.D., Esch, G.L., Faris, R.A., Hixson, D.C., Smith, G.J. and Grisham, J.W. (1997) Evaluation of the differentiation potential of WB-F344 rat liver epithelial stem-like cells *in vivo*: Differentiation to hepatocytes after transplantation into dipeptidylpeptidase-IV-deficient rat liver. *Am. J. Pathol*., **151**, 353-359.
5) Couchie, D., Holic, N., Chobert, M.-N., Corlu, A. and Laperche, Y. (2002) *In vitro* differentiation of WB-F344 rat liver epithelial cells into the biliary lineage. *Differentiation*, **69**, 209-215.
6) Dabeva, M.D., Petkov, P.M., Sandhu, J., Oren, R., Laconi, E., Hurston, E. and Shafritz, D.A. (2000) Proliferation and differentiation of fetal liver epithelial progenitor cells after transplantation into adult rat liver. *Am. J. Pathol*., **156**, 2017-2031.
7) Nagai, H., Terada, K., Watanabe, G. *et al*. (2002) Differentiation of liver epithelial (stem-like) cells into hepatocytes induced by coculture with hepatic stellate cells. *Biochem. Biophys. Res. Commun*., **293**, 1420-1425.
8) Li, D. and Friedman, S.L. (2001) Hepatic stellate cells: Morphology, Function, and Regulation. In Arias, I.M., Boyer, J.L., Chisari, F.V., Fausto, N., Schachter, D. and Shafritz, D.A. (eds.) *The Liver: Biology and Pathobiology*. Lippincott Williams & Wilkins, Philadelphia, pp. 455-468.
9) Rojkind, M. and Greenwel, P. (1994) The extracellular matrix of the liver. In Arias, I.M., Boyer, J.L., Fausto, N., Jakoby, W.B., Dchachter, D.A. and Shafritz, D.A. (eds.) *The Liver: Biology and Pathobiology*. Raven Press, New York, pp. 843-868.
10) Kleinman, H.K., Klebe, R.J. and Martin, G.R., (1981) Role of collagenous matrices in the adhesion and growth of cells. *J. Cell Biol*., **88**, 473-485.
11) Mooney, D., Hansen, L., Vacanti, J., Langer, R., Farmer, S. and Ingber, D. (1992) Switching from differentiation to growth in hepatocytes: control by extracellular matrix. *J. Cell Physiol*., **151**, 497-505.
12) Maher, J.J. and Bissell, D.M. (1993) Cell-matrix interactions in liver. *Semin. Cell Biol*., **4**, 189-201.
13) Brill, S., Holst, P.A., Zvidel, I., Fiorino, A.S., Sigal, S.H., Somasundaran, U. and Reid, L.M. (1994) Extracellular Matrix Reguration of Growth and Gene Expression in Liver Cell Lineages and Hepatomas. In Arias, I.M., Boyer, J.L., Fausto, N., Jakoby, W.B., Dchachter, D.A. and Shafritz, D.A. (eds.) *The Liver: Biology and Pathobiology*. New York: Raven Press, 869-897.
14) Dunn, J.C., Tompkins, R.G. and Yarmush, M.L. (1992) Hepatocytes in collagen sandwich: evidence for transcriptional and translational regulation. *J. Cell Biol*., **116**, 1043-1053.
15) Awata, R. Sawai, H., Imai, K., Terada, K., Senoo, H. and Sugiyama, T. (1998) Morphological comparison and functional reconstitution of rat hepatic parenchymal cells on various matrices. *J. Gastroenterol. Hepatol*., **13** (Suppl.), S 55-61.
16) Lee, J.E., Park, J.C., Hwang, Y.S., Kim, J.K., Kim, J.G. and Sub, H. (2001) Characterization of UV-irradiated dense/porous collagen membranes: morphology, enzymatic degradation, and mechanical properties. *Yonsei Med. J*., **42**, 172-179.
17) Kuroyanagi, Y., Yamada, N., Yamashita, R. and Uchinuma, E. (2001) Tissue-engineered product: allogeneic cultured dermal substitute composed of spongy collagen with fibroblasts. *Artif. Organs*, **25**, 180-186.
18) Shalaby, F. and Shafritz, D.A. (1990) Exon

skipping during splicing of albumin mRNA precursors in Nagase analbuminemic rats. *Proc. Natl. Acad. Sci. USA*, **87**, 2652-2656.

19) Kaneko, T., Shima, H., Esumi, H., Ochiai, M., Nagase, S., Sugimura, T. and Nagao, M. (1991) Marked increases of two kinds of two-exon-skipped albumin mRNAs with aging and their further increase by treatment with 3′-methyl-4-dimethylaminoazobenzene in Nagase analbuminemic rats. *Proc. Natl. Acad. Sci. USA*, **88**, 2707-2711.

20) Esumi, H., Takahashi, Y., Sato, S., Nagase, S. and Sugimura, T. (1983) A seven-base-pair deletion in an intron of the albumin gene of analbuminemic rats. *Proc. Natl. Acad. Sci. USA*, **80**, 95-99.

21) Lazaro, C.A., Rhim, J.A., Yamada, Y. and Fausto, N. (1998) Generation of hepatocytes from oval cell precursors in culture. *Cancer Res*., **58**, 5514-5522.

22) Watt, F.M. and Hogan, B.L. (2000) Out of Eden : stem cells and their niches. *Science*, **287**, 1427-1430.

23) Schuppan, D., Schmid, M., Somasundaram, R., Ackermann, R., Ruehl, M., Nakamura, T. and Riecken, E.O. (1998) Collagens in the liver extracellular matrix bind hepatocyte growth factor. *Gastroenterology*, **114**, 139-152.

24) Martins-Green, M. (1997) The dynamics of cell-ECM interactions with implications for tissue engineering. In Lanza, R.P, Langer, R. and Chick, W.L. (eds.) *Principles of Tissue Engineering*. Academic Press, London, pp. 23-46.

25) Demetriou, A.A., Levenson, S.M., Novikoff, P.M., Novikoff, A.B., Chowdhury, N.R., Whiting, J., Reisner, A. and Chowdhury, J.R. (1986) Survival, organization, and function of microcarrier-attached hepatocytes transplanted in rats. *Proc. Natl. Acad. Sci. USA*, **83**, 7475-7479.

26) Takeshita, K., Bowen, W.C. and Michalopoulos, G.K. (1998) Three-dimensional culture of hepatocytes in a continuously flowing medium. *In Vitro Cell Dev. Biol. Anim*., **34**, 482-485

27) Makino, M., Esumi, H., Sato, S., Takahashi, Y., Nagase, S. and Sugimura, T. (1982) Elevation of serum albumin concentration in analbuminemic rats by administration of 3′-methyl-4-dimethylaminoazobenzene. *Biochem. Biophys. Res. Commun*., **106**, 863-870.