Deactivation of Hepatic Stellate Cells by Culturing on VECELL Inserts

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Abstract
Hepatic stellate cells play a cardinal role in the development of liver fibrosis. Quiescent hepatic stellate cells isolated from normal liver are activated by plating on a plastic culture dish. Therefore, a culture method that maintains hepatic stellate cells in a quiescent state is required for studies of fibrosis. We attempted to deactivate human hepatic stellate cells by culturing on VECELL® culture inserts (Preset VECELL). VECELL is a cell culture scaffold consisting of an expanded polytetrafluoroethylene mesh that is coated with collagen type I. Cryopreserved human hepatic stellate cells and LI90 cells, which is a cell line established from an outgrowth of a human hepatic mesenchymal tumor, were cultured on Preset VECELL. The expression of activation markers α-SMA and COL1A was decreased by VECELL cultivation. In addition, actin filaments (markers of activated hepatic stellate cells) were not detected in LI90 cells on VECELL inserts. These results suggest that human hepatic stellate cells were deactivated by VECELL cultivation, which could provide a model system for the analysis of deactivated human hepatic stellate cells. Thus, Preset VECELL will be a useful in vitro tool for the clarification of underlying mechanisms and the development of drugs to treat liver fibrosis. This study will contribute to provide alternative methods to animal tests that have been mainly carried out in studies of hepatic stellate cell, liver fibrosis, and liver cirrhosis.

Key words: Preset VECELL®, hepatic stellate cells, deactivation, liver fibrosis, cell morphology

Introduction
Experimental animal such as rat and mouse has been mainly used in studies on liver fibrosis (Weiler-Norman et al., 2007, Kim et al., 2017, Wang et al., 2018), but alternative methods to animal test are preferable in the drug development in recent years. Hepatic stellate cells (HSCs) are pericytes residing in small areas between the sinusoids and hepatocytes. HSCs play an important role in the storage of retinol (vitamin A) in normal liver. HSCs in normal liver are not active in functions for inducing fibrosis (“quiescent” state), since they are seldom proliferate and produce little secretory proteins such as extracellular matrix components and cytokines. Following liver injury, quiescent HSCs are activated and start to proliferate. Activated HSCs transdifferentiate into α-smooth muscle actin (α-SMA)-positive cells (Blomhoff and Wake, 1991, Benyon and Athur, 1998) and secrete excessive extracellular matrix (ECM) such as collagen type I, leading to scar formation and fibrosis. HSCs play a cardinal role in the development of liver fibrosis. HSCs are frequently used for fibrosis studies in vitro. However, when quiescent HSCs which are isolated from normal liver are cultured on plastic plates, they are spontaneously activated. Therefore, a method to culture HSCs in a quiescent state is required for fibrosis studies. Benyon et al. (1998) found that the quiescence of rat HSCs was maintained by culturing on basement membrane-like matrix (Gaça et al., 2003). Shimada et al. (2010) reported that the LI90 cell
line, which was established from an outgrowth of a human hepatic mesenchymal tumor, was deactivated when cultured on Matrigel (Shimada et al., 2010). However, basement membrane-like matrices and Matrigel include bioactive factors that might interfere with various assays. Therefore, there is a significant need to maintain deactivated HSCs on a surface without bioactive factors.

In this study, VECELL® culture inserts (Preset VECELL) were used for the culture of human HSCs. VECELL is a cell culture scaffold consisting of an expanded polytetrafluoroethylene (ePTFE) mesh coated with collagen type I derived from salmon (Furutani et al., 2010). Cells placed on VECELL adhere to the mesh surface where there is no excess scaffold for cells to stretch. Thus, VECELL allows the culture of cells under physiological conditions without mechanical stress unlike the surface of normal plastic cell culture plates. As a result, VECELL cultures should maintain their native properties. Previously, we found that the human hepatic progenitor cell line HepaRG maintained the undifferentiated state (Kubo et al., 2016) during cultivation on Preset VECELL. Therefore, we attempted to maintain deactivated HSCs by culture on VECELL, expecting that the transdifferentiation into activated HSCs would be suppressed. In this study, we investigated the effect of VECELL culture on the activation of human HSCs, examining morphological changes, actin filament state and the expression of HSCs activation-related genes.

Materials and Methods
Cell culture
Cryopreserved human hepatic stellate cells (cryo-hHSCs) were obtained from Lonza (Basel, Switzerland). The L190 cell line was established from an outgrowth of a human hepatic mesenchymal tumor (Murakami et al., 1995); it was obtained from the Japanese Collection of Research Bioresources, Osaka, Japan. L190 cells in plate culture show the characteristics of activated HSC including well developed alpha-smooth muscle actin filaments and production of ECM etc (Murakami et al., 1995). Therefore, L190 cells have been used for in vitro studies on liver fibrosis (Sugimoto et al., 2005, Shimada et al., 2010, Kawano et al., 2014, Tang et al., 2016). L190 cells were maintained on standard culture plates, or cultured on VECELL inserts (Vessel Inc., Kitakyushu, Japan). Cryo-hHSCs were maintained on collagen-coated culture plates, or cultured on VECELL inserts. The seeding densities for L190 cells and cryo-hHSCs were 4.0 x 10^4 cells/cm^2. L190 cells were cultured in Dulbecco’s modified Eagle Medium (DMEM low glucose, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Life Technologies), 100 units/mL penicillin, and 10 μg/mL streptomycin (Life Technologies); cryo-hHSCs were cultured in Cryo Human Stellate Cell growth media (Lonza). All cells were incubated at 37°C in a humidified atmosphere containing 5% CO2.

Cell staining with Diff-Quik
Diff-Quik (Dade Behring, Newark, NJ, USA) is modified based on the Wright- Giemsa stain and used as a rapid cytological staining method. Cells cultured for 7 days after seeding were stained with Diff-Quik according to the manufacturer’s instructions.

LPS stimulation
L190 cells cultured on plates were detached with trypsin-EDTA and suspended in medium. Different concentrations of lipopolysaccharide (LPS; Sigma-Aldrich, Darmstadt, Germany, 10, 30, 100, 300 ng/mL) were added to the cell suspension and cells were seeded on the VECELL inserts. The cells were cultured for 3 days and cell morphology and actin filaments were observed.

Actin filament staining with phalloidin
Cells were fixed for 15 min by 3.7% formaldehyde solution after 3 washes in PBS, and cells were permeabilized with 0.1% Triton X-100/PBS for 10 min. The cells were incubated with Alexa Fluor 594 Phalloidin (Thermo Fisher Scientific, San Jose, CA, USA) in PBS (10 U/mL) for 2 h at room temperature after three washes in PBS. Coverslips were mounted by ProLongR Diamond Antifade reagent (Thermo Fisher Scientific). The sample was observed using an excitation wavelength of 540/250 nm with a fluorescence microscope (BIOREVO BZ-90000, KEYENCE, Osaka, Japan).
RNA isolation
After culture for 4 days or 7 days, cells were washed twice with PBS, and total RNA was isolated from cells by using the RNeasy Mini total RNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.

TaqMan real-time RT-PCR
Reverse transcription was performed with 1.0 µg total RNA using TaqMan Reverse Transcription Reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions. Gene expression was measured using the QuantStudio 7 Flex (Thermo Fisher Scientific) and the following primer and probe sets were used for detection of each gene transcript: ACTA2 (α-SMA: Hs00426835_g1), COL1A1 (Hs00164004_m1) and BAMBI (Hs03044164_m1). Expression of genes on standard culture plates was normalized to 1.0. Measurements for each sample were performed in triplicate and averaged.

Statistical analysis
Statistical analysis of the expression data was performed using a Student’s t-test. Values of P < 0.01 were considered statistically significant.

Results
Effect of cultivating LI90 cells on VECELL inserts: cell morphology and expression of activation markers
VECELL inserts allow the investigator to culture cells such that they maintain their physiologic shape because of the limited amount of scaffold for cells to stretch. We found that the human hepatic progenitor cell line HepaRG maintained the undifferentiated state (Kubo et al., 2016) during VECELL insert culture. Thus, we expected that deactivated HSCs could be maintained by culturing them in VECELL inserts. When activated HSCs are deactivated by Matrigel cultivation, the cells adopt a compact spherical morphology (Gaça et al., 2003, Shimada et al., 2010). This suggests that morphological change could be related to deactivation of HSCs. Therefore, to evaluate the association of cell morphological changes and activation, we observed cell morphology with phase-contrast microscopy and measured the expression of activation markers by RT-qPCR in LI90 cells. Fig. 1A shows the morphology of unstained cells, and Fig. 1B show that of cells stained with Diff-Quik. The stained area in Fig. 1B (right) shows LI90 cells on the VECELL insert. LI90 cells cultured on standard culture plate exhibited the typical myofibroblast-like appearance of activated HSC one day after seeding, whereas LI90 cells cultured on a VECELL insert congregated on day 1 and formed spheroids 2 days after seeding (Fig. 1A, B) similar to HSCs cultured on Matrigel (Shimada et al., 2010). Expression of α-SMA and collagen type I alpha 1 chain (COL1A1), both of which are upregulated by the activation of HSCs, was measured on Days 4 and 7. In LI90 cells cultured on VECELL inserts, the expression of α-SMA and COL1A1 was downregulated at days 4 and 7.

Effects of VECELL culture on cell morphology and expression of activation markers in cryo-hHSCs
During culture of LI90 cells on VECELL inserts, spheroid formation and downregulation of activation markers were observed, similar to the culture of HSCs on Matrigel. Therefore, we examined the cell morphology and the expression of activation markers in VECELL cultures of cryo-hHSCs. In VECELL cultures of cryo-hHSCs, cell extension was inhibited (Fig. 2A). The expression of α-SMA and COL1A1 in cryo-hHSCs on VECELL inserts was downregulated by days 4 and 7, similar to the results obtained with LI90 cells (Fig. 2B), although the degree of downregulation was smaller than that in LI90 cells.

Effect of VECELL culture on actin filaments in LI90 cells
Deactivation of human HSCs on VECELL inserts was suggested by the RT-qPCR measurements that showed downregulation of activation marker expression. When HSCs are activated, polymerization of actin filaments is accelerated (Yee, 1998). To evaluate the state of LI90 cells during VCELL culture, actin filaments were stained with Alexa Fluor 594 Phalloidin and observed by fluorescence microscopy. In LI90 cells cultured on standard culture plates, actin filaments were detected from Days 4 to 7 (Fig. 3). On the other hand, the actin filaments were not detected in LI90 cells cultured on VECELL inserts.
Figure 1
Cultivation of LI90 cells on VECELL inserts: effects on cell morphology and expressions of HSCs activation markers. (A), (B) LI90 cells were cultured on a standard culture plate (left) or on a VECELL insert (right), and cell morphology was observed by phase contrast microscopy at 10X magnification 7 days after seeding. Cells were not stained (A) or stained with Diff-Quik (B). (C) Expression of HSCs activation markers: α-smooth muscle actin (α-SMA : left) and collagen type I (COL1A1 : right) on days 4 and 7 after seeding was measured by real-time RT-PCR. The relative expression was calculated by setting the expression in standard plate culture to 1.0. Error bars indicate the standard deviation (n=3). * P < 0.01, significantly different when compared with plate culture.

Figure 2
The effect of VECELL insert culture on cryo-hHSC morphology and expression of HSCs activation markers. (A), (B) Cryo-hHSCs were cultured on a standard culture plate (left) or on a VECELL insert (right) and cell morphology was observed by phase contrast microscopy at 10X magnification 7 days after seeding. Expression of HSCs activation markers (C): α-smooth muscle actin (α-SMA : left) and collagen type I (COL1A1 : right) 4 and 7 days after seeding was measured by real-time RT-PCR. The relative expression was calculated by setting the expression in standard plate culture to 1.0. Error bar indicates the standard deviation (n=3). * P < 0.01, significantly different when compared with plate culture.

Figure 3
Effect of cultivating LI90 cells on VECELL inserts on actin filaments. LI90 cells were cultured on a standard culture plate (left) or on a VECELL insert (right). After 4 (A) or 7 days (B), actin filaments were stained with phalloidin labeled with Alexa Fluor 594. Actin filaments stained with phalloidin were observed by fluorescence microscopy at 20X magnification; red: actin filaments.
Figure 4
Concentration-dependent effects of LPS on the morphology of LI90 cells on VECELL inserts. LI90 cells were treated with different concentrations of LPS (10, 30, 100, 300 ng/mL) for 3 days. Cell morphologies were observed by phase contrast microscopy at 10X magnification.
Deactivation of HSCs by VECELL cultivation was suggested from the above results: (i) morphological change such as when LI90 cells were cultured on Matrigel, (ii) downregulation of activation markers, and (iii) dissipation of actin filaments. To observe the effect of an activator of HSCs on deactivated LI90 cells on VECELL, LI90 cells culturing on the VECELL were stimulated by LPS, which is a general activator of HSCs derived from microflora in the intestine (Paik and Seki, 2010). LPS was added to a suspension of LI90 cells and the suspension was seeded on VECELL inserts. Formation of spheroids on the VECELL was suppressed in a LPS concentration-dependent manner (Fig. 4). Actin filaments in LI90 cells exposed to LPS for 2 days were observed (Fig. 5).

Expression of BAMBI, which relates to the suppression of HSCs activation, in LI90 cells LPS stimulation activated LI90 cells on VECELL inserts. This result may indicate that factor(s) downstream from LPS stimulations are related to deactivation of LI90 cells during VECELL culture. Therefore, we measured gene expression of BMP and activin membrane bound inhibitor (BAMBI), which is downstream from LPS stimulation and suppresses activation of HSCs via TFG-β signaling (Seki et al., 2007, Paik and Seki, 2010, Sun et al., 2018). TFG-β signaling is a critical pathway involved in HSCs

Figure 5
Effect of LPS treatment (300 ng/mL) on actin filaments of LI90 cells cultured on VECELL inserts. Polymerization of actin filaments in LI90 cells treated with LPS for 2 days on VECELL was observed. Actin filaments were stained with Alexa Fluor 594 Phalloidin and observed by fluorescence microscopy at 20X magnification. Cell morphologies were observed by phase contrast microscopy at 10X magnification. Control (A), LPS treatment (B), phase contrast microscopy (left), fluorescence microscopy (right), red : actin filaments.
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activation, and it upregulates the expression of \( \alpha\text{-SMA} \) and \( \text{COL1A1} \) (Meng et al., 2016). The expression of \( \text{BAMBI} \) in LI90 cells on VECELL inserts was upregulated on days 4 and 7 compared to that on the plate (Fig. 6).

**Discussion**

In normal liver, HSCs are in a quiescent state within a small area between the sinusoids and hepatocytes. Following liver injury, HSCs transform to an activated state and secrete a wide array of cytokines and chemokines that are involved in both liver fibrosis, regeneration and collagen deposition (Blomhoff and Wake, 1991, Benyon and Athur, 1998, Yin et al., 2013). Therefore, analysis of HSCs is an important approach to better understand the mechanism of fibrosis and regeneration in the liver. However, culture of HSCs in a quiescent state is difficult owing to the activation induced by culturing on standard culture plastics. Therefore, a method to culture HSCs in a quiescent state is required for fibrosis studies. In preceding study, HSCs were deactivated by culturing on a basement membrane-like matrix or Matrigel (Gaça et al., 2003, Shimada et al., 2010, Bility et al., 2016), at which point they formed spheroids. But basement membrane-like matrices and Matrigel include bioactive factors that might interfere with various assay results. In this study, we made the following observations. (1) Expression of activation markers, \( \alpha\text{-SMA} \) and \( \text{COL1A1} \), in cryo-hHSCs and LI90 cells was downregulated during VECELL culture. (2) Actin filaments, which are used as a marker of activated HSCs, were not detected in LI90 cells during VECELL cultivation. These results suggest that VECELL culture, which does not include bioactive factors, deactivated human HSCs as was seen with Matrigel culture (Shimada et al., 2010).

Therefore, the two states of human HSCs, “deactivated” and “activated,” can be modulated reversibly by culturing on VECELL inserts or on plastic plates.

When LI90 cells and cryo-hHSCs were deactivated by VECELL culture, spheroid formation or extension inhibition was observed. This result suggests that deactivation of human HSCs could be related to morphological change. Further, we observed that the deactivation of LI90 cells was suppressed and spheroid formation was inhibited by the addition of LPS (an activator of stellate cells) in a concentration-dependent manner. From these results, it could be possible to screen activators of human HSCs by observing spheroid
formation in VECELL inserts. In the future, it is need to study about the effect on human HSC activation in various three-dimensional cultures to clarify the relationship between deactivation and morphological change.

TGF-β signaling is a major cause of HSCs activation and it requires the binding of TGF-β to the TGF-β type I / type II receptor complex. BAMBI binds to the TGF-β type I receptor and inhibits the formation of the TGF-β type I / type II receptor complex (Seki et al., 2007, Paik and Seki, 2010). As a result, BAMBI negatively regulates TGF-β signaling. Importantly, the expression of BAMBI in LI90 cells was upregulated by culture on VECELL inserts. This observation suggests that TGF-β signaling may have been inhibited in the LI90 cells on VECELL insert. Expression of the HSC activation markers α-SMA and COL1A1, which are upregulated by activation of TGF-β signaling, was downregulated by VECELL culture. From the above, deactivation of LI90 cells on the VECELL surface may be caused via deactivation of TGF-β signaling by upregulation of BAMBI.

In this study, we found that human HSCs were deactivated by VECELL culture. Further, cell morphology and TGF-β signaling may relate to the deactivation of human HSCs during VECELL culture. Activation of HSCs is a cause of fibrogenesis, and the drugs that deactivate HSCs have been examined for treatment of liver fibrogenesis (Vilaseca et al., 2017a, 2017, Shi et al., 2017). Our findings regarding deactivation of HSCs could be important for therapy of liver fibrogenesis. Non-alcoholic steatohepatitis (NASH) is becoming a major cause of liver cirrhosis. In NASH, HSCs are activated by pro-inflammatory cytokines, and the cells transdifferentiate into myofibroblast-like cells that cause fibrosis (Friedman et al., 2018). Therefore, HSCs are used in the study of NASH in vitro, and the change of the activation - deactivation state is observed (Ryan et al., 2016, Davidson et al., 2017). There are drugs that induce NASH (Rabinowich et al., 2015, Dash et al., 2017) and liver fibrogenesis (Cheng and Rademaker, 2018) as a side effect. When these side effects are evaluated, analysis requires HSCs that are deactivated. VECELL could be used to screen drugs that induce NASH and liver fibrogenesis because human HSCs are deactivated. In addition, the formation of spheroids on VECELL was suppressed by LPS exposure in a concentration-dependent manner. For this reason, drugs that activate human HSCs might be evaluated by observation of cell morphology on VECELL inserts. Thus, VECELL will be a useful tool for cell-based drug safety tests on liver fibrosis. This study will contribute to provide alternative methods to animal tests that have been mainly carried out in studies of hepatic stellate cell, liver fibrosis, and liver cirrhosis.

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