Upregulations of metallothionein gene expressions and tolerance to heavy metal toxicity by three dimensional cultivation of HepG2 cells on VECELL 3-D inserts

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ABSTRACT — The VECELL 3-D insert is a new culture scaffold consisting of collagen-coated ePTFE (expanded polytetrafluoroethylene) mesh. We analyzed the effects of VECELL 3-D inserts on the functionality of HepG2, a human hepatocellular carcinoma cell line. HepG2 cells cultured on VECELL 3-D inserts maintained a round shape, while those cultured on a standard culture plate or collagen-coated cell culture plate showed a flattened and cubic epithelial-like shape. HepG2 cells cultured on VECELL 3-D inserts had showed upregulated expression of metallothionein genes and in turn a higher tolerance to toxicity induced by heavy metals. These results suggest that HepG2 cell functions were changed by the cell morphology that is induced by culturing on a VECELL 3-D insert.

Key words: VECELL 3-D insert, HepG2 cell, 3-D culture, Metallothionein, Heavy metal toxicity, Global gene expression analysis

INTRODUCTION

Several studies have shown changes in cell functions following three-dimensional (3-D) culture, including formation of glycosaminoglycan in NIH3T3 cells (Lee et al., 2004), cell growth and differentiation of rat dorsal prostatic epithelial cells (Tokuda et al., 1999), albumin production in human fetal hepatocytes (Akiyama et al., 2004), and albumin and cytochrome P450 (CYP) enzyme expression in HepG2 cells (Nakamura et al., 2011).

HepG2 cell line, a human hepatocellular carcinoma-derived cell line, is used for drug metabolism and toxicology test. Because the drug metabolism activities in HepG2 cells are extremely lower than those in primary hepatocyte when cells are cultured in standard culture plates, 3-D culture is often conducted for enhancing the drug metabolism activities (Godoy et al., 2013). We previously reported that 3-D culture of HepG2 cells in a radial-flow bioreactor (RFB) upregulated expression of a number of genes (Hongo et al., 2005, 2006; Horiuchi et al., 2009). A careful mechanistic study using global gene expression analysis revealed that the changes in expression of microtubule molecules might be responsible for these functional changes. When microtubules were stabilized by treating HepG2 cells with a sublethal dose of docetaxel (a microtubule stabilizing agent), effective induction of drug metabolism-related genes altered in RFB culture was observed (Horiuchi et al., 2009). Moreover, HepG2 cells treated with a sublethal dose of docetaxel assumed a round shape as observed by phase-contrast microscopy (Ishida, 2012). We hypothesized that if the effects of docetaxel were able to be reproduced in the absence of the drug, the cells would show similar functional changes. Reproducing the effects of docetaxel without the drug would be important if the culture were used for the safety evaluation of chemicals.

The VECELL 3-D insert (VECELL) is a cell culture scaffold consisting of an expanded polytetrafluoroethylene (ePTFE) mesh that is coated with salmon collagen. Cells placed on VECELL adhere to the mesh surface where there is no excess scaffold for cells to stretch. Thus, cells maintain their round shape during cultivation.
In this study, we compared changes in gene expression patterns in HepG2 cells cultured on VECELL with that of HepG2 cells cultured on standard culture plates. The assessment of global gene expression elucidated the effect(s) of morphological changes induced by VECELL culture of HepG2 cells.

MATERIALS AND METHODS

Cell cultivation

HepG2 cells (American Type Culture Collection, Rockville, MD, USA) were seeded on standard culture plates (BD, Franklin Lakes, NJ, USA), collagen-coated cell culture plates (BD) or on VECELL (Vessel Inc., Fukuoka, Japan) at densities of \(1.3 \times 10^5\) cells/cm\(^2\); and cultured in Dulbecco’s modified MEM (DMEM high glucose, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Life Technologies), 100 units/mL penicillin, and 10 \(\mu\)g/mL streptomycin (Life Technologies) at 37°C in a humidified atmosphere containing 5% CO\(_2\).

Immunostaining

HepG2 cells were cultured on cover slips or on VECELL for two days, and then fixed with 4% paraformaldehyde before staining with 4’,6-diamidino-2-phenylindole, dihydrochloride (DAPI, Sigma-Aldrich, St. Louis, MO, USA) and treatment with anti-human albumin antibody (Bethyl Laboratory Inc., Montgomery, TX, USA). After washing, the sample was treated with FITC-conjugated donkey anti-goat IgG antibody (Jackson ImmunoResearch, West Grove, PA, USA) and observed with a Nikon A1 Confocal Laser Microscope System (Nikon Instech, Tokyo, Japan).

Global gene expression analysis

Cells were cultured for three days and then washed twice with Dulbecco’s phosphate buffered saline (PBS, Sigma-Aldrich) before RNA isolation using RNeasy Mini Total RNA Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Extracted RNA (100 ng) was converted to fragmented aRNA according to the manufacturer’s instructions. The hybridization cocktails containing 15 \(\mu\)g of the fragmented aRNA were hybridized to human genome U133A GeneChip DNA microarrays in duplicate (Affymetrix, Santa Clara, CA, USA) for 16 hr at 45°C. After hybridization, the DNA microarrays were washed and stained on a Fluidics Station (Affymetrix) according to the manufacturer’s protocol. The DNA microarrays were scanned and the resulting images were analyzed by GeneChip Operating Software (GCOS, Affymetrix). The complete dataset was submitted to the NCBI GEO (Gene Expression Omnibus) database (GSE41270). Gene expression data of duplicate measurements were averaged first, and averaged values were filtered according to the following criteria: (i) genes with signals above 500 in either culture; (ii) genes with \(p\)-values less than 0.05 when a \(t\)-test was performed between the triplicate measurements of RNA isolated from cell grown on VECELL or standard culture plates; and (iii) genes with \(\geq 2.0\)-fold increase or \(\leq 0.5\)-fold decrease in RNA expression from VECELL culture compared to cells from standard culture plates.

TaqMan real-time PCR

Reverse transcription was performed with 1.0 \(\mu\)g total RNA using TaqMan Reverse Transcription Reagent (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. The following primer and probe sets were used for detection of each gene transcript using the Prism 7900 Real-Time PCR System (Applied Biosystems): MT1F (Hs00744661_sH), MT1G (Hs02578922_gH), MT2A (Hs01591333_g1). The expression level of each gene was normalized against the \(\beta\)-actin expression level. Measurements for each sample were performed in triplicate and averaged.

Toxicity test

HepG2 cells were cultured for one day on either standard culture plates or VECELL. Various concentrations of CuCl\(_2\) (100 \(\mu\)M-1,200 \(\mu\)M, Wako Pure Chemical Industries, Osaka, Japan) were then added to the medium and the culture was continued for another three days. At the end of the culture period, the relative cell numbers were measured with an MTT assay. Each culture condition was performed in triplicate as follows: upon culture medium removal, MTT solution (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Wako Pure Chemical Industries, 0.5 mg/mL in culture medium) was added to each culture and incubated for four hours. After the incubation, 300 \(\mu\)L isopropanol was added and the extracted MTT formazan measured with a microplate reader at 570 nm. The ratio of MTT formazan formed in each culture to that in the corresponding non-treated culture ([CuCl\(_2\)] = 0 \(\mu\)M) was calculated (relative cell viability) for each concentration. The same experiment was performed three times and the average values were plotted.

Measurement of copper ion concentration

The copper ion concentration in the medium was
measured by NANOCOLOR Copper photometric determination reagent with cuprizone (MACHEREY-NAGEL, Düren, Germany) according to the manufacturer’s instructions.

Statistical analysis
Statistical comparisons were performed using a Student’s t-test and the level of significance was set at $p < 0.05$ or $p < 0.01$.

RESULTS

The effect of VECELL culture on cell morphology
Cell morphologies following culture under three conditions (VECELL, collagen, standard) were observed by phase-contrast microscopy two days after seeding. HepG2 cells cultured on standard culture plates or collagen-coated cell culture plates showed a flattened and cubic epithelial-like shape. In contrast, those cultured on VECELL maintained a round shape as observed by phase-contrast microscopy (Fig. 1A). To confirm this observation, cell nuclei were stained with DAPI and the cell cytoplasm was stained with anti-albumin antibody in HepG2 cells cultured on VECELL or standard culture plates two days after seeding, and the samples were observed by confocal microscopy. Images of different focal planes within the sample were obtained and stacked according to the z-axis to reconstitute 3-D morphologies of nuclei and cytoplasm (Fig. 1B). Reconstituted 3-D morphologies indicated that cells on VECELL retained a rounder shape compared to

![Cell morphology differences following culture on standard culture plates, collagen-coated cell culture plates, and VECELL.](image)

**Fig. 1.** Cell morphology differences following culture on standard culture plates, collagen-coated cell culture plates, and VECELL. (A) Cell morphologies were observed by phase contrast microscopy at 100X magnification; Scale Bar 100 μm. (B) Cell nuclei were stained with DAPI and the cell cytoplasm was stained with an anti-albumin antibody. Images at different focal planes within the sample were obtained and stacked according to the z-axis to reconstitute 3-D morphologies of nuclei and cells. Distance between grids is 100 μm.
those grown on standard culture plates. These observations support the schematic model of VECELL cell culture shown in Supplementary Fig. 1.

**The effect of VECELL culture on cell proliferation**

Cell growth was then measured to check the effect of VECELL culture on cell proliferation. For this purpose, cells from both culture conditions were harvested by trypsinization one, two, three, four and seven days after seeding and number of cells attached to standard plate or VECELL were counted with a hemacytometer (Fig. 2). Floating cells in VECELL culture were observed one day after seeding. These cells were not included as the attached cell number, thus attached cell number reduced in VECELL culture. Most floating cells attached to VECELL during the first two days, and the attached cell number became nearly equal in both culture. Cell growth continued for four days after seeding and then reached a plateau.

**The effect of VECELL culture on metallothionein gene family expression**

Next, functional changes induced by VECELL culture were analyzed by global gene expression analysis. HepG2 cells were cultured in triplicate on standard culture plates or on VECELL for four days. Genes that showed altered expression levels were selected as described in the Materials and Methods. In VECELL cultures 108 and 22 genes were up- and down-regulated, respectively (Supplementary Table 1). The genes remaining after filtering were categorized based on gene ontology using DAVID Bioinformatics Resources (National Institute of Allergy and Infectious Diseases (NIAID), NIH, http://david.abcc.ncifcrf.gov/) (Huang da et al., 2009a, 2009b). Only one gene group related metallothionein (metallothionein 1F, 1H, 1 G, 1E/1L, 1X, 2A) was found to be enriched among the gene list by the DAVID Gene Functional Classification Tool (enrichment score of 5.23), thus, this study focused on metallothionein gene family and tolerance to heavy metal toxicity. All of the metallothionein genes that showed significantly altered expression levels were upregulated in HepG2 cells cultured on VECELL (Fig. 3A). To confirm the DNA microarray results, expression of metallothionein 1F (MT1F), metallothionein 1G (MT1G) and metallothionein 2A (MT2A) was measured by real-time PCR. RNAs from the cells on standard culture plates, collagen-coated cell culture plates and VECELL were prepared in triplicate and the same amount of RNA was used for real-time PCR measurements. To check the effects of collagen, which is coated on the surface of the VECELL ePTFE mesh, we examined the expression of metallothionein genes in HepG2 cells cultured on collagen-coated cell culture plates (Fig. 2B). The collagen coating on the surface of the culture plates showed no significant effect on the expression of the genes tested. As expected from the DNA microarray analysis results, the expression of the three metallothionein genes that were analyzed was highly upregulated in HepG2 cells cultured on VECELL compared to those grown on standard culture plates (MT1F, 9.9-fold; MT1G, 41.7-fold; MT2A, 13.4-fold, p < 0.01) (Fig. 3B). These results indicate that the expression of the metallothionein gene family was upregulated by VECELL culture.

**The effect of VECELL culture on tolerance to heavy metal toxicity**

Metallothionein is a cysteine-rich, metal-binding protein that plays a role in the detoxification of heavy metals such as cadmium, mercury, zinc and copper by chelating these metals with its cysteine residues (Sato and Kondoh, 2002; Thirumoorthy et al., 2007). Specific expression upregulation of the metallothionein gene family by VECELL culture indicated that HepG2 cells cultured on VECELL may be more tolerant to heavy metal toxicity than cells cultivated on standard culture plates. To investigate this hypothesis, HepG2 cells were cultured either on standard culture plates or VECELL with various concentrations of CuCl₂ (100 μM-1,200 μM) for three days, and relative cell numbers were measured with...
an MTT assay. When HepG2 cells grown on standard culture plates were incubated with various concentrations of CuCl₂, the number of viable cells was reduced in a CuCl₂ concentration-dependent manner (Fig. 4). The LC₅₀ was 930 μM, which was consistent with the previous report by Schilsky et al. (750 μM, Schilsky et al., 1989). For HepG2 cells cultured on VECELL, more than 50% of cells survived even at the highest copper concentration assayed (1,200 μM CuCl₂, Fig. 4). Moreover, the relative cell viability at a concentration > 800 μM in VECELL culture was higher than on standard culture plates (P < 0.05, Fig. 4). These results show that HepG2 cells cultured on VECELL were more resistant to toxicity induced by copper than those cultured on standard culture plates and are consistent with predictions based on gene expression analyses.

**DISCUSSION**

In this study we used global gene expression analyses to determine the effects of VECELL culture on HepG2 cell gene expression and found that expression of the metallothionein gene family was upregulated (Fig. 3). This upregulation in turn induced functional changes in HepG2 cells that were manifested as a higher tolerance to toxicity induced by heavy metals (Fig. 4). As indicated in the schematic culture model (Supplementary Fig. 1), HepG2 cells grown on VECELL showed a rounded mor-
phology (Fig. 1). Based on observations obtained in this study, we propose that the morphological changes induced by VECELL culture promoted the observed changes in HepG2 cell functionality. Besides the effects of VECELL culture on cell morphology, supplies of nutrition and oxygen are different from the standard plate culture. Nutrition and oxygen are supplied to cells both top and bottom side of cells on VECELL. This might also affect cell functionality and further study is required to elucidate these points.

We previously used global gene expression analysis to demonstrate the upregulation of drug metabolism-related genes and microtubule molecule genes in 3-D HepG2 cultures by RFB. These results suggested that microtubule molecules might be responsible for upregulating drug metabolism-related genes. When microtubules were stabilized by treating HepG2 cells with a sub-lethal dose of the microtubule stabilizing agent docetaxel, effective induction of drug metabolism-related genes was observed (Horiuchi et al., 2009). Meanwhile, docetaxel-treated HepG2 cells showed a round shape as observed by phase-contrast microscopy (Ishida, 2012). These observations support our hypothesis that altered morphology induced changes in HepG2 cell function. However, expression of drug metabolism-related genes in HepG2 cells cultured on VECELL showed no induction compared to those cultured on standard cell culture plates (Ishida, 2012). Elucidating these differences in gene expression will contribute to a better understanding of the effects of cell morphology on cell function. Compared to docetaxel treatment, the VECELL culture system is simpler because it does not require additional chemical substances. These findings are important for developing assay systems for use in evaluating chemical substances, and further refinement of the VECELL culture system for this application is ongoing.

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**Conflict of interest**——The authors declare that there is no conflict of interest.

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