RAT HEPATOCYTE CULTURE PHYSIOLOGY SHOWS ENHANCED CYTOCHROME P450 ACTIVITY ON A SYNTHETIC EXTRACELLULAR MATRIX

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We examined the impact of a new three-dimensional synthetic matrix on hepatocyte physiology. Hepatocytes were plated on Corning® labware with Ultra-Web™ and Ultra-Web™ polyamine surfaces and on Becton Dickinson (BD) BioCoat™ collagen I microplates. The samples were then challenged for 48 hours with two known modulators of cytochrome P450, and P450 activity was measured using the P450-Glo™ Assay. The data show enhanced physiologic function of primary rat hepatocytes on microplates coated with the Ultra-Web™ synthetic matrix.

Introduction

A key issue facing scientists performing cell-based assays is whether the physiology observed in two-dimensional culture systems truly reflects that observed in vivo. In vivo, cells interact with the extracellular matrix (ECM) to form a dynamic three-dimensional structure. It is in this interaction that many cellular functions occur including development, proliferation and communication (1,2). The ECM is multifunctional; it provides support, anchorage and separation between tissues and aids in cellular communication by sequestering growth factors and other ligands. Therefore, there is a need for in vitro surfaces that can mimic the cell basement membrane/ECM interactions found in intact tissues. Any technology (cell surface, vessel, growth medium or combinations thereof) that allows cells to maintain their physiologic function (including developmental and metabolic functions) will be of great importance for basic research and drug development studies (3).

There are a variety of ECM-like products available, including Matrigel™ (BD) and collagen. These products can be problematic because they are derived from animal sources, are unstable at room temperature, exhibit batch-to-batch variability and possess growth factors in the matrix. A new line of products incorporating the Ultra-Web™ synthetic matrix, which bypasses all of these issues, has been developed by Corning Incorporated, Donaldson Co., and SurModics, Inc.

Corning labware with the Ultra-Web™ surface provides a nanofibrillar growth scaffold that mimics the ECM architecture and provides nanotopography for cell attachment, growth and organization. The Ultra-Web™ nanofibers (Figure 1, Panel A) are close in size to those of collagen (Figure 1, Panel B) but are totally synthetic, thus stabilizing the surface and providing an ideal technology for many drug development applications. Studies using various cell types have demonstrated that the unique Ultra-Web™ structure promotes in vivo-like cellular responses (see **www.corning.com/lifesciences/** for more information). The Ultra-Web™ matrix is also available in a form modified by covalently linked polyamines, creating a charged surface that increases cell attachment. The nanofibers can be further modified by the attachment of various biomolecules (e.g., growth factors, peptide sequences, carbohydrate moieties, etc.), allowing the user to develop a cell/tissue-specific microenvironment (4).

Hepatocytes provide a key example of the need for an alternative in vitro surface because they rapidly lose their morphologic and metabolic phenotype when plated on a two-dimensional tissue culture surface. However, hepatocytes plated on coated surfaces that mimic the internal ECM retain their phenotype for a much longer period of time (1,5). A key function of hepatocytes is the oxidative metabolism of drug compounds by cytochrome P450 enzymes (CYPs), making

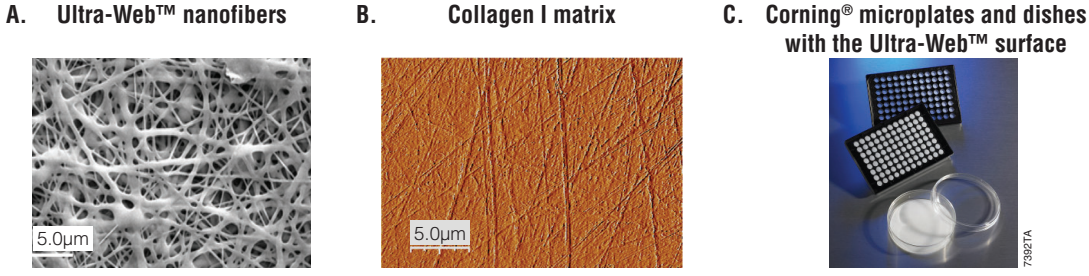

Figure 1. Ultra-Web™ and collagen I surfaces. Panel A. Electron micrograph showing the Ultra-Web™ surface, which is slightly hydrophilic and uncharged. The Ultra-Web™ polyamine surface (not shown) is also slightly hydrophilic but has a positive charge. Microscopically, both Ultra-Web™ surfaces appear identical. **Panel B.** Collagen I nanofibers at the same scale as Panel A. **Panel C.** Corning® microplates and dishes with the Ultra-Web™ surface.

drugs more water soluble and more easily excreted. Fluorescence-, luminescence- and LC/MS-based CYP enzyme assays are widely used in the pharmaceutical industry to screen for CYP inhibition and induction by test compounds. CYP1A and CYP3A activities are induced by numerous drugs, and screening for these effects is common because of the impact they may have on the metabolism of the inducing or co-administered drug(s).

We compared the functional activity of primary rat hepatocytes plated on three different materials using the P450-Glo™ CYP3A4 Assay[a,b] (Cat.# V8901). The three surfaces tested were Ultra-Web™, Ultra-Web™ polyamine (Corning Cat.# 3872xx1 and 3873xx1, respectively) and collagen I (BD). Two known inducers of rodent CYP3A activity, dexamethasone (Dex) and pregnenolone-16α–carbonitrile (PCN) were used. Inducible CYP3A enzyme activity is a reasonably stringent marker of hepatocyte phenotype because it is particularly sensitive to culture conditions.

Assay Method

The P450-Glo™ Assays use derivatives of beetle luciferin that are luminogenic substrates of cytochrome P450 enzymes (6). These substrates are inactive until metabolized by CYP enzymes to luciferin, which then reacts with luciferase to produce an amount of light proportional to CYP enzyme activity. To measure CYP3A induction in rat hepatocytes, the cells were cultured as monolayers, and untreated control cells were compared to cells treated with the CYP3A inducers Dex and PCN. After treatment, the luminogenic CYP3A enzyme substrate, luciferin-PFBE, was added to the culture medium and incubated with the cells. In rat hepatocytes the substrate is selective for CYP3A1 and CYP3A2. Intracellular CYP3A converts the luciferin-PFBE to luciferin, which passes out of cells into the medium. A sample of the culture medium was removed and combined with a luciferin detection reagent containing luciferase, and the resulting light signal was read on a luminometer. The cells remained intact and were tested further for viability using the CellTiter-Glo® Assay (Cat.# G7570), which correlates ATP content with viable cells. Luminescent signals for the P450-Glo™ CYP3A assay were divided by signals from the viability assay to express CYP activity in terms of viable cell number. A compound was scored as a CYP3A inducer if it caused a significant increase in CYP-dependent luminescence compared to an untreated control.

Results

Primary rat hepatocytes seeded onto 96-well collagen I-coated plates and Ultra-Web™ or Ultra-Web™ polyamine microplates had the morphological appearance characteristic of cultured hepatocytes (5) when examined microscopically (data not shown). The hepatocytes were challenged for 48 hours with either Dex or PCN and assayed for CYP3A activity using the P450-Glo™ Assay. Background luminescence from wells

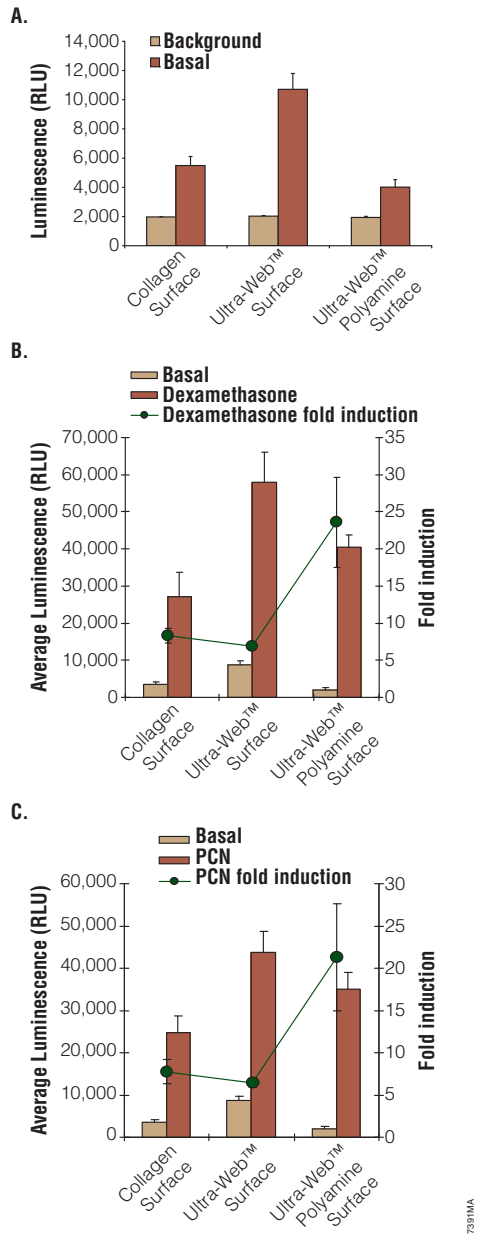

Figure 2. CYP3A activity of primary rat hepatocytes on collagen and Ultra-Web™ surfaces. Cryopreserved primary rat hepatocytes (Xenotech, LLC) were seeded on 96-well collagen-coated plates (BioCoat™ Collagen I, Discovery Labware) and Ultra-Web™ or Ultra-Web™ polyamine microplates (Corning) at a density of 1.5×10^5 cells per cm^2 (4.8×10^5 cells/well) in Hepatocyte Culture Medium (HCM, Xenotech). The following day, treatment was initiated by replacing HCM with HCM containing 10µM dexamethasone (**Panel B**), 30µM pregnenolone-16α-carbonitrile (PCN, **Panel C**) or vehicle (0.1% DMSO, **Panel A**). Fresh treatments were applied after 24 hours. After 48 hours of treatment, CYP3A activity was measured using the P450-Glo™ CYP3A4 Assay (Cat.# V8901). Treatment medium was replaced with medium containing 50 µM Luciferin-PFBE. Four hours later, CYP3A-dependent conversion of Luciferin-PFBE to luciferin was determined by combining a sample of medium in a separate plate with luciferin detection reagent. Luminescence was measured on a GloMax® 96 Microplate Luminometer (Cat.# E6501). Last, cell number was determined using the CellTiter-Glo® Luminescent Cell Viability Assay (Cat.# G7570), and relative luminescence units (RLU) from the P450-Glo™ Assay were normalized to cell number.

without cells was identical among the three surfaces. Importantly, basal luciferase activity was approximately twofold higher on the Ultra-Web™ surface than on collagen or Ultra-Web™ polyamine surfaces (Figure 2, Panel A) and fivefold higher than background. Having a higher differential between basal and background signals gives greater reliability and sensitivity in an assay format that is hampered by variability in hepatocyte sources and preparations.

Induced CYP3A activity was higher on the Ultra-Web™ and Ultra-Web™ polyamine surfaces than on the collagen surface, with the Ultra-Web™ surface showing the greatest induced activity (Figure 2, Panels B and C). Dex (Figure 2, Panel B) and PCN (Figure 2, Panel C) treatment resulted in similar induced CYP3A activity and fold-induction over basal. Cells on the Ultra-Web™ polyamine surface gave the highest induced:basal activity ratio (23:1) of the three surfaces. Despite having higher induced CYP activity on the Ultra-Web™ surface, the higher basal activity seen on that surface (Figure 2, Panel A) resulted in a fold induction that was not significantly different from that on collagen I. Thus the Ultra-Web™ surface provides a synthetic alternative to collagen with a more robust basal activity and equivalent fold induction by known rat CYP inducers. The Ultra-Web™ polyamine surface provides the greatest difference in basal and induced CYP activity with these two modulators. Recent FDA guidance for drug interaction studies (7) suggests that positive controls for CYP induction in hepatocytes should cause basal CYP enzyme activity to increase more than twofold, a value that was observed in this study and improved upon with the Ultra-Web™ surface. The FDA guidance also favors CYP enzyme activity measurement over mRNA measurement for monitoring CYP induction. The P450-Glo™ Assay provides a convenient approach to accommodate this preference.

Summary and Conclusions

Corning microplates with the Ultra-Web™ surface provide a convenient synthetic nanofibrillar surface for cell culture and cell-based assays. This unique microenvironment is able to promote proliferation, differentiation, and in vivo-like physiologic responses (3,8–11). The surface can be used in a number of different plate formats, making it ideal for cell-based assays, including FLIPR® calcium flux, luciferase and imaging assays. This study examined the impact of three surfaces on rat hepatocyte physiology by measuring cytochrome P450 function in a luminescence-based assay format.

- Using the P450-Glo™ Assay we showed enhanced cytochrome activity in hepatocytes cultured on Ultra-Web™ and Ultra-Web™ polyamine surfaces compared to collagen I surfaces.
- Basal CYP3A activity in rat hepatocytes grown on the Ultra-Web™ surface was more than twofold greater than that of cells grown on collagen and fivefold above background.
- Fivefold induction of CYP3A by Dex and PCN was observed on Ultra-Web™ surfaces, a value that exceeds FDA recommendations for efficacious, positive control inducers.
- Ultra-Web™ and Ultra-Web™ polyamine dishes and microplates provide an excellent surface to culture and retain hepatocyte morphology and physiology. In a related study, we have observed enhanced CYP1A activity for >14 days in rat and human primary hepatocytes (in press).

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Protocol

P450-Glo™ CYP450 Assay Systems Technical Bulletin #TB325 (www.promega.com/tbs/tb325/tb325.html)

Ordering Information

Product	Size	Cat.#
P450-Glo™ CYP3A4 Assay (Luciferin-PFBE) Cell-Based/Biochemical Assay	10ml	V8901
CellTiter-Glo® Luminescent Cell Viability Assay*	10ml	G7570
GloMax® 96 Microplate Luminometer	1 each	E6501

*For Laboratory Use. Additional sizes available.

[a]U.S. Pat. Nos. 6,602,677 and 7,241,584, Australian Pat. Nos. 754312 and 785294 and other patents and patents pending.

[b]The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.

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