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Effects of ECM on Cell Proliferation and Differentiation in Hepatocytes

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Abstract

The cellular microenvironment *in vivo* is characterized by specific interactions between cells, extracellular matrix (ECM), and soluble factors, such as cytokines and hormones. To create physiologically relevant *in vitro* models thatsupport normal cell structure and function, the *in vitro* microenvironment has to incorporate the major components of the *in vivo* microenvironment. By optimizing the medium and ECM, we have constructed a cell culture system that supports the maintenance of differentiated hepatocytes for up to 3 weeks *in vitro*. Hepatocytes were isolated from adult male Wistar rats and plated in Hepato-STIM medium onto amorphous collagen I, collagen I gel, or Corning® Matrigel® Basement Membrane Matrix. Cells were assessed for morphology (by light and electron microscopy), growth rates, and differentiated function (cytochrome P450 1A1 activity). Cells plated onto amorphous collagen I demonstrated a flattened, squamous morphology and rapidly lost function within 3-5 days. Hepatocytes on collagen I gels exhibited a mixed morphology of squamous and spherical cells and lost function within 7-10 days. Hepatocytes on Corning Matrigel Matrix demonstrated a spherical, clustered morphology and maintained well-differentiated function for up to 22 days. Electron microscopy at 1 and 4 weeks demonstrated healthy, functioning cells with ultrastructural characteristics typical of differentiated hepatocytes. The cells contain a complex cytoplasm and are arranged in clusters of 6-12 cells, in a single layer enclosing a lumen. Adjacent cells have interdigitation of cytoplasmic extensions, tight and gap junctions as well as intercellular spaces suggestive of bile canaliculi. In conclusion, we have demonstrated that the combination of an optimized culture medium (Hepato-STIM medium) and ECM (Corning Matrigel Matrix) provides an optimized microenvironment (Corning BioCoat™ Hepatocyte Differentiation Environment) that supports long-term maintenance of differentiated function in hepatocytes *in vitro*.

Materials and Methods

Cell Isolation: Primary rat hepatocytes were isolated from adult, male Wistar rats (180-200 gms) following a two-step collagenase perfusion modified from published procedures[1,2,3]. Cellular viability was assessed by trypan blue exclusion. Cells were seeded at 8 x 10^5 cells/well into 6-well plates containing Corning Matrigel Matrix, collagen I, or a collagen I gel (Corning). Culture medium was Hepato-STIM medium supplemented with 50 µ/mL penicillin, 50 µg/mL streptomycin, 100mM L-glutamine, and 10 ng/mL EGF (Corning. Cultures were incubated at 37ºC, 5% CO_2 -95% air, and medium was changed every 2-3 days.

Cell Proliferation: Cell growth was assessed at various time points using hemacytometer cell counts after a 60 minute Dispase treatment of cells grown on Corning Matrigel Matrix or collagen I, and a 30 minute 0.05% Collagenase treatment of cells grown in a collagen gel.

Cytochrome Expression: Cytochrome P450 1A1 activity was measured using a modified metabolic fluorescent assay4. Briefly, cultured cells were incubated with a noncytotoxic concentration (8 µm) of 7-ethoxyresorufin for 30 minutes. Dicumarol (10 µm) was added to the assay media to prevent further metabolism of the resorufin formed by the cytosolic enzyme diaphorase. Culture supernatents were incubated with b-glucuronidase for 2 hours at 37ºC, and dealkylated resorufin released was quantified by spectrofluorimetry using 530 nm excitation and 590 nm emission filters.

Light Microscopy: Samples were examined using an Olympus phase contrast microscope at a 10x magnification and photographed with Polaroid 667 film.

Scanning Electron Microscopy (SEM): Cells were fixed with 5% glutaraldehyde in a sucrose/cacodylate (0.1 M each) buffer, followed by 1% OsO4 in buffer. Samples were dehydrated in graded ethanol, dried with HMDS, sputter-coated with gold palladium, and examined using an Amray 1800I microscope.

Transmission Electron Microscopy (TEM): Cells were fixed for 1.5 hours in K-II at 4ºC, rinsed in 0.1 M cacodylate buffer, centrifuged, and suspended in 2% Bacto agar; 1 mm cubes were post-fixed in 2% OsO4 in 0.1 M cacodylate for 2 hours. Samples were dehydrated in graded ethanol, infiltrated with propylene oxide and epon, and embedded in epon. Thick sections (1 µm) were stained with 0.5% toluidine blue (figure 5). Thin sections (70-90 nm) were stained with saturated uranyl acetate and Sato's lead stain (figures 6-9). Sections were examined and photographed using a Philips CM-10 microscope.

Results

Effect of ECM on hepatocyte proliferation

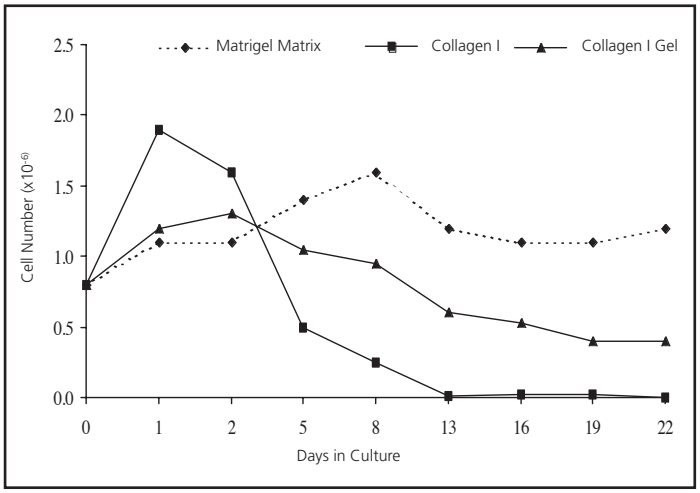


Figure 1: Effects of ECM on cell numbers. Note the decrease in the number of cells cultured on amorphous collagen I between days 2 and 13, and the gradual loss in the number of cells cultured in collagen I gels. In contrast, hepatocytes cultured on Corning Matrigel Matrix maintain a constant cell number through day 22.

Effect of ECM on cytochrome P450 expression

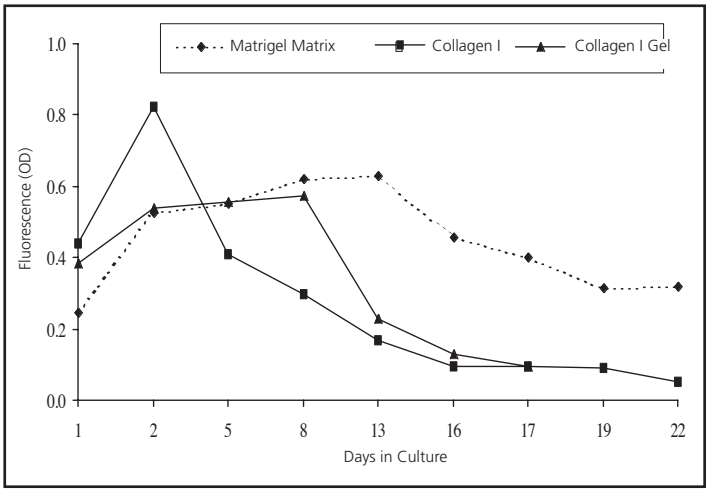


Figure 2: Effects of ECM on cytochrome P450 1A1 activity. Note the decrease in cytochrome P450 activity within 8-13 days for cells cultured on amorphous collagen I or in collagen I gels. In contrast, hepatocytes cultured on Corning Matrigel® Matrix maintain cytochrome P450 expression through day 22.

Effects of ECM on cell morphology: micrographs of hepatocytes cultured on various culture substrata

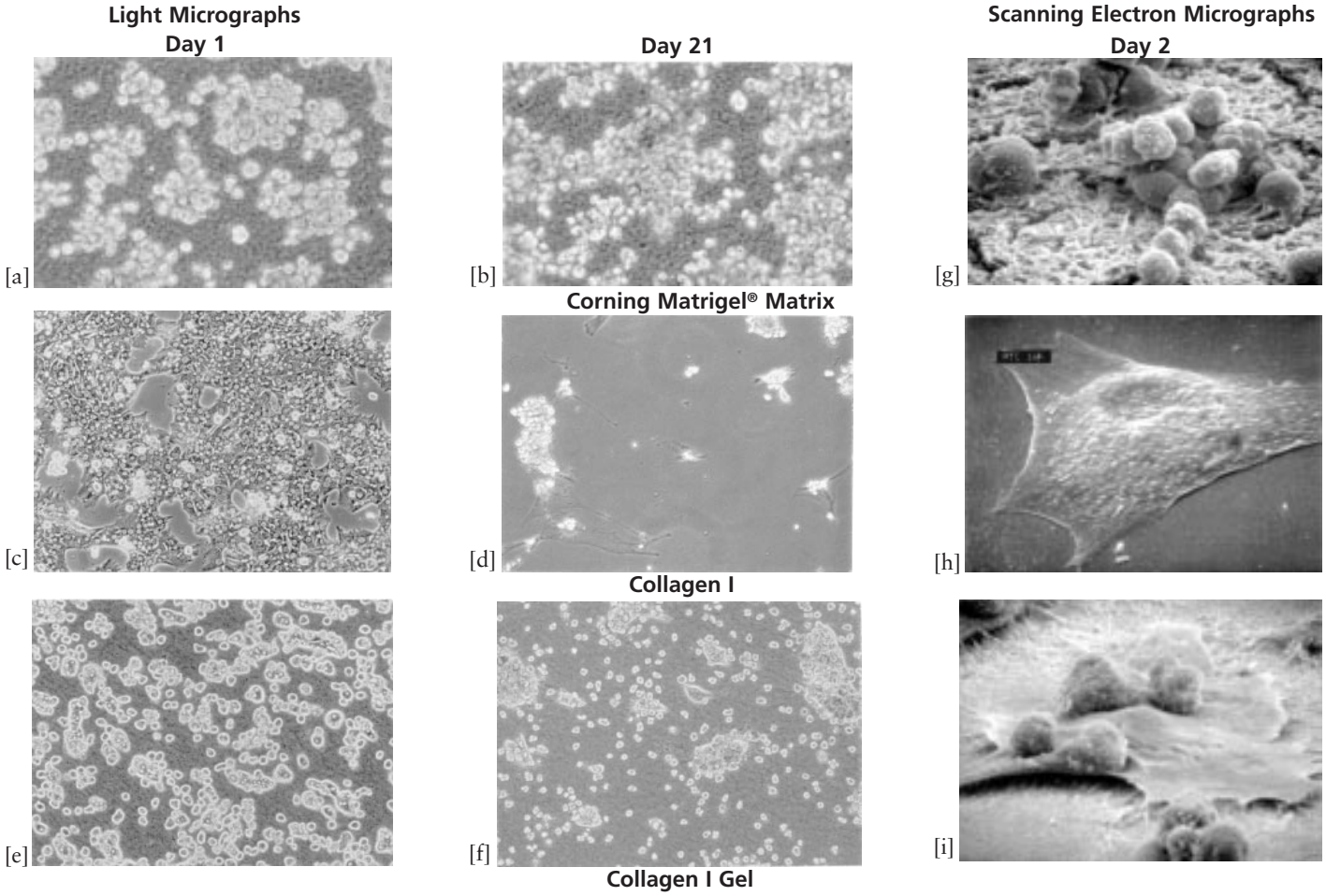


Figure 3: Light micrographs of primary rat liver epithelial cells cultured in a serum-free, supplemented medium for one day (a, c, e) or three weeks (b, d, f) on polystyrene plates containing Corning Matrigel Matrix (Corning BioCoat™ Hepatocyte Differentiation Environment) (a and b), collagen I (c and d), or collagen I gels (e and f). Note the lack of tight clusters of cells grown in collagen I gels, despite their spherical cell shape (magnification = 100x).

Figure 4: SEMs of hepatocytes cultured for two days on Corning Matrigel Matrix (g), collagen I (h), or collagen I gels (i). Note the clusters of spherical cells for hepatocytes cultured on Corning Matrigel Matrix, typical of differentiated cells (g).

Analyses of cell morphology by light and scanning electron microscopy demonstrate that a culture system, in which key components of the microenvironments (ECM and culture medium) have been optimized, can support a differentiated phenotype of primary rat hepatocytes. Furthermore, the results indicate that the combination of Corning Matrigel matrix and Hepato-STIM medium (forming the Corning BioCoat Hepatocyte Differentiation Environment) outperforms other ECM and medium combinations examined. Using this system, differentiated cell morphology can be maintained for at least three weeks.

Transmission Electron Micrographs of Primary Rat Hepatocytes Cultured on Corning® Matrigel® Matrix and on Optimized Serum-Free Medium

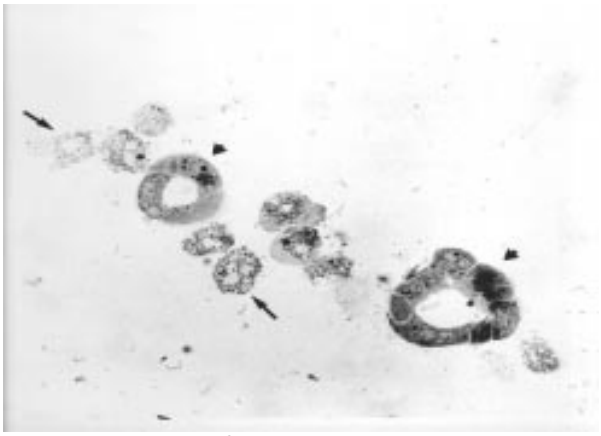
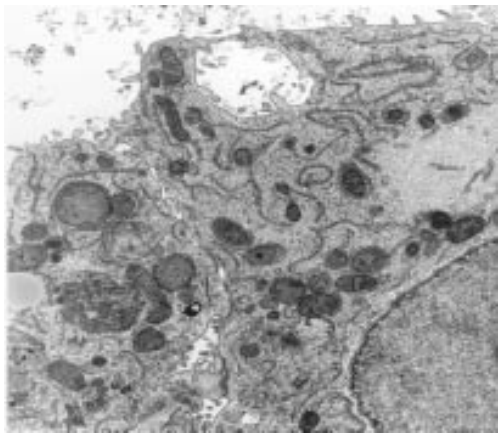


Figure 5: Thick sections of hepatocytes cultured on Corning Matrigel Matrix in a serum-free medium (Corning BioCoat™ Hepatocyte Differentiation Environment). Healthy cells are arranged in clusters (arrowheads) of 6-12 cells in a single layer (compare to figure 4a), enclosing a lumen, giving the appearance of a duct. In contrast, single cells (arrows) are dead or dying.



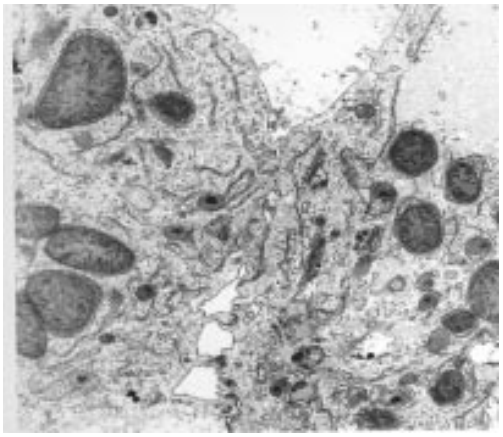
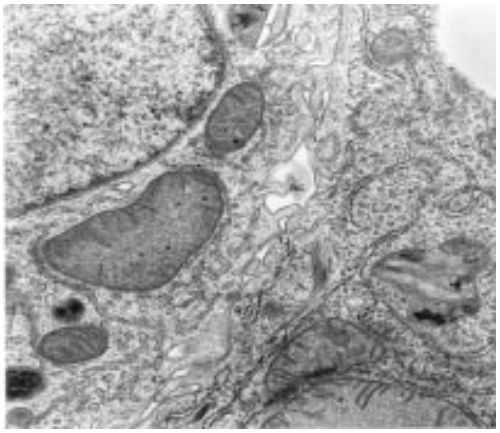


Figure 6 and 7: Thin sections of hepatocytes cultured for one week in the Corning BioCoat Hepatocyte Differentiation Environment show healthy, functioning cells containing a complex cytoplasm, typical of differentiated cells Nuclei are active with a single large nucleolus and the cytoplasm contains many mitochondria with few cristae and occasional calcium deposits as in normal hepatocytes. Rough ER is arranged in regular stacks of single strands, occasionally arranged near the lumen, outer cell surfaces, and surrounding mitochondria. The cytoplasm also contains golgi complexes, lysomes, ribosomal rosettes, vesicles, lipid droplets, microfilaments, and microtubules. Hepatocytes contain large pools of flocculant material representing lost glycogen rinsed from tissue during dehydration.



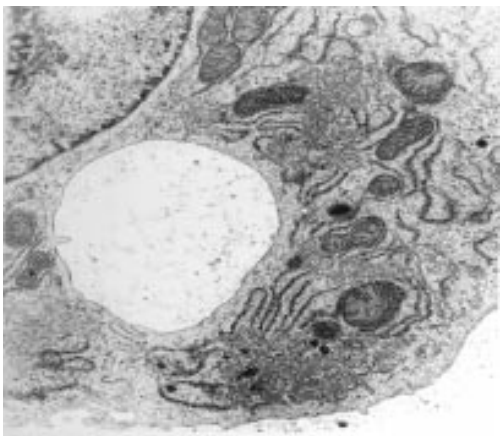


Figure 8 and 9: Thin sections of hepatocytes cultured in the Corning BioCoat Hepatocyte Differentiation Environment for four weeks show healthy, functioning cells with frequent interdigitation of cytoplasmic extensions. Apposing cell membranes show tight junction as well as spaces or lumens between cells with microville present, suggestive of bile canaliculi.

Conclusions

• During a three-week culture period in the Corning® BioCoat™ Hepatocyte Differentiation Environment, the cell number of the hepatocytes changes only slightly indicating absence of cell proliferation (**Figure 1**), characteristic of differentiated cells[5,6,7,10].

• Cellular functions typical of differentiated hepatocytes are maintained for at least three weeks as shown by continued expression of cytochrome P450 (**Figure 2**), a hemoprotein involved in the oxidative metabolism of various compounds, an important function of the liver.

• Tight clusters of spherical cells with a lumen appear within the first day of culture in the Corning BioCoat Hepatocyte Differentiation Environment. Cells will not spread, but remain in clusters throughout the entire culture period of three weeks (**Figures 3a, 3b, and 4a**).

• TEMs at one and four weeks of hepatocytes cultured in the Corning BioCoat Hepatocyte Differentiation Environment show healthy functioning clusters of 6-12 cells in a singlelayer surrounding a lumen. Junctional complexes between apposing cell membranes are suggestive of bile canaliculi.

Discussion

The study of hepatocytes in culture is of great importance to research investigating mechanisms that control liver-specific functions. However, hepatocytes often dedifferentiate and die in response to inadequate culture environments, precluding such systems from being used as relevant *in vitro* models.

Corning Matrigel® Matrix, a reconstituted basement membrane, has been shown to suppress cell growth and prevent growthassociated dedifferentiation, as evidenced by the disappearance of growth-related transcription factors and appearance of liver-specific transcription factors in cells cultured on Corning Matrigel Matrix[8]. Hepatocytes cultured in Corning Matrigel Matrixbased systems have been shown to maintain liver-specific functions *in vitro* for longer periods of time than do hepatocytes cultured in most collagen-based systems[9-12]. Hepatocytes cultured in Corning Matrigel Matrix-based systems have been shown to express high levels of plasma proteins, such as albumin[9] and the multidrug resistance-associated plasma membrane glycoprotein gpg[10], and retain the ability to respond to a number of xenobiotics[9,11] and hormones[12] with increased expression of members of the cytochrome P450 supergene family or metabolic enzymes, respectively.

Changes in cell shape as modulated by ECM have been shown to regulate expression of growth and differentiation in rat hepatocytes[8,13,14]. Culture of hepatocytes on collagen I induces expression of Immediate-Early Growth Response genes (Cjun, jun-B, C-myc) expressed in proliferating cells, and downregulates expression of transcription factors (e.g., C/EBPa) shown to be expressed in differentiated cells. In a collagen I sandwich, albumin mRNA expression is maintained, but C/EBPa expression is downregulated. In contrast, hepatocytes cultured on a reconstituted basement membrane (Corning Matrigel Matrix) show restored C/EBPa expression and low-level expression of C-jun, jun-B, and C-myc, indicating that the cells are growth-arrested in GO phase[8,14].

The Corning BioCoat Hepatocyte Differentiation Environment is an integrated culture system of Corning BioCoat Matrigel Matrix plates (6-well) and a fully defined, serum-free optimized medium that is supplemented with hormones (dexamethasone and insulin), growth factors (EGF), and other defined metabolites. We have shown that this system has been optimized to support cultures of differentiated hepatocytes in a serum-free environment for at least 3 weeks. Due to its ability to support a liver-specific phenotype, this Environment can be used to create *in vitro* models to study various liver functions, such as liver-specific gene expression, metabolism and transport of drugs or natural compounds, drug toxicity, and altered or impaired liver function due to disease.

References

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Corning acquired the Discovery Labware Business including the BioCoat™ and Matrigel® brands. For information, visit www.corning.com/discoverylabware.

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