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ENGINEERED SUBSTRATES FOR CONTROLLING CELL-CELL INTERACTIONS

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INTRODUCTION

Biomaterials have been previously engineered to serve a variety of different functions: precise degradation in vivo (Kimura, 1993), modulation of cell physiology via binding to specific ligands (Hubbell et al. 1992), and selective permeability of certain solutes (Lysaght et al. 1994). However, in complex tissues, where cell-cell interactions strongly influence tissue function, biomaterials which modulate this fundamental parameter have not been available. In this study, we describe a technique which allows control over cell-cell interactions by using semiconductor-based microfabrication, silane derivatization of borosilicate, and immobilization of specific biomolecules. We have focused on liver tissues due to the clinical significance of liver disease and the potential utility of a highly functional in vitro substitute for the liver (Rozga et al. 1994). Previous studies have reported that co-culture of primary hepatocytes with mesenchymal cells induces up-regulation of liver-specific functions in the hepatocyte population (Guguen-Guillouzo et al. 1983). Using microfabricated techniques to pattern co-cultures, we have demonstrated the importance of local cell-cell interactions in overall tissue function. Specifically, we show that the level of heterotypic interaction modulates the kinetics of up-regulation of liver-specific functions when compared to unpatterned controls. This approach will have applications in many areas including tissue engineering, developmental biology, and transplantation.

MATERIALS AND METHODS

Microfabrication techniques were used to modify borosilicate substrates with biomolecules. These modified substrates were utilized to micropattern co-cultures of hepatocytes and fibroblasts and allowed control over both homotypic and heterotypic cell-cell interactions. Biochemical assays were performed to assess the functionality of the resulting 'micropatterned co-cultures'.

Substrate Preparation

Detailed procedures for microfabrication of substrates and subsequent modification were previously described (Bhatia et al. 1997). Briefly, 2" borosilicate wafers were spin-coated with positive photoresist, baked, exposed to ultraviolet light through chrome masks of the desired dimensions, and immersed in developer solution. Discs were then baked and exposed to oxygen plasma to remove unwanted resist in areas to be subsequently modified.

Substrates were modified using experimental methods derived from Lom et al (1993) and Britland et al (1992). Modification of bare glass with 3-[(2-aminoethyl)amino]propyl trimethyoxysilane (AS), and glutaraldehyde allowed the immobilization of collagen I in specified areas. Finally, the residual photoresist was removed by sonication in acetone.

Cell Culture

Hepatocytes were isolated from 2- to 3-month-old adult female Lewis rats. Detailed procedures for isolation and purification of hepatocytes were previously described by Dunn et al (1989). Culture medium was Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 0.5 U/mL insulin, 7 ng/mL glucagon, 20 ng/mL epidermal growth factor, 7.5 μg/mL hydrocortisone, 200 U/mL penicillin, and 200 μg/mL streptomycin. Serum-free culture medium was identical except for the exclusion of serum.

NIH 3T3-J2 fibroblasts were the gift of Howard Green, Harvard Medical School. Cells were grown in DMEM with high glucose, supplemented with 10% bovine calf serum and passaged at pre-confluency no more than 10 times.

Substrates were sterilized in ethanol, rinsed, incubated in a solution of bovine serum albumin in water to deter non-specific cell attachment, and seeded with hepatocytes in serum-free media. Hepatocytes attached primarily to collagen-modified regions. The following day, 3T3-J2 cells were trypsinized, plated, and allowed to attach overnight. Fibroblast adhesion to outlying areas resulted in 'micropatterned co-cultures'. Subsequently, hepatocyte culture media was sampled and replenished daily.

Experimental Design and Biochemical Assays

Spatial configuration of micropatterned co-cultures were manipulated by varying mask dimensions. In order to achieve identical hepatocyte numbers across varying micropatterned configurations the total surface area of collagen-modified glass (area dedicated to hepatocyte attachment) was kept constant despite changes in pattern dimensions. In this study, we utilized 490 μm diameter circles, hexagonally-packed, with 1230 μm center-to-center spacing, a single circle of 17800 μm, and an unpatterned control surface. Unpatterned controls were uniformly modified with collagen as described above and seeded with 250,000 hepatocytes, resulting in randomly-distributed cell attachment (this number corresponds to the approximate number of spread hepatocytes on patterned substrates). Subsequently, both micropatterned and unpatterned wafers were processed identically.

Urea synthesis, monitored as a marker of liver-specific metabolic function, was assayed using a commercially available kit (Sigma). Reaction with diacetyl monoxime under acid and heat yields a color change detected at 540 nm.

RESULTS

Co-cultures were performed in both unpatterned and micropatterned configurations. Micropatterned co-cultures were generated with variations in heterotypic interface yet identical surface area (i.e. cell numbers) dedicated to both hepatocyte and fibroblast adhesion. Variation in spatial configuration were utilized to generate differences in total perimeter of hepatocyte islands (primary location of heterotypic contact) from 235.2 cm to 5.6 cm. Micropatterned hepatocytes were found to adhere homogeneously to collagen-modified areas whereas unpatterned controls displayed heterogeneous homo- and heterotypic contacts (Figure 1).

All co-culture conditions led to up-regulation of liver-specific functions; however, differences in both the level of function and kinetics of up-regulation of urea synthesis (as a marker liver-specific function) were observed. In this study, we focused on the variations in the kinetics of up-regulation of liver-specific functions between unpatterned (conventional) co-culture and micropatterned co-cultures of various configurations. Figure 2 shows that, in certain micropatterned configurations (490 μm islands), the increase in urea synthesis preceded that of the unpatterned, conventional controls by almost one week. Larger micropatterns (17800 μm islands), which contained less initial heterotypic interaction, displayed lower, steady-state levels of urea synthesis. Interestingly, while unpatterned, randomly-distributed cultures produced low levels of urea initially (as in the larger micropattern), the level of urea synthesis was observed to increase over the next week. These results indicated that certain micropatterned co-cultures have kinetic advantages over conventional cultures. We hypothesize that initial cell-cell interactions contribute significantly to these responses.

DISCUSSION

Optimization of tissue function for use in a hepatocyte-based bioreactor would require precise definition of the kinetics of 'recovery' of liver-specific functions after initiation of co-culture. Typically, co-culture with mesenchymal cells induced hepatic functions on the order of 7-10 days (Figure 2, unpatterned control); however, in order to utilize a

bioreactor soon after cell seeding, we examined the utility of controlling initial cellular microenvironment in accelerating this up-regulation. We found that certain functions can be induced up to 1 week earlier by use of micropatterning. Specifically, a ~2-fold improvement in urea synthesis was noted early in culture in smaller hepatocyte island configurations (490 μm). This level of urea synthesis reached a plateau by day 4 whereas unpatterned, control cultures steadily increased the amount of daily urea production until day 10. Subsequently, both cultures performed similarly. In contrast, large micropatterned island (17800 μm) had no improvement in initial levels of urea production, suggesting that cellular microenvironment played a role in modulating these kinetics.

The differences in kinetics observed between unpatterned co-cultures and smaller micropatterned co-cultures, have a number of potential causes. The most likely modulator of this response is the initial cell-cell interactions- indeed, randomly-distributed cultures reorganize over 7-10 days into cord-like structures (data not shown) and could be achieving more favorable cell-cell interactions as time progresses. In contrast, 490 μm micropatterns and 17800 μm micropatterns did not reorganize over time, therefore a fixed cellular microenvironment led to relatively constant levels of urea synthesis with higher degree of heterotypic interaction (490 μm) leading to increased urea synthesis at the onset of co-cultures.

While cell-cell interactions are a likely candidate for modulators of this response, the remodeling of the substrate by extracellular matrix (EMC) deposition of both cell types must also be considered. Note that unpatterned co-cultures consisted of both hepatocyte and fibroblast adhesion directly on collagen I (and proteins adsorbed from serum) whereas micropatterned co-cultures consisted of hepatocyte localization on collagen I and fibroblast adhesion on bare glass (and proteins adsorbed from serum). Fibroblasts in unpatterned co-cultures are likely to modify the extracellular matrix environment by secretion of local ECM. Matrix is well-known to modify cellular responses of all kinds, notably integrin expression in response to soluble growth factors (Xu and Clark, 1995). In addition, collagen I binding is integrin mediated (Hynes, 1992). Therefore, matrix deposition and/or cellular reorganization may play important roles in the kinetics of up-regulation.

Finally, we address the selectivity of this response (improved kinetics of up-regulation) to urea synthesis. Other liver-specific markers such as albumin did not display this behavior (data not shown). Indeed, the pattern of recovery for different markers of liver-specific function is known to vary in other in vitro hepatocyte systems (Dunn et al. 1989). We hypothesize that improved kinetics of albumin up-regulation are not observed in micropatterned co-cultures due to the extended recovery after isolation for this particular function: therefore, differences in level of hepatic function would not be clearly manifested until the requisite cellular machinery is intact. Dunn et al. 1992, hypothesized that albumin secretion is dependent on average polyribosomal size and showed that polyribosomal assembly requires approximately 1 week to recover from hepatocyte isolation. In addition, this and other protein secretion pathways require intact packaging and vesicular trafficking pathways, which may also recover over this time frame. In contrast, small biochemicals like urea can be easily synthesized from amino acids in the presence of the necessary enzymes without polyribosomal translation, packaging, or vesicular trafficking. The pattern of up-regulation which other liver-specific functions (such as detoxification via P450 enzymes) display will need to be empirically determined.

In summary, we have demonstrated the ability to modulate liver-specific functions by varying parenchymal/mesenchymal interactions in vitro. Certain configurations displayed an increased rate of up-regulation of at least 1 liver-specific function over conventional cultures. Future studies will examine the kinetics of "recovery" of other clinically relevant markers of liver-specific function such as detoxification, carbohydrate metabolism, and synthesis of plasma proteins. The ability to quantitatively examine the role of cell-cell interaction on tissue function in vitro will have applications in other areas of tissue engineering as well as cell physiology in vivo.

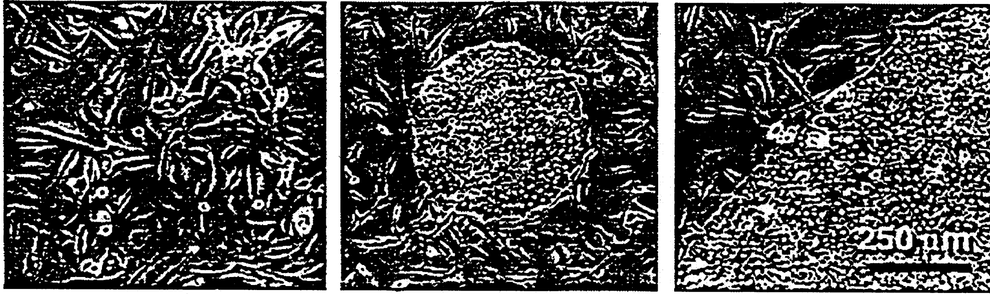

Figure 1. Phase Contrast Micrographs of A)Unpatterned, Randomly-Distributed Co-Culture, B) Micropatterned Co-Culture of Hepatocyte Island Diameter 490 μm, and C) Micropatterned Co-Culture of Hepatocyte Island Diameter 17800 μm. All three culture conditions contain approximately identical numbers of hepatocytes and fibroblasts.

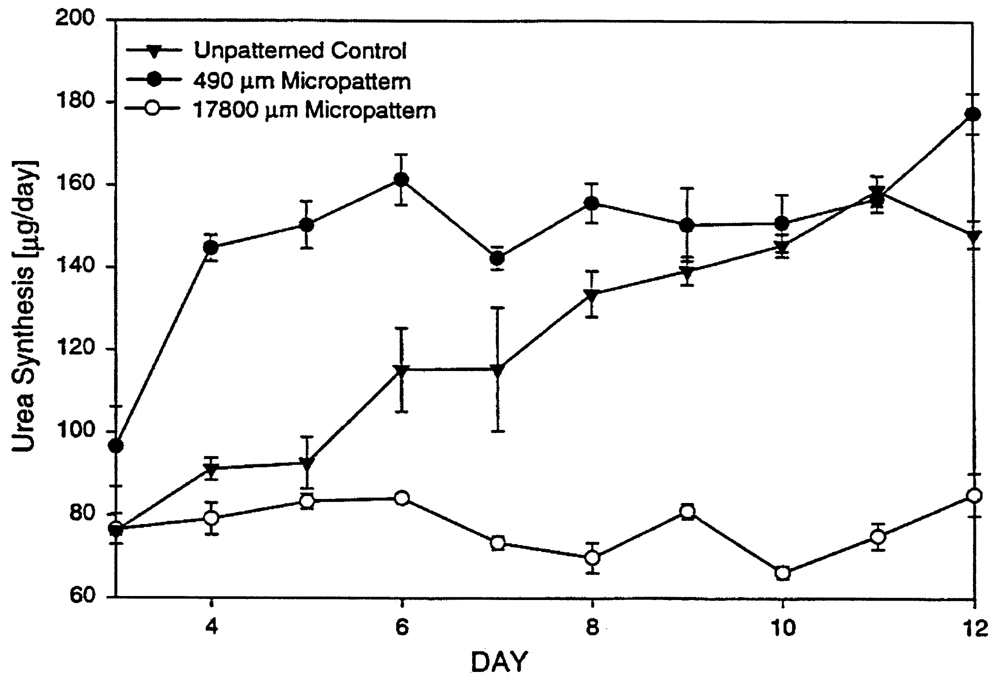

Figure 2. Urea synthesis in unpatterned and micropatterned co-culture configurations. Unpatterned controls contained randomly-distributed hepatocytes and fibroblasts whereas micropatterned co-cultures contained approximately equal numbers of both cell types in different spatial configurations. Hepatocyte island dimensions were 490 μm with 1230 μm center-to-center spacing and a single island of 17800 μm diameter.

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