INDUCTION OF HEPATOCYTE DIFFERENTIATION BY THE EXTRACELLULAR MATRIX AND AN RGD-CONTAINING SYNTHETIC PEPTIDE

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ABSTRACT

To design novel biomaterials for hepatocyte transplantation it will be necessary to determine whether specific extracellular matrix (ECM) molecule(s) or the adhesive interactions between the surface and hepatocytes are responsible for regulation of hepatocyte function. Purified ECM molecules (laminin, fibronectin, types I and IV collagen) and a synthetic peptide containing the arginine-glycine-aspartate (RGD) cell-binding sequence were pre-coated at defined densities to non-adhesive polystyrene dishes. Hepatocytes cultured on dishes coated with a low density of ECM molecules (1 ng/cm^2) maintained a round morphology, and high liver-specific protein secretion rates. In contrast, culturing hepatocytes on increasing ECM densities (50-1000 ng/cm^2) resulted in extensive cell spreading, a loss of liver-specific protein secretion, and cell growth. Hepatocytes cultured on dishes coated with the RGD-containing peptide did not spread even on a high density of the peptide (10,000 ng/cm^2), and albumin secretion remained high for hepatocytes cultured on all peptide densities (1-10,000 ng/cm^2). These results suggest that a variety of ECM molecules and synthetic peptides are capable of inducing hepatocyte differentiation *in vitro*, and these effects depend on their ability to promote cell spreading.

INTRODUCTION

One proposed therapy to replace lost or deficient tissue function is to engineer new tissue by transplanting selected cell populations attached to a polymer scaffold [1]. However, to induce the proper function in the transplanted cells it will be necessary to understand how the attachment matrix controls cell function, and to design the biomaterial appropriately. Hepatocytes are an ideal cell type to study this question as there is a great deal of interest in utilizing them in tissue engineering [2], and both their growth and expression of liver-specific functions are very responsive to the attachment surface [3].

Hepatocyte viability and function *in vitro* appear to largely depend on the adhesive interactions between the cells and naturally occurring extracellular matrix (ECM) molecules [3]. However, these molecules present difficulties for large-scale use. They must be isolated from human or animal tissue, and thus are quite expensive and suffer from large batch to batch variations. In addition, they are often not amenable to processing, and typically have poor structural properties. If the mechanisms by which ECM molecules control cell function can be determined, it may be possible to design synthetic analogues to these molecules. These synthetic analogues could be coated onto polymer scaffolds, or covalently bound to polymer scaffolds (i.e., utilizing reactive side-chains of the polymer) for use in cell transplantation.

Past studies have indicated that specific ECM molecules promote hepatocyte growth, while others promote liver-specific function [4,5]. The concept that specific chemical structures exert particular effects on cell function is supported by the identification of cell surface receptors that recognize and bind specific amino acid sequences present in ECM molecules [6]. However, other studies have suggested that specific regulation is

dependent on the mechanical properties (malleability) of the attachment matrix [7]. It has been previously hypothesized that the biomechanics of the cell adhesion to the attachment surface control cell spreading and regulate endothelial cell function [8].

In this study we have developed an experimental system to independently alter the specific ECM molecule utilized for cell attachment, and the ability of the attachment surface to promote hepatocyte spreading. We have found that a variety of ECM molecules control hepatocyte liver-specific function and growth in a similar manner, and this regulation is based on the ability of these molecules to promote hepatocyte spreading. Parallel studies with a synthetic peptide containing the arginine-glycine-aspartate (RGD) amino acid sequence, a common cell-binding sequence of ECM molecules, support these findings and further indicate that synthetic peptides are capable of inducing a high level of tissue-specific function in cultured hepatocytes.

MATERIALS AND METHODS

Hepatocyte Culture

Collagenase perfusion of adult Wistar rats was used to obtain liver cells [9], and a purified hepatocyte population was obtained by utilizing a Percoll gradient technique [10]. Hepatocytes were cultured in William's E medium (Gibco, Grand Island, NY) supplemented with insulin (20 mU/ml), epidermal growth factor (10 ng/ml), dexamethosone (5 nM), sodium pyruvate (20 mM), ascorbic acid (50 ug/ml), and penicillin/streptomycin (100 U/ml).

Hepatocytes were cultured on non-adhesive polystyrene dishes (Becton Dickinson, Lincoln Park, NJ) that were pre-coated with a defined density of purified ECM molecules with a carbonate buffer adsorption technique [11]. Previous quantitation of protein adsorption confirmed that this method yields surfaces with defined and quantitatively reproducible ECM molecular densities [11, 12]. In this study, laminin (LM - Collaborative Research), fibronectin (FN - Cappel, Malvern PA), type I collagen (Collagen Corp., Palo Alto, CA), type IV collagen (Collaborative Research), and a 2000 molecular weight peptide containing the RGD sequence (Telios Inc.) were applied at 1, 50 and 1000 ng/cm^2 (RGD peptide applied at 1, 50, and 10,000 ng/cm^2). Throughout this manuscript ECM densities will be reported in mass densities instead of molecular densities as this format is typically followed in the hepatocyte literature, and will thus make the findings of this report easier to compare to earlier studies. Hepatoctyes were plated at densities of 5, 7.5 and 10 X 10^3 cells/cm^2 on plates of low, moderate, and high ECM density, respectively, to obtain similar densities of adherent cells. Hepatocytes were plated at a density of 10 X 10^3 for all experiments utilizing RGD coated plates.

Morphological Analysis

Photomicrographs of glutaraldehyde-fixed cells were recorded on Kodak Pan-X film. Cell spreading was quantitated by staining fixed cells with Coomassie brilliant blue, and measuring projected cell areas as previously described [11]. A minimum of 30 randomly selected cells were used for each measurement.

DNA Synthesis

DNA synthesis was measured by ^{3}H-thymidine autoradiography. Hepatocytes were fed with medium containing ^{3}H-thymidine (1 μCi/ml, NEN, Boston, MA) 48 hours after plating, and glutaraldehyde fixed 18 hr. later. Seven days after being overlaid with Kodak NTB-2 emulsion, the plates were developed with Kodak D-19 developer. The percentage of cells actively synthesizing DNA, and thus entering S phase of the cell cycle, was determined by analyzing a minimum of 80 cells per experimental condition.

Albumin Secretion

Secretion of albumin from cultured hepatocytes was measured using a quantitative dot-blot assay. Media samples were collected over a 24 hr. period, serially diluted, and loaded onto nitrocellulose paper (0.1 μm pore size; Schleicher and Schuell, Keene, NH) using a 96 well minifold apparatus (Schleicher and Schuell). Serial dilutions of rat albumin (Cappel) were loaded in parallel on each blot. Blotted proteins were exposed to rabbit antibodies directed against rat albumin (Cappel) for 1 hr at room temperature, followed by exposure to ^{125}I-conjugated donkey anti-rabbit Fab^2 (Amersham; Arlington Heights, IL). The individual dots were then cut out and counted in a gamma counter. The amount of secreted albumin in the medium was determined by comparison with values from the linear portion of the calibration curve calculated using the albumin protein standard. Albumin secretion was normalized for cell number by counting the cells in the dish from which the sample was taken. Quantitation of cell number was achieved by exposing the cells to a trypsin-EDTA solution, and counting the released cells with a coulter counter.

RESULTS

Control of Hepatocyte Spreading and Growth by ECM Density

To determine if the extent of hepatocyte spreading could be controlled independently of the particular ECM molecule utilized for attachment, hepatocytes were plated onto dishes coated with varying densities of laminin (LM), fibronectin (FN), type I collagen (CI), and type IV collagen (CIV). These molecules are naturally occurring ECM molecules present in a variety of tissues. The efficiency of hepatocyte adhesion varied from 25 % on dishes coated with a low density of ECM molecules (1 ng/cm^2), to 55 % on dishes coated with a high density of the ECM molecules (1000 ng/cm^2). Thus, the differing cell plating densities yielded equivalent densities of adherent cells under all experimental conditions (approximately 3000 cells/cm^2). Dishes that did not contain pre-adsorbed ECM molecules did not support significant hepatocyte adhesion, indicating that hepatocytes adhered only to the adsorbed ECM molecules, and did not adhere in a non-specific manner. The attachment efficiencies observed here are typical for primary hepatocytes, and the fact that less than 100% of the cells attached to even the dishes coated with a high ECM density may reflect damage to cell-surface receptors or other cell components during the isolation procedure.

Inhibition of cell spreading was found when a low density (1 ng/cm^2) of any of these molecules was utilized. In contrast, hepatocyte spread and achieved an epithelial-like morphology when the ECM density was raised to a high density (1000 ng/cm^2), independently of the particular ECM molecule utilized for cell adhesion. Quantitation of projected cell area with computerized image analysis confirmed that hepatocyte spreading increased in direct proportion as the ECM coating density was raised (shown in Fig. 1a for hepatocytes cultured on plates coated with increasing densities of LM), until a plateau was reached at a saturating ECM density. Thus, the extent of cell spreading could be varied independently of the particular ECM molecule utilized for cell attachment.

Furthermore, this control over hepatocyte spreading was paralleled by a similar regulation of cell growth. The percentage of cells entering S phase of the cell cycle (as measured by ^{3}H-autoradiography) increased in proportion to the LM coating density on the culture dishes (Fig. 2b) until once again, a saturating level of LM was reached. A similar relation has been found for the other four ECM molecules, and analysis of cell number over 4 days in culture confirmed that those cells entering S phase of the cell cycle did go on to divide (not shown). Thus, the ability of ECM coated surfaces to promote hepatocyte growth depends on their regulation of the extent of hepatocyte spreading, not the presence of specific ECM molecules.

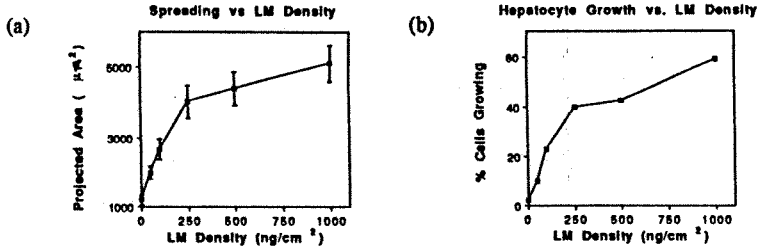

Figure 1. Parallel control over hepatocyte spreading and growth by the density of adsorbed LM on dishes used for cell culture. (a) The extent of hepatocyte spreading increased in an approximately linear fashion with increasing densities of adsorbed LM until a saturating LM density is obtained. (b) The percentage of hepatocytes entering S phase of the cell cycle, and thus stimulated to grow, similarly increased with the LM coating density until a saturating LM density is again reached.

Control of Liver-Specific Function by the ECM

Secretion of albumin, a major liver product, was then measured to determine the liver-specific function of hepatocytes cultured on dishes coated with the varying densities of ECM molecules. A high secretion rate was found for hepatocytes cultured on dishes coated with a low density of ECM molecules. However, down regulation of albumin secretion was found as the ECM coating density was raised, and hepatocyte spreading promoted, regardless of the ECM molecule utilized for hepatocyte adhesion (Fig. 2). When these experiments were continued for eight days the albumin secretion from hepatocytes cultured on a high LM density decreased to 53 ± 4 ng. albumin/1000 cells/24 hr. In contrast, hepatocytes cultured on the low LM density showed an increase of albumin secretion during this period, with a peak secretion rate of 277 ± 24 ng. albumin/1000 cells/24 hr.

Utilization of a Synthetic, RGD-containing Peptide for Hepatocyte Adhesion

These studies were then further extended to dishes coated with a 2000 dalton synthetic peptide containing the RGD cell-binding sequence common to many ECM molecules. Hepatocytes attached to the RGD coated surfaces with an efficiency of 32 ± 3 %, independently of the RGD density. Cell adhesion to the RGD coated plates was apparently due to a specific binding interaction between hepatocytes and the RGD peptide, as soluble RGD (a GRDGSP peptide was used) could effectively inhibit cell adhesion and spreading on plates coated with the bound RGD peptide (not shown). Interestingly, while surfaces coated with varying densities of the RGD peptide (1 - 10,000 ng/cm^2) promoted hepatocyte adhesion, they did not promote extensive hepatocyte spreading. The extent of

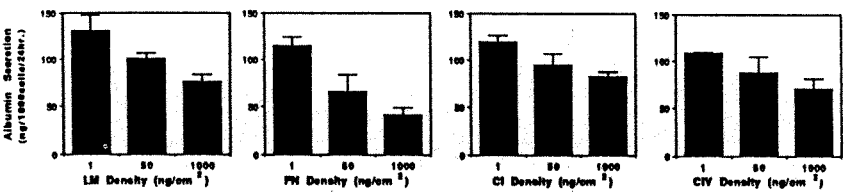

Figure 2. The rate of albumin secretion at day 2 was controlled by varying the density of ECM molecules, independently of the particular ECM molecule utilized for cell adhesion.

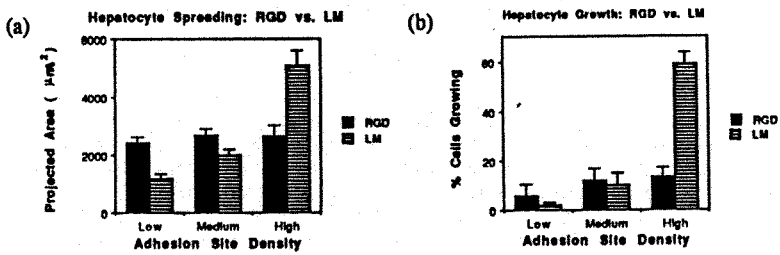

Figure 3. Both hepatocyte spreading (a) and growth (b) was constant on dishes coated with varying densities of the RGD-containing peptide, in contrast to hepatocytes cultured on varying LM densities. Low adhesion site density was 1 ng/cm^2, and medium was 50 ng/cm^2 for both molecules. High adhesion site density was 1000 ng/cm^2 for LM, and 10,000 ng/cm^2 for RGD.

spreading on these surfaces was similar to that found on an intermediate LM density (50 ng/cm^2) (Fig. 3a), even though the different molecular weights of LM and the RGD peptide result in approximately 100X higher densities of adhesion sites for the RGD coated surface with the same weight density as a LM coated dish. The constant cell adhesion efficiency and cell spreading with the varying RGD densities indicate that a saturating density of adhesion sites is present even at the lowest RGD density.

The growth of hepatocytes cultured on these RGD-coated surfaces was found to be relatively low, as one would expect from their inability to promote hepatocyte spreading (Figure 3b). Furthermore, hepatocytes maintained a high level of albumin secretion when cultured on RGD coated plates. At day 2 the secretion rate from hepatocytes on all RGD densities is similar to that found on ECM coated dishes which promote a similar extent of cell spreading, and this rate continued to increase through the eight days of the experiment.

CONCLUSIONS

In this study we have independently varied the ability of the cell adhesion surface to promote hepatocyte spreading, as well as the specific ECM molecule utilized for adhesion. Sparse and constant cell culture densities were utilized under all experimental conditions to minimize cell-cell adhesion, and focus on the role of the attachment matrix in regulating hepatocyte function. We have found that all four ECM molecules utilized in this study control hepatocyte spreading, growth, and liver-specific function. The regulation of hepatocyte gene expression by the ECM appears to be related to the ability of the ECM molecules to promote hepatocyte spreading, which in these studies was controlled by varying the ECM molecule density on the attachment surface. Studies utilizing an RGD containing synthetic peptide for hepatocyte adhesion support these findings, as dishes coated with the RGD peptide do not promote extensive hepatocyte spreading or growth, but do promote a high level of liver-specific function.

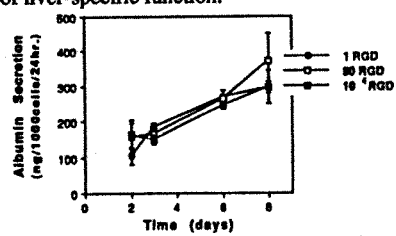

Figure 4. The albumin secretion rate was high for hepatocytes cultured on all 3 RGD densities, and increased over the 8 days of the experiment

While these results indicate that ECM molecules (and synthetic peptides) regulate hepatocyte function based on their ability to promote or prohibit hepatocyte spreading, they do not rule out other explanations. For example, clustering of cell-surface ECM receptors may be effected by the ECM density [12], or the varying ECM densities may differentially present growth factors to the cell [13]. However, these explanations are not likely to explain the findings presented here, as large-scale changes in cell shape have been found to be required for cells to progress through the cell cycle [11]. If alterations of cellular morphology are indeed responsible for the alterations of cellular function, these effects may be mediated by the cellular cytoskeleton [14]. The alterations of cytoskeletal organization and polymerization which accompany cell shape changes may effect cell function by alterating nuclear structure or the cytoplasmic organelles physically connected to the cytoskeleton, by changing the availability or number of cell-surface hormone receptors, or by a variety of other mechanisms.

These findings indicate that to design biomaterials for use in tissue engineering it will be necessary to consider both the chemical composition of the surface, and the biomechanics of the cell-surface adhesion. In addition, they suggest that it may be possible to construct completely synthetic polymer scaffolds that will promote specific cell functions. In the present study we have determined that a synthetic, RGD-containing peptide is capable of inducing liver-specific function in cultured hepatocytes. As the structure and function of ECM molecules becomes better understood it will be possible to synthesize different peptide sequences of these molecules. Eventually, one will be able to choose specific peptide sequences which are capable of inducing cell spreading and growth, and others which suppress growth and promote tissue-specific function.

ACKNOWLEDGEMENTS

The authors wish to thank Drs. Aiken, Gilbert, Johnson and Stein for their help with hepatocyte isolations. This work was supported by a grant from Neomorphics Inc., and the Whitaker foundation.

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