Improved performance of primary rat hepatocytes on blended natural polymers

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Abstract: Alginate (Alg), chitosan (Chi), collagen (Col), gelatin (Gel), and the mixtures of every two of them were screened to determine their suitability for hepatocyte culture. The test materials were fabricated as films and then evaluated on the basis of their abilities to promote the attachment and functions of rat hepatocytes cultured on them. Cellular attachment on Col and Gel was favorable. However, cellular viability, cytoskeleton organization and function, as evaluated by albumin production, ureagenesis, and enzyme activity of cytochrome P450 as well as expression levels of hepatocyte nuclear factor 4α deteriorated. Reverse cellular behavior was observed on Alg and Chi. Two blends, composed of Chi and Col or Gel, were found to be superior to other materials and sustained viability and differentiated functions of hepatocyte. © 2005 Wiley Periodicals, Inc. J Biomed Mater Res 75A: 268–274, 2005

Key words: alginate; chitosan; collagen; gelatin; hepatocyte

INTRODUCTION

The development of tissue-engineered hepatic devices requires the selection of the appropriate biomaterials for cultivation of hepatocytes. To reproduce the characteristics of the hepatic tissue, these materials should help to maintain the number, viability, and function of hepatocytes.[1] Compared with synthetic polymers, natural polymers attract much attention because of their good biocompatibility. Desirable biomaterials include specific interaction with, or mimicry of, extracellular matrix (ECM) components, growth factors, or cell-surface receptors; however, none of natural materials can cover all of these features.[2]

Cellular functions are controlled by components in ECM,[3] ECM density,[4] and the mechanical determinant of the substrate surface.[5] ECM molecules can influence cellular shape, cytoskeleton organization, and transcription activity by conveying mechanical and chemical stimuli.[6,7] In an ECM environment rich in glycosaminoglycans, collagen (Col), fibronectin, and elastin, tissue repair occurs naturally.[8] Hepatocyte activities *in vitro* are often limited by the lack of the liver specificity of the environment where they are cultured. Natural polymer usually contains a distinct ECM component or its analog, which interacts with specific cell-surface receptors. Hepatocytic function expression may depend on a collective interaction of cell receptors binding to extracellular ligands.[9] Therefore, it may be facilitated for hepatocytic survival by blending different natural polymers to create an optimal extracellular environment. Because different polymers possess respective chemical properties, their blending may result in a mixture with improved strength or physiological characteristics. This is conducive to developing a suitable substrate for optimal hepatocyte attachment and functional maintenance.

With this purpose, and, in order to study the hepatocytic compatibility of single or blended natural polymers as possible selected biomaterials for liver tissue engineering, we have evaluated *in vitro* the capabilities of interesting polymers to support the survival of rat hepatocytes. Four polymers, namely, alginate (Alg), chitosan (Chi), Col, and gelatin (Gel), were chosen to represent conventional scaffold materials. The former two are polysaccharides, and the later two are polypeptides. Although previous investigators have

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examined the applicability of these polymers for hepatocyte culture, they seldom made comparisons among these materials or tested their combinations.

MATERIALS AND METHODS

Materials

Alg and fluorescein diacetate (FDA) were purchased from ICN Biomedical Inc. Chi (relative molecular mass ~400,000) and Gel (pH 5.0, 180 Bloom) were from Fluka. Collagenase type IV and Dulbecco's modified Eagle medium were from Gibco BRL. Lactate dehydrogenase (LDH) kit, rat albumin enzyme-linked immunosorbent assay kit, and urea UV detection kit were from Boehringer Mannheim, Bethyl Laboratories, and Centronic GmbH, respectively. Anti-hepatocyte nuclear factor 4α (HNF4α) polyclonal antibody (α445 antibody) was provided by Sladek FM (University of California, Riverside, CA). SuperSignal West Pico Chemiluminescent Substrate was from Pierce. All other materials used were from Sigma unless otherwise specified.

Preparation of films

Col was extracted from rat tail tendon by the procedure of Dunn et al.[10] and its final concentration was adjusted to 0.1% (w/v) in 1 m*M* HCl. Alg and Gel were dissolved in double-distilled water except Chi in 20 m*M* acetate acid. Material blending was performed by mixing every two components at 1:1 ratio under mechanical stirring. For the fabrication of films, 0.1% (w/v) solution was poured into 24-well culture plates, Petri dishes, or coverslips and then allowed to incubate at room temperature for 1 h. The surplus fluid was discarded and culture ware was dried in air. The films were then crosslinked by immersion into 0.2% (v/v) glutaraldehyde for Chi and polypeptides or by 20 m*M* $CaCl_2$ for Alg. The crosslinking was developed for 5 h and nonreacted aldehyde groups were blocked for 1 h by 0.1*M* glycine. The acidity of the film was neutralized for 30 min by adding 1*N* NaOH solution. The films were repeatedly washed with Hanks balanced salt solution until the film pH returned to a physiologic range. All material membranes were incubated in the culture medium for 30 min before cell culture.

Isolation and culture of hepatocytes

Primary rat hepatocytes were obtained by collagenase perfusion of adult Sprague-Dawley rat liver,[11] followed by purification through a Percoll gradient.[10] Viability of the freshly isolated hepatocytes was 92–98% as determined by trypan blue exclusion assay. Nonparenchymal cell contamination was <1% as judged by their size and morphology. Hepatocytes were inoculated at 7×10^4 cells/cm^2 in a chemically defined serum-free medium.

Cell-adhesion assay

After hepatocytes were inoculated onto all sorts of material films, attachment was allowed to proceed for 6 h. The plates were then washed twice with phosphate-buffered saline (PBS), and the cell adhesion was quantified based on the DNA content of the attached hepatocytes.[12] The attached hepatocytes were lysed by incubation with 0.1*M* phosphate buffer (pH 7.0) containing 0.5% Triton X-100. The lysis solution was collected and centrifuged at 10,000*g* for 10 min. The supernatant was analyzed to quantify DNA by Hoechst dye 33258. Standard DNA solutions were prepared by a serial dilution of calf thymus DNA.

Viability studies

An *in situ* fluorescence viability assay with FDA/EB was performed to assess the cell viability on the membrane surfaces.[13] The final concentration of FDA and EB were 5 and 20 μg/mL in PBS, respectively. After 12 h of culture, the hydrogel films were rinsed with PBS twice to remove unattached cells, and then immersed in the FDA/EB solution for 5 min at room temperature and then washed in PBS for 5 min. Fluorescence in the cells was examined and photographed under a fluorescent microscope. These images were "merged" with matching fluorescence images in order to visualize the numbers of responding cells. A quantitative assessment of cell viability was conducted by measuring the LDH activity in cell culture supernatants.

Hepatocytic functional assays

After the inoculation of hepatocytes for 6 h, medium was refreshed to remove unattached cells. The 24-h supernatant from different cultures was analyzed for albumin and urea content using the kits according to manufacturer's guidance. Cytochrome P450-dependent 7-ethoxyresorufin *O*-deethylase (EROD) and 7-ethoxycoumarin *O*-deethylase (ECOD) activity assays were measured by the reported procedures.[14,15] The DNA content of cultured hepatocytes was determined with a fluorometric DNA assay.[12]

Fluorescence staining of cytoskeleton

Hepatocytes cultured on coated coverslips were fixed using 2% paraformaldehyde in PBS for 30 min followed by permeabilization for 15 min in 0.1% Triton X-100. The cells were then washed and held in PBS until stained. Microtubule staining was conducted for 30 min with mouse anti-α-tubulin monoclonal antibody (F-2168, Sigma) in PBS and washed twice in PBS for 30 min. For localization of F-actin, the anti-actin antibody (C-5838, Sigma) was used. After addition of secondary antibody, the coverslips were washed four times in PBS (15 min each) and then overnight in PBS. After staining, to minimize quenching of fluorescence, a

drop of 10 mg/mL *n*-propyl gallate (in a mixture of glycerol and 10 m*M* Tris buffer, pH 8.5) was added to each coverslip before placing it on a glass slide and sealing with nail polish. The fluorescence staining was imaged using confocal laser scanning microscopy.

Western blotting

Hepatocytes on material films were digested and solubilized in lysis buffer containing 1% protease inhibitor cocktail. The samples were adjusted to equal protein concentration, diluted in equal volumes of 2× sample buffer, and boiled for 5 min. Samples were run on a 10% sodium dodecyl sulfate–polyacrylamide gel, and proteins were transferred to nitrocellulose membrane in 25 m*M* tris, 192 m*M* glycine, pH 8.3, containing 20% methanol, using a Bio-Rad Transblot Apparatus at 100 V for 2 h. The filter was blocked in 5% nonfat milk for 1 h followed by incubation overnight at 4°C with α445 antibody diluted in Tris-buffered saline. The antibody was detected using a horseradish peroxidase-labeled anti-rabbit immunoglobulin G antibody. Peroxidase activity was detected by SuperSignal substrate.

RESULTS

Compatibility of materials

The aqueous solution of Alg was incompatible with the acid solution of Chi. The two solutions formed colloidal complex when they were mixed, as reported by Polk et al.[16] The mixing of Alg and Col resulted in a fibrous complex. A trial to dissolve the complex anew by down-adjusting pH failed. The two complexes were hard to prepare as even films on the surface of culture ware. All the other mixing gave rise to homogeneous solutions.

Cellular attachment and morphology on different materials

When inoculated onto Col and Gel films, hepatocytes attached on them within 3–4 h; however, the attachment of hepatocytes on Alg and Chi occurred at 1–2 h later. The attachment rate of hepatocytes on all tested materials is illustrated in Figure 1. The Gel film was the most adhesive substrate for hepatocytes with an attachment efficiency of 80%. The number of hepatocytes adhered to Alg was less. Hepatocytes sparsely adhered to Chi but cell adherence was improved significantly when Col or Gel was introduced to it; nevertheless, introduction of Col or Gel to Alg did not acquire similar results. Hepatocytes spread more extensively on films of polypeptide than on polysaccharide. Typical

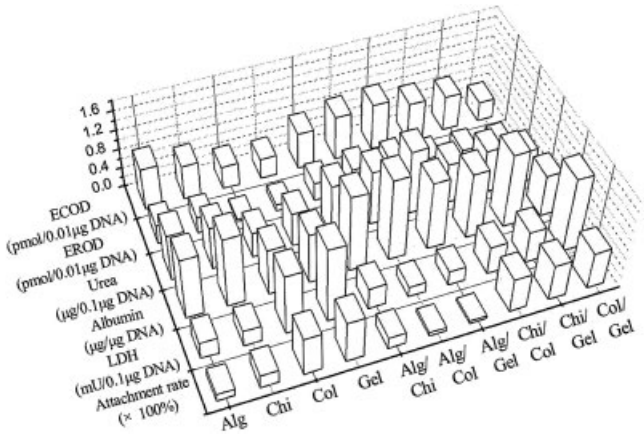

Figure 1. Attachment rate, LDH release, and functions of primary rat hepatocytes cultured on various material films. Attachment-rate determination was conducted after inoculation of the cells for 6 h. LDH release and function assays were conducted in the 24-h supernatant from different cultures.

hepatocyte diameter in suspension is about 20 μm, whereas upon attachment and spreading on Col and Gel, cell diameters increase to 30–40 μm. This morphology was in contrast to cells cultured on Chi membrane, which was mostly cubical (Fig. 2). Most of cells cultured on Alg-based membrane remained single cells without extension, and had a higher tendency to detach from the membrane surface.

Hepatocyte viability

Hepatocyte viability is compared in Figure 3. The number of live cells on polysaccharides or their mixtures, indicated by green staining, was far more than those on polypeptides. The cells on polypeptides contained many dying cells, which were stained red by EB. Incorporation of Col or Gel to Alg increased the viability of hepatocytes on their blends. Col or Gel blending with Chi did not significantly change the viability of hepatocytes on the two mixtures. The viability of the hepatocytes was also reflected qualitatively by marked differences in the LDH release as shown in Figure 1.

Cytoskeleton organization

Microtubules and actin microfilaments have been shown to have an important role in both the structural and functional integrity of hepatocytes.[17,18] To demonstrate the effect of different materials on cytoskeleton organization, the distribution of intracellular microtubule and actin filament were localized by anti-tubulin and anti-actin antibodies, respectively. Anti-tubulin staining revealed amorphous fluores-

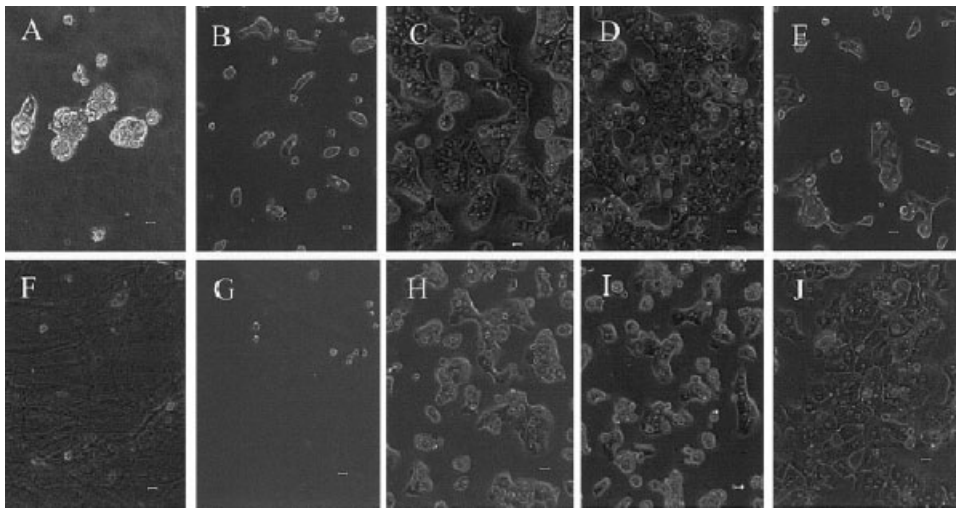

Figure 2. Phase-contrast micrographs of primary rat hepatocyte cultures on (A) Alg, (B) Chi, (C) Col, (D) Gel, (E) Alg/Chi, (F) Alg/Col, (G) Alg/Gel, (H) Chi/Col, (I) Chi/Gel, and (J) Col/Gel films after 36 h of culture. Bar = 20 μm.

cence filling the cytoplasm of hepatocytes cultured on Alg and Alg/Gel, presumably because of the presence of tightly packed microtubules. This indicated that there was no reorganization of microtubules. In the hepatocytes on Alg/Chi and Chi, the majority of microtubules nucleated from a centrosome or microtubule-organizing center that is located next to the nucleus in nonpolarized hepatocytes. A few individual microtubules were also visible at the cell border together with a sparse network close to the basolateral membrane, whereas in the hepatocytes on rest materials, a highly extended array of individual microtubules appeared, extending radially from the perinuclear area to the cell periphery (Fig. 4).

Labeling of actin in hepatocytes cultured on Alg and Alg/Gel revealed homogeneous staining. This staining pattern was presumably the result of a high density of actin fibers in the round cells. On Alg/Chi and Chi films, hepatocytes exhibited a marginal actin band. The conspicuous actin structure was the circular compact bundle along the outer edge of cells, especially at lateral intercellular contacts. Actin in hepatocytes on Col and Gel was evenly distributed in cytoplasm and less peripheral staining of actin was observed (Fig. 5).

Polymer effects on liver-specific function

Functions of hepatocytes cultured on various substrates were assessed by their albumin and urea synthesis level as well as cytochrome P450 biotransforma-

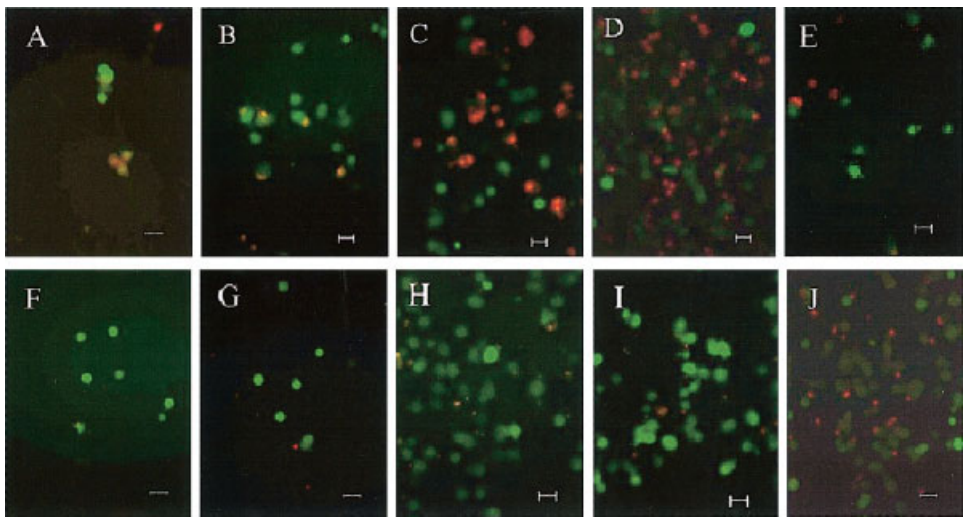

Figure 3. Merged images of the staining for FDA/EB in hepatocytes cultured for 12 h on (A) Alg, (B) Chi, (C) Col, (D) Gel, (E) Alg/Chi, (F) Alg/Col, (G) Alg/Gel, (H) Chi/Col, (I) Chi/Gel, and (J) Col/Gel films. Bar = 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

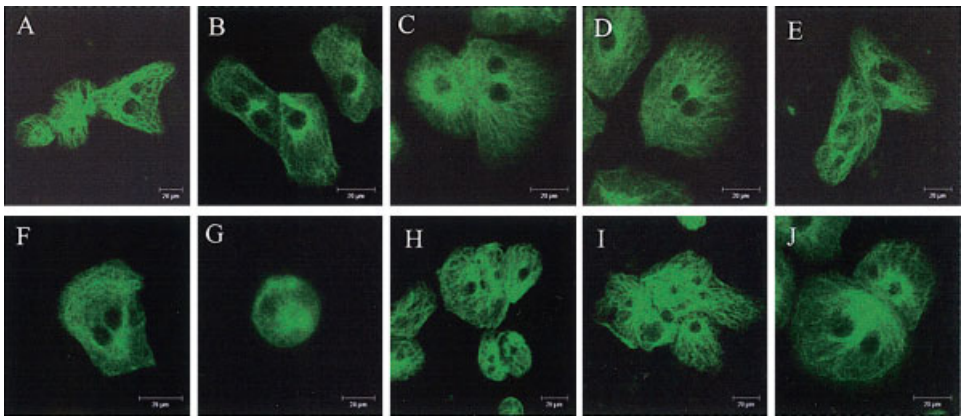

Figure 4. Immunofluorescence microscopic view of the staining for α-tubulin in hepatocytes cultured for 36 h on (A) Alg, (B) Chi, (C) Col, (D) Gel, (E) Alg/Chi, (F) Alg/Col, (G) Alg/Gel, (H) Chi/Col, (I) Chi/Gel, and (J) Col/Gel films. Bar = 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

tion function. The effects of material formulation on metabolic activity in primary cultures of rat hepatocytes are summarized in Figure 1. In general, hepatocytes cultured on polysaccharide-based materials exhibited higher functions than those on polypeptide-based materials. The constituent of substratum affected albumin synthesis least. Urea genesis and biotransformation function, as indicated by EROD and ECOD activity, displayed bigger fluctuation corresponding to different materials. Whatever hepatocytic function is concerned, Chi/Col and Chi/Gel cultures yielded slightly lower and comparable level as compared with pure Chi, which suggests that polypeptide incorporation does not impair the characteristics of Chi.

HNF4α controls transcription of genes that are preferentially expressed in liver. HNF4α target genes include enzymes involved in lipid, amino acid, and glucose metabolism.[19] To further disclose the transcriptional mechanism behind hepatocytic phenotype, a Western blot was performed to quantify the HNF4α level. As shown in Figure 6, the content of HNF4α proteins in the hepatocytes on Chi/Col and Chi/Gel was slightly lower than that on Alg-based materials, but was markedly higher than that on simple Col, Gel, and their mixture.

DISCUSSION

The data presented above together suggest that Alg and Chi are apt to sustain hepatocytic functions, but they are poor substrata for hepatocyte attachment. Col and Gel are excellent matrices for hepatocyte attachment, but cellular differentiation functions suffered from damage caused by excess spreading. Chi combination with either Col or Gel yielded hepatocytic compatible composite materials through simple blending. The Chi/Col and Chi/Gel materials are capable of maintaining both hepatocytic number and functions,

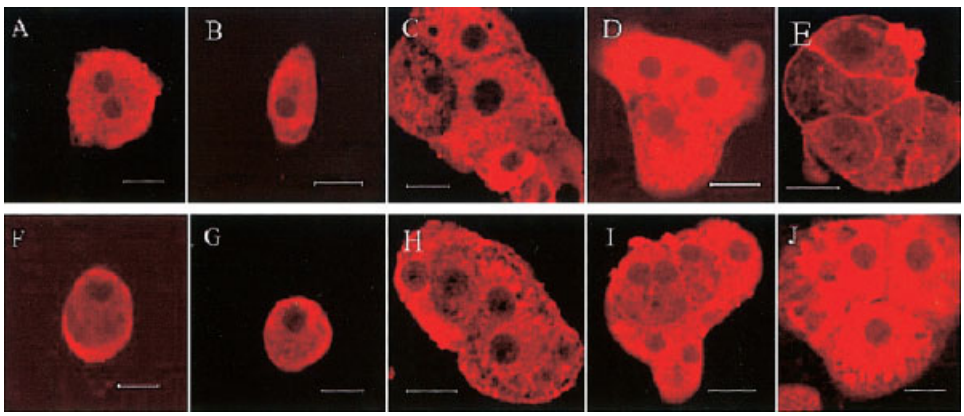

Figure 5. Immunofluorescence microscopic view of the staining for actin in hepatocytes cultured for 36 h on (A) Alg, (B) Chi, (C) Col, (D) Gel, (E) Alg/Chi, (F) Alg/Col, (G) Alg/Gel, (H) Chi/Col, (I) Chi/Gel, and (J) Col/Gel films. Bar = 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

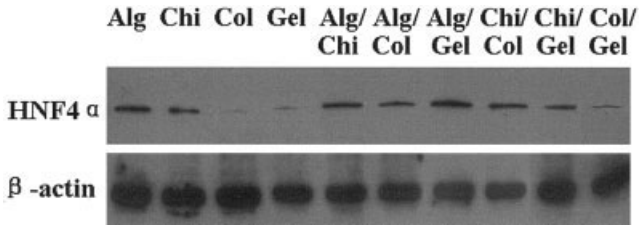

Figure 6. HNF4α protein levels in hepatocytes on various material films. Cell lysates were collected and processed at 24 h after inoculation. The whole cellular protein was electrophoresed on 10% sodium dodecyl sulfate–polyacrylamide gel. Western blot was performed using α445 antibody. β-Actin was used as a lane-loading control.

which are essential for the efficiency of tissue-engineered hepatic devices.

The molecular components of ECM are physically interconnected with proteins of cytoskeleton via transmembrane receptors such as integrins.[20] These mechanical interactions seem important in determining cell shape and cytoskeleton configuration.[21] Because they contain different ECM components or their analogs, Alg, Chi, Col, Gel, and their mixtures possess different densities of simplex ECM and mechanical characteristics. It has been reported that cell shape may act to regulate cell functions.[22,23] Hepatocytes exhibiting a flattened morphology possess lower differentiated activity than cells maintained in a more spherical morphology. Rounded cells on Matrigel were able to retain extended cytochrome P450 and other liver-specific gene expressions compared with flattened cells plated on Col.[24] The variation of cellular shape is derived from the difference of cytoskeleton. There exists an inverse relationship between cytoskeletal and liver-specific protein expression.[25] The importance of the cytoskeleton and cell shape for control of cell function was also confirmed by Mooney et al.[26] They found that a brief exposure of hepatocytes to nocodazole resulted in both decreased cell size and enhanced production of a liver-specific product many hours later.

The biophysical nature and composition of ECM have been consistently shown to have significant effects on hepatic specific function expression and induction *in vitro*.[25,27] ECM, especially Col and proteoglycans, greatly influenced hepatocyte growth and the stability of mRNA for expression of liver-specific functions.[28] Woods et al.[29] reported that a proteoglycan-binding substratum exhibits a reduced ability to support initial cell attachment and spreading, fewer cells attach, and cell spreading and organization of an actin cytoskeleton are impaired when compared with the same cells on an integrin-binding substratum. Cells easily attach and spread on a substratum made of an integrin-binding fragment of fibronectin, whereas they hardly attach and spread on a proteoglycan-binding fragment. Moreover, cells on either of these substrata did not form a well-organized actin cytoskeleton or focal adhesion. However, cells on a mixed substratum of the two fragments formed an organized actin cytoskeleton and focal adhesion. Hepatocytic functions could thus be manipulated by the overall ECM composition.

The blending of different materials may change the density of material ingredient. A low density of ECM supported hepatocyte attachment, but did not promote cell spreading or growth. Round hepatocytes on low density of ECM maintained increased secretion rates for multiple liver-specific proteins. The regulatory signals conveyed by contained ECM molecules depend on the density at which they are presented and, thus, on their ability to either prohibit or promote cell spreading.[30] In general, higher levels of liver-specific functions were exhibited by hepatocytes that were prevented from extending by modulating cell-ECM binding. We conclude that compatibility of substrate materials, complexity of ECM kinds, and appropriate density of ECM, all contribute to the maintenance of hepatocytic functions.

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