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Adipocyte-derived basement membrane extract with biological activity: applications in hepatocyte functional augmentation *in vitro*

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ABSTRACT **Natural and synthetic biomaterials utilized in tissue engineering applications require a dynamic interplay of complex macromolecular compositions of hydrated extracellular matrices (ECMs) and soluble growth factors. The challenges in utilizing synthetic ECMs is the effective control of temporal and spatial complexity of multiple signal presentation, as compared to natural ECMs that possess the inherent properties of biological recognition, including presentation of receptor-binding ligands, susceptibility to cell-triggered proteolytic degradation, and remodeling. We have developed a murine preadipocyte differentiation system for generating a natural basement membrane extract (Adipogel) comprising ECM proteins (collagen IV, laminin, hyaluronan, and fibronectin) and including relevant growth factors (hepatocyte growth factor, vascular endothelial growth factor, and leukemia inhibitory factor). We have shown the effective utilization of the growth factor-enriched extracellular matrix for enhanced albumin synthesis rate of primary hepatocyte cultures for a period of 10 d as compared to collagen sandwich cultures and comparable or higher function as compared to Matrigel cultures. We have also demonstrated comparable cytochrome P450 1A1 activity for the collagen-Adipogel condition to the collagen double-gel and Matrigel culture conditions. A metabolic analysis revealed that utilization of Adipogel in primary hepatocyte cultures increased serine, glycine, threonine, alanine, tyrosine, valine, methionine, lysine, isoleucine, leucine, phenylalanine, taurine, cysteine, and glucose uptake rates to enhance hepatocyte protein synthesis as compared to collagen double-gel cultures. The demonstrated synthesis, isolation, characterization, and application of Adipogel provide immense potential for tissue engineering and regenerative medicine applications.—Sharma, N. S., Nagrath, D., Yarmush, M. L. Adipocyte-derived basement membrane extract with biological activity: applications in hepatocyte functional augmentation *in vitro*. *FASEB J.* 24, 000–000 (2010). www.fasebj.org**

Key Words: Adipogel · natural biomaterials · metabolic analysis

SYNTHETIC BIOMATERIALS AND natural extracellular matrix (ECM)-derived basement membrane (BM) complexes represent state-of-the-art tools for drug delivery, cellular engineering, and 3-dimensional scaffold generation to mimic *in vivo* tissue architecture for biomedical applications (1). The main challenges of developing an ideal biomaterial are host biocompatibility; batch-to-batch variability; ease of availability; ability to form scaffolds, powders, and gels; biodegradability; and defined ECM and growth factor composition. Both biologically derived and synthetic biomaterials have been extensively used in regenerative medicine and tissue-engineering applications that require a dynamic interplay of complex macromolecular compositions of hydrated ECMs, soluble growth factors, and protein molecules expressed by cells. Recently, cell matrix biologists and bioengineers have used natural ECM-derived biomaterials for 3-dimensional scaffold formation for cell and tissue morphogenesis, growth, migration, and differentiation (2–4). The development and utilization of methodologies to modify synthetic biomaterials such as self-assembly oligopeptide nanofibers, RGD-grafted collagen, and synthetic ECM analogs include enhancing protein-protein interactions using polymeric cross-linkers; modulation of fibrillar structures, such as kinked, wavy, or branched fibers that modify scaffold architecture; and synthetic hydrogel modulation by presentation of cell adhesion ligands, proteolytic susceptibility, and biologically relevant elasticity.

The main challenges of developing and utilizing synthetic ECMs is the effective control of dynamics and spatial organization of multiple signal presentation, as compared to natural ECMs, which possess the inherent properties of biological recognition, including presentation of receptor-binding ligands, susceptibility to cell-triggered proteolytic degradation, and remodeling implicated in tissue morphogenesis. Thus, synthetic ECM

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analogs represent oversimplified mimics of natural ECMs, lacking the spatial and temporal complexity.

Numerous techniques have been developed to isolate natural ECMs from a variety of sources, such as decellularized submucosal intestine (5), urinary bladder (6), liver (7), and skeletal muscle (8) for tissue engineering and regenerative medical applications. The novelty of development of mammalian cell-derived natural ECMs supersedes both previously established synthetic analogs and tissue-based ECMs because of various advantages, such as obviation of chemical and enzymatic procedures to isolate basement membrane extract (BME), hence obviating disruption of protein-protein interactions; ease of generating BME using a less cumbersome procedure; animal-free extraction procedures and minimal batch to batch variability; reduction of pathogen transmission; and ability to modulate the supramolecular composition of the BME utilizing various *in vitro* biochemical perturbations.

To develop a naturally cross-linked BME that meets the above criteria, there is a pressing need to develop a novel *in vitro* cell culture system that has the ability to generate substantial amounts of ECM with defined growth factor and ECM protein composition. In the present work, we have developed a novel murine preadipocyte cell differentiation system to derive a natural ECM (termed Adipogel) with growth factors, cytokines, and hormones implicated in investigating cell behavior *in vitro*. We have shown proof of concept of the cell-derived BM-like extract for culture of hepatocytes and improvement of their differentiated function in the presence of soluble Adipogel as compared to conventional methods. In addition, we have investigated the effect of the soluble matrix supplementation on hepatic metabolism.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/L glucose, fetal bovine serum (FBS), penicillin, streptomycin, and trypsin-EDTA were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Dexamethasone, isobutyl-methylxanthine, epidermal growth factor, insulin, glucagon, rosiglitazone, and hydrocortisone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Protein centrifugal filters (10- and 100-kDa cutoff) were purchased from Millipore Technologies (Billerica, MA, USA). Matrigel at high concentration was purchased from BD Biosciences (Franklin Lakes, NJ, USA).

Adipogel generation using preadipocyte differentiation

3T3-L1 murine preadipocytes purchased from American Type Culture Condition (Manassas, VA, USA) were cultured in T-175 cm^2 flasks in DMEM supplemented with 10% FBS and 2% penicillin and streptomycin until the cells attained confluency. Then, at 48 h postconfluency, the cells were differentiated in culture medium supplemented with 1 μM dexamethasone, 0.1 μM isobutyl-methylxanthine, and 1 μM rosiglitazone for 2 d with medium changes every 2 d. On the second day postdifferentiation, cells were exposed to culture medium supplemented with 10 μg/ml rosiglitazone for an additional 2 d. Medium supernatant was collected on d 2 and 4 of differentiation and stored at 4°C prior to further processing.

To purify the ECM-rich material, the differentiated preadipocyte-conditioned medium was centrifuged at 4000 *g* for 1.5 h using an Amicon 100- or 10-kDa centrifugal filter. The concentrate, primarily composed of medium constituents with molecular mass cutoff of 100 and 10 kDa, comprised the cell culture supernatant-derived protein concentrate, including ECM. About 250 μl of protein concentrate was obtained at the end of the purification step per 15 ml of conditioned medium, with a yield of ~60 fold. Because the concentrate was derived from an adipocyte-related cell type and had a gel-like configuration, it was termed "Adipogel."

Scanning electron microscopy (SEM) analysis of Adipogel

Adipogel was plated at 100 μl/well in a 96-well plate. After 24 h incubation at 37°C, Adipogel was fixed with 4% paraformaldehyde for 15 min, followed by 1 h incubation with 95% alcohol, followed by absolute alcohol. The postfixed gels formed in 96-well plates were gold/palladium coated using a Balzers SCD004 sputter-coating unit (Balzers Hochvakuum GmbH, Wiesbaden-Nordenstadt, Germany) followed by SEM imaging using an Amray 1830 I unit (SEMTech Solutions, North Billerica, MA, USA) equipped with an EDAX 9800 X-ray system (EDAX, Mahwah, NJ, USA), a Robinson backscatter detector (SPI Supplies, West Chester, PA, USA), an Acorn computer-controlled stage, and image analysis software (Acorn Technology Systems, Devan, MA, USA).

Adipogel composition determination using protein arrays

The protein composition was determined using Biotin-Labeled Antibody Arrays (Ray Biotech, Norcross, GA, USA) for simultaneous detection of 308 mouse proteins in d 4 Adipogel. Through a simple process, the diluted Adipogel samples were biotinylated and dialyzed overnight in preparation for incubation with the array. The biotinylated sample was added onto the glass slide antibody arrays and incubated at room temperature with gentle shaking. After incubation with fluorescent dye streptavidin, the signals were visualized by either chemiluminescence or fluorescence. Protein concentrate purified from FBS-supplemented basal medium was utilized as negative control. A normalization of up-regulated proteins in Adipogel *vs.* controls was performed to detect fold changes. Fold changes >1.5 were identified and categorized into different protein subtypes.

Fibronectin, collagen IV, hyaluronan, and laminin ELISA assays

Mouse fibronectin concentrations were determined using a quantitative sandwich enzyme immunoassay technique (Assay-Pro, St. Charles, MO, USA). Fibronectin in standards and samples were sandwiched by the immobilized antibody specific for fibronectin precoated on microplates and biotinylated polyclonal antibody specific for fibronectin, which was recognized by a streptavidin-peroxidase conjugate. Unbound material was then washed away, and a peroxidase enzyme substrate was added. The color development was stopped, and the intensity of the color was measured and quantified against standards.

For detection of murine collagen IV, prediluted Adipogel samples, controls, and assay standards were added to murine collagen IV-coated wells (Exocell, Philadelphia, PA, USA) followed by addition of rabbit anti-collagen IV antibody. The

antibody interacts and binds with the collagen IV immobilized to the stationary phase or with the antigen in the fluid phase, hence the notion of competitive binding. After a suitable incubation period, the plates were washed, and an anti-rabbit HRP conjugate was used to detect bound rabbit antibody. After washing, only the antibody-conjugate bound to the stationary phase remains in the well, and this is detected using a chromogenic reaction. Color intensity was inversely proportional to the logarithm of collagen IV in the fluid phase.

Mouse laminin was determined by the Laminin enzyme-linked immunosorbent assay (ELISA) kit (Insight Genomics, San Diego, CA, USA) as per the manufacturer's instructions. Hyaluronan was determined using a commercially available kit (Echelon Biosciences, Salt Lake City, UT, USA).

Primary rat hepatocyte isolation

Female Lewis rats (Charles River Laboratories, Wilmington, MA, USA) weighing 180–200 g (2–3 mo old) were used as a hepatocyte source and were maintained in accordance with National Research Council guidelines. Experimental protocols were approved by the Subcommittee on Animal Care, Committee on Research, Massachusetts General Hospital. Using a modification of the 2-step collagenase perfusion method (9–11), which involves purification of the cell suspension by means of centrifugation over Percoll, ~1–2 $\times$ 10^6 cells were routinely isolated from one rat with viability between 85 and 98%, as judged by Trypan blue exclusion.

Hepatocyte culture in collagen sandwich, Adipogel, and Matrigel

Type 1 collagen was prepared by extracting acid-soluble collagen from Lewis rat-tail tendons (12). To create a thin layer of collagen gel in 12-well tissue culture plates, 400 μl of an ice-cold mixture of 1 part of 10× concentrated DMEM and 9 parts of 1.25 mg/ml rat tail tendon type I collagen were evenly distributed over the bottom of each well. The plates were incubated at 37°C for 60 min to induce collagen gelation before cell seeding. Each well of the 12-well culture plates received 5 $\times$ 10^5 primary hepatocytes in suspension in 0.5 ml standard hepatocyte culture medium, which consisted of DMEM supplemented with 14 ng/ml glucagon, 7.5 μg/ml hydrocortisone, 0.5 U/ml insulin, 20 ng/ml EGF, 200 U/ml penicillin, 200 μg/ml streptomycin, and 10% FBS. Cultures were incubated in 90% air/10% CO_2 at 37°C. Cells were rinsed with 1× PBS to remove nonadherent cells 4–6 h after seeding. For the double-collagen-gel culture configuration, a second layer of 250 μl collagen was laden on top of the cells 48 h postseeding. Medium was changed every 24 h and collected from d 3 onwards until d 10. Three additional culture conditions were utilized as described below. For the Adipogel conditions, the BME isolated on d 4 was utilized for the entire set of hepatocyte experiments. For the soluble Adipogel condition, 100 μl of Adipogel was solubilized in 400 μl of culture medium by continuous pipeting. The supplemented medium was added to cell cultures on d 0, 1, 2, 5, 7, and 9 of culture. For the second condition, to form the adipocyte-derived gel, 100 μl of Adipogel was uniformly spread over each well by slow dripping along the wall. To promote gelation, the plates were incubated at 37°C for 60 min, followed by addition of culture medium. For Matrigel cultures, a single layer of 200 μl Matrigel was added to each well of a 12-well plate, as described previously (13). The coated plates were incubated for 30 min at 37°C for gelation. Following this, hepatocytes were seeded on top of the single-gel Matrigel cultures.

Hepatocyte functional assessment

Albumin concentration in the collected medium samples was analyzed using a competitive ELISA. Albumin protein and the antibody were purchased from MP Biomedicals (Solon, OH, USA). Urea concentration was determined *via* its specific reaction with diacetyl monoxime with a commercially available assay kit (Fisher Scientific, Pittsburgh, PA, USA). The absorbance was measured with a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Cytochrome P450 function was evaluated based on ethoxyresorufin 0-deethylase activity, which is chiefly catalyzed by the isoenzyme CYP4501A1, as described previously (13). The activity was measured as the rate of resorufin formation the ethoxyresorufin substrate (Invitrogen, Molecular Probes). Briefly, on d 10 of culture, 2 μM of the CYP4501A1 inducer 3-methylcholanthrene (3MC) was added to phenol red-free cell culture medium 48 h before the assay. The substrate solution (600 μl) containing 10 μM ethoxyresorufin and 90 μM dicumarol in Earle's balanced salt solution was added to each well after washing with PBS. After 5, 10, 25, and 45 min of incubation, 50 μl of substrate solution was taken from each culture well and dispensed in 2 wells of a 96-well plate. Fluorescence intensity was measured with an fMax fluorescence plate reader (excitation wavelength, 530 nm; emission wavelength, 590 nm; Molecular Devices). Fluorescence intensity was converted to the resorufin concentration by comparison with a standard curve of resorufin fluorescence *vs.* concentration ranging from 0 to 1000 nM. The slope (nM/min) of the fitted resorufin synthesis curve, corresponding to the rate of resorufin formation, was used as the metric of cytochrome P450 activity.

Biochemical assays

The biochemical assays were performed on d 10 medium samples. Amino acids were fluorescently labeled using the AccQ-Tag system (Waters, Milford, MA, USA), separated by high-performance liquid chromatography (HPLC model 2690; Waters) and quantitated by a fluorescence detector (model 474; Waters) (14). Glucose and lactate levels were measured with commercially available kits (Sigma), the former based on the reaction of glucose catalyzed by glucose oxidase, and the latter based on the conversion of lactate to pyruvate catalyzed by lactate oxidase.

Statistical analysis

Each data point represents the mean of 2 or 3 experiments (each with 3 biological replicates), and the error bars represent the SEM. Statistical significance was determined using Student's *t* test for unpaired data. Differences were considered significant at values of $P \leq 0.05$.

RESULTS

Determination of surface characteristics and composition of Adipogel

We have performed a preliminary characterization of the ECM components derived as a BME from preadipocytes during the differentiation process. The preadipocytes are cultured in T-175 cm^2 flasks in DMEM supplemented with 10% FBS and 2% penicillin and

streptomycin until the cells attain confluency. At 48 h after confluency, the cells are differentiated in culture medium supplemented with 1 μM dexamethasone, 0.1 μM isobutyl-methylxanthine, and 1 μM rosiglitazone for 2 d with medium changes every 2 d. On d 2, the differentiation medium is supplemented with rosiglitazone only.

During the differentiation process, cell-exposed medium is collected and processed further for generation of cell-derived ECMs. We have identified a highly viscoelastic material on d 2 and 4 of adipocyte differentiation resembling ECM components secreted by preadipocytes to maintain adipose tissue cell-cell contact, morphological induction of adipocytes, and functional and gene expression indicative of mature adipocyte lineage. To purify the ECM-rich material, the cell-exposed medium is centrifuged at 4000 *g* for 1.5 h using Amicon 100- or 10-kDa centrifugal filters. The concentrate, primarily composed of medium constituents with molecular mass cutoff of 100 or 10 kDa, comprises the cell culture supernatant-derived protein-rich ECM (**Fig. 1**).

An SEM analysis of the gel was performed to evaluate the structural and morphological characteristics. As shown in **Fig. 2**, both the d 2 and 4 Adipogel comprise a dense, complex network with nonuniform peeling of edges in the cross section. The texture of the biomaterial in general comprises surfaces of 2 size ranges. The larger configuration represents the size and orientation of the cross section, and the smaller configuration represents etched ridges and grooves on the surface.

The ECM has been characterized as composed of collagen IV, laminin, hyaluronan, and fibronectin. As shown in **Fig. 3**, although the fibronectin concentration is comparable, the collagen IV concentration is ~3 fold higher for midstage (d 4 Adipogel) as compared to early-stage BME (d 2 Adipogel). The laminin concentration is also significantly higher for d 4 as compared to d 2 Adipogel samples. In addition to characterization of ECM proteins, we have identified the protein composition of the d 4 Adipogel as shown in **Fig. 4**. The protein content of the gel consists of 27 up-regulated proteins as compared to controls (basal medium protein concentrate). In addition, we have categorized the proteins in a pie chart (Fig. 4); as shown, >22% of the up-regulated proteins correspond to growth factors and receptor proteins, whereas ~19% represent cytokines. The growth factors with tissue-engineering potential that were up-regulated for d 4 Adipogel are hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and leukemia inhibitory factor (LIF). These growth factors have numerous biological functions, ranging from cell proliferation, differentiation, and migration to phenomena such as tissue-specific tumorigenesis, angiogenesis and wound healing, tumor suppression, and embryogenesis.

Effect of Adipogel on hepatic differentiated function

Routine culture of primary hepatocytes is difficult and cumbersome because of their ability to develop compromised function. We have developed a primary hepatocyte culture system that supersedes the traditional methodol-

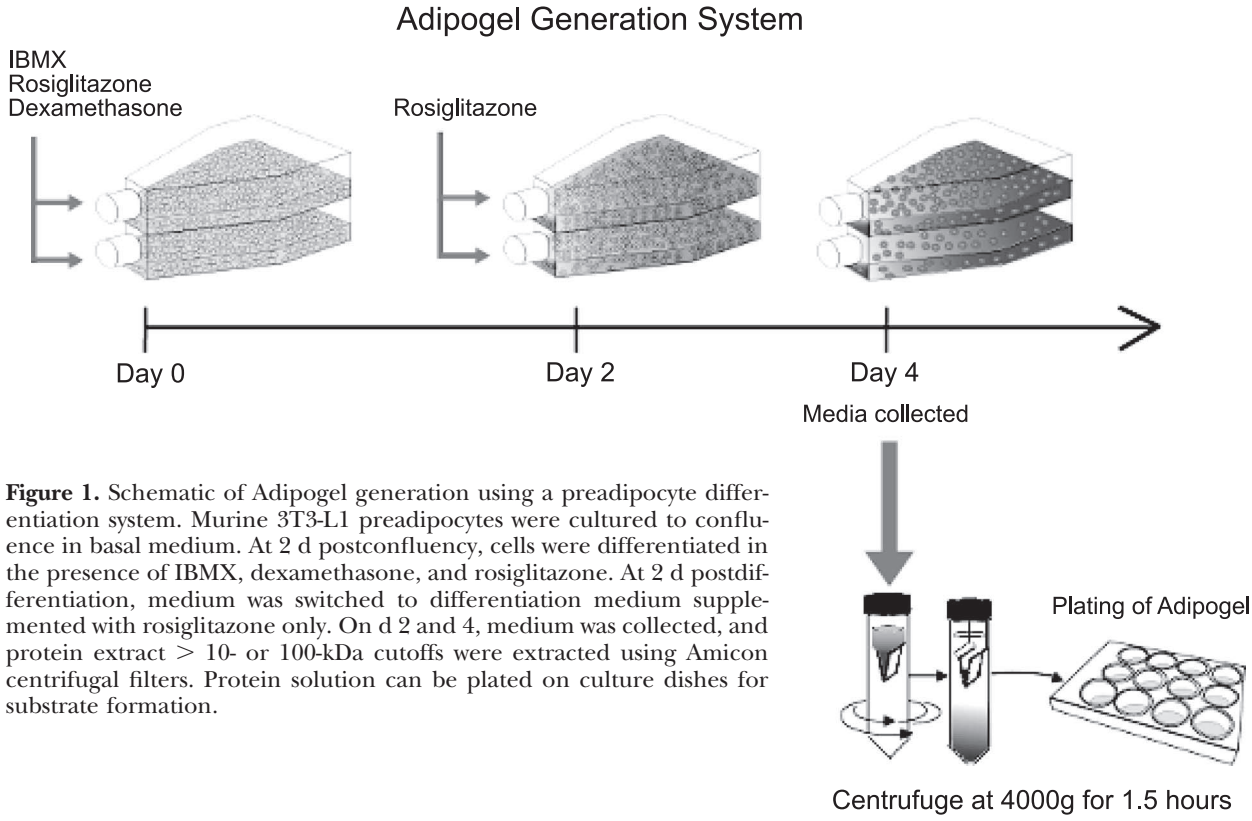


Figure 1. Schematic of Adipogel generation using a preadipocyte differentiation system. Murine 3T3-L1 preadipocytes were cultured to confluence in basal medium. At 2 d postconfluency, cells were differentiated in the presence of IBMX, dexamethasone, and rosiglitazone. At 2 d postdifferentiation, medium was switched to differentiation medium supplemented with rosiglitazone only. On d 2 and 4, medium was collected, and protein extract > 10- or 100-kDa cutoffs were extracted using Amicon centrifugal filters. Protein solution can be plated on culture dishes for substrate formation.

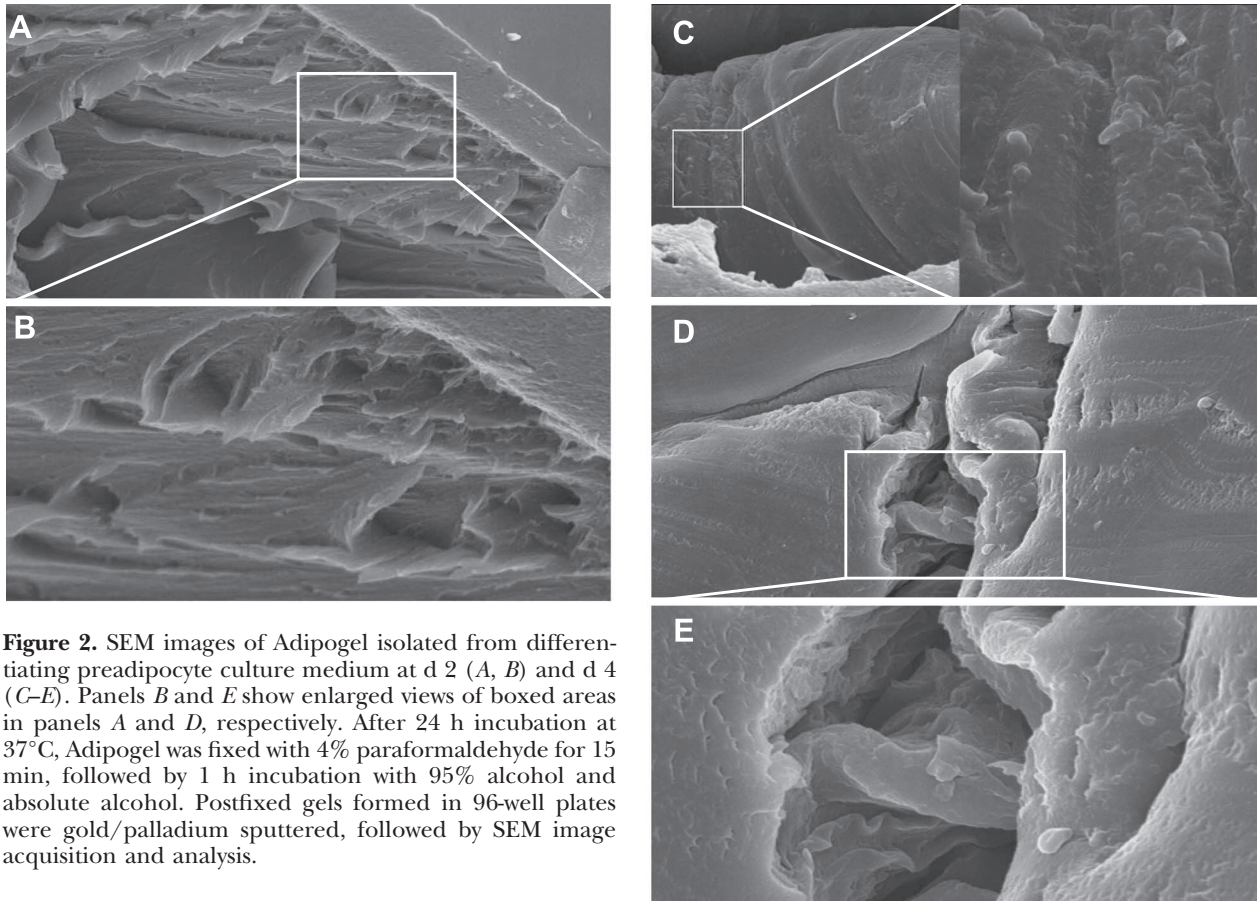


Figure 2. SEM images of Adipogel isolated from differentiating preadipocyte culture medium at d 2 (*A*, *B*) and d 4 (*C–E*). Panels *B* and *E* show enlarged views of boxed areas in panels *A* and *D*, respectively. After 24 h incubation at 37°C, Adipogel was fixed with 4% paraformaldehyde for 15 min, followed by 1 h incubation with 95% alcohol and absolute alcohol. Postfixed gels formed in 96-well plates were gold/palladium sputtered, followed by SEM image acquisition and analysis.

ogy of maintaining hepatocyte function and polarity in collagen double-gel sandwich systems (15). As shown in **Fig. 5**, hepatocytes cultured on single collagen gel with a soluble matrix of Adipogel in the culture medium showed comparable urea secretion rates, but significantly higher albumin secretion rates, from d 4 to 10 of culture, as compared to collagen double-gel (CDG) cultures. The albumin secretion rate for collagen single gel (CSG) + soluble Adipogel (solASG) was significantly higher on d 6, 7, and 10 of culture, as compared to Matrigel cultures. A comparison of the cytochrome P450 1A1 activity revealed that hepatocytes in CSG + solASG exhibited similar activity to Matrigel and CDG cultures, as shown in **Fig. 6**.

A comparison of the morphology of the hepatocytes in culture showed that CSG cultures have elongated morphology; on the other hand, collagen sandwich and collagen-Adipogel sandwich (CSG+ASG) cultures result in uniform polygonal morphology. Hepatocytes cultured in collagen-Adipogel soluble matrix configuration results in morphologies that are intermediate to collagen single-gel and collagen sandwich cultures (data not shown), while hepatocytes in Matrigel cultures exhibit spheroids.

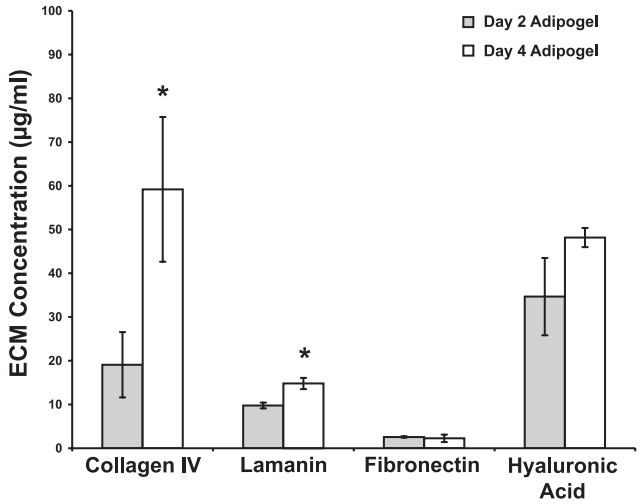
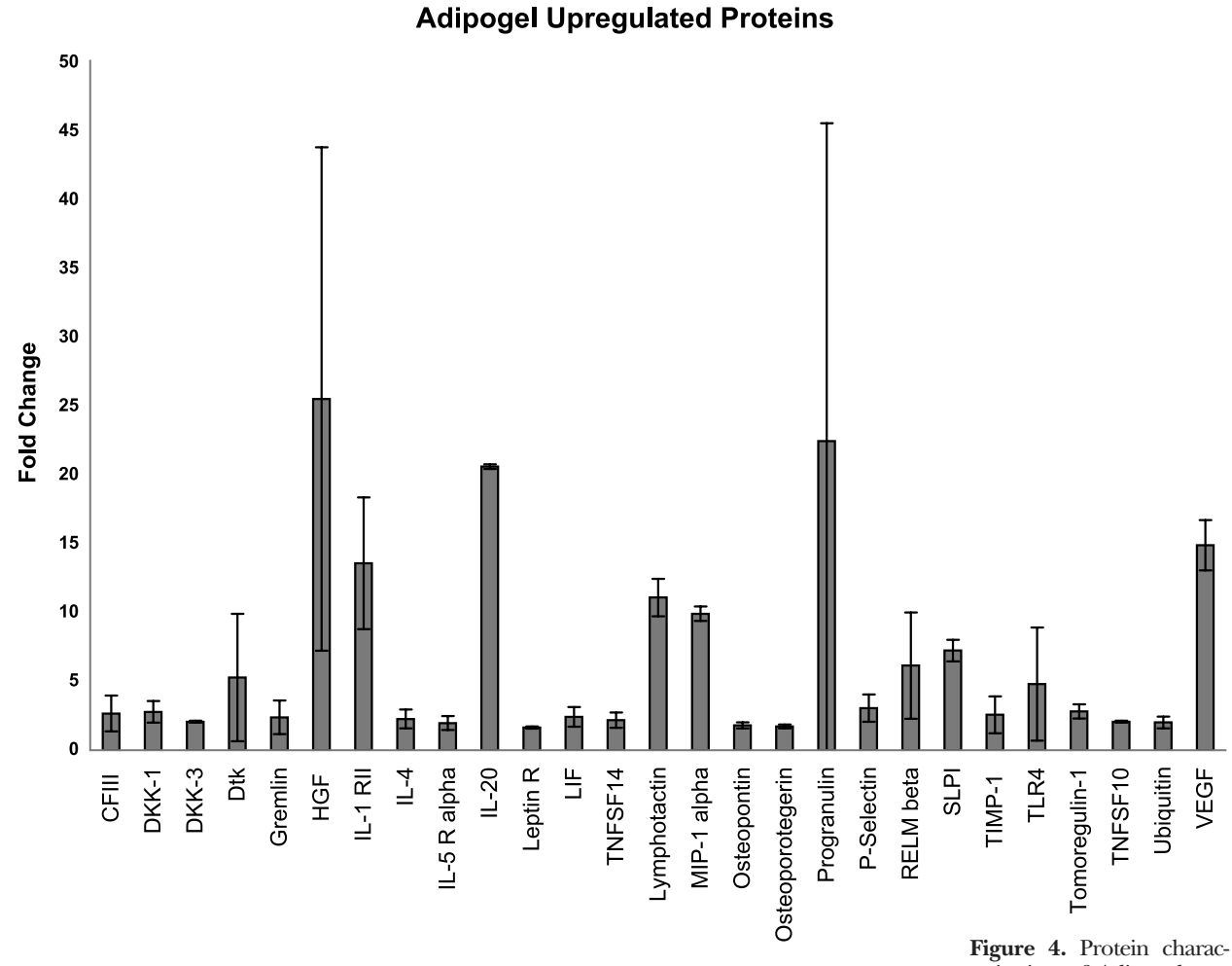


Figure 3. ECM characterization of Adipogel: collagen IV, laminin, fibronectin, and hyaluronan concentration determination using ELISA. As shown, fibronectin concentration is comparable in ECMs derived at early stage (d 2) and midstage (d 4). Collagen IV and laminin concentrations are significantly higher Adipogel at d 4 as compared to d 2. $^*P < 0.05$ *vs.* d 2.

Effect of Adipogel on hepatic metabolism

We have performed a metabolic analysis of the effect of the different culture configurations *viz.* the CSG, CDG, CSG + ASG, and CSG + solASG. As shown in **Table 1**, whereas hepatocytes in CDG are gluconeogenic with lactate synthesis probably *via* glycogenolysis, there is a significant higher glucose consumption rate for the remaining experimental conditions.

Serine, glycine, threonine, alanine, tyrosine, valine,



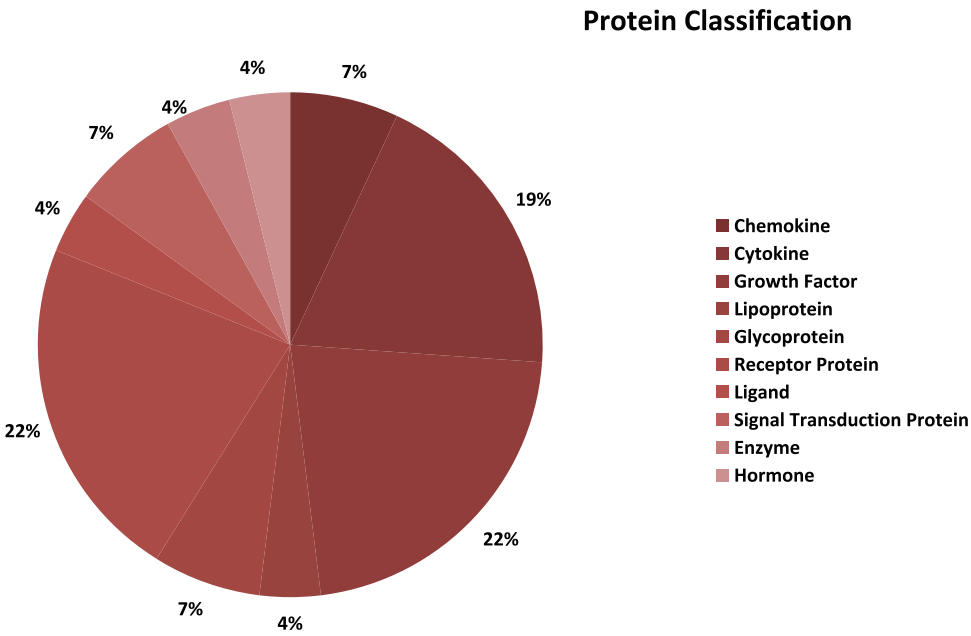


Figure 4. Protein characterization of Adipogel: upregulated protein composition of Adipogel compared to DMEM basal medium at d 4; 10-kDa cutoff. Adipogel with >10-kDa protein cutoff was isolated using Amicon centrifugal filters on d 4 of differentiation. Protein extract was isolated, and protein composition was determined using Protein Array (Ray Biotech). Protein composition was determined using a 5-d process wherein biotinylated protein antibody was added to samples in a glass slide chip. Signal detection and quantification were performed using label antibody-based protein array glass chip slides. Pie chart shows distribution of proteins up-regulated in Adipogel. Numbers in each section indicate percentage of proteins belonging to each category. CFIII, coagulation factor III; DKK-1, Dickkopf-1; DKK-3, Dickkopf-3; Dtk, developmental receptor tyrosine kinase; HGF, hepatocyte growth factor; IL-1RII, interleukin-1 receptor II; IL-4, interleukin-4; IL-5 R alpha: interleukin-5 receptor α; IL-20, interleukin-20; Leptin R, leptin receptor; LIF, leukemia inhibitory factor; TNFSF14, tumor necrosis factor SuperFamily 14; MIP-1 alpha, macrophage inflammatory protein-1α; RELM beta, Resistin-like molecule β; SLPI, secretory leukocyte peptidase inhibitor; TIMP-1, tissue inhibitor of metalloproteinases 1; TLR4, Toll-like receptor 4; TNFSF10, tumor necrosis factor SuperFamily 10; VEGF, vascular endothelial growth factor.

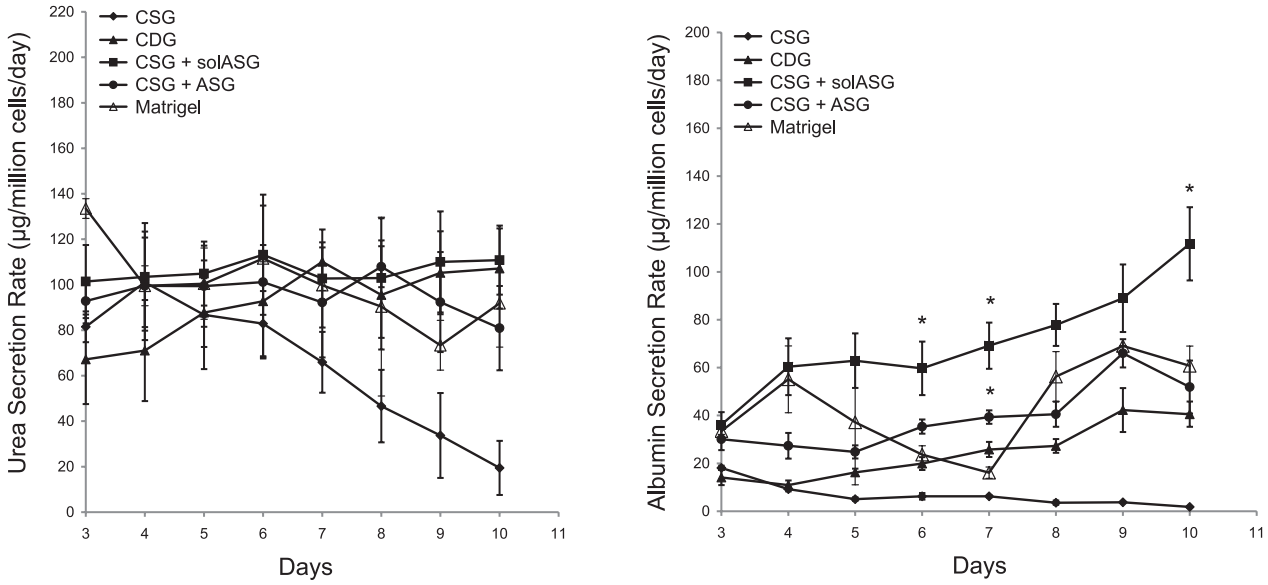


Figure 5. Functional analysis of rat hepatocytes *in vitro* using Adipogel: urea (left panel) and albumin (right panel) secretion rate of hepatocytes cultured in 5 different configurations at a density of 5×10^5 cells/well in a 12-well plate. CSG, culture on single collagen gel; CDG, culture in collagen double-gel sandwich configuration; CSG + solASG, hepatocytes cultured on collagen single gel with soluble Adipogel in medium; CSG + ASG, culture on collagen single gel overlaid with Adipogel; Matrigel, single-gel culture on Matrigel. Adipogel was utilized at a 1:5 ratio with culture medium; medium was changed on d 0, 1, 2, 5, 7, and 9. Although urea secretion rates are similar for CDG (positive control) and CSG + solASG conditions, albumin secretion rate is significantly higher for CSG + solASG condition on d 6, 7, and 10 of culture period as compared to Matrigel cultures. $^*P < 0.05$ *vs.* Matrigel.

methionine, lysine, isoleucine, leucine, phenylalanine, taurine, and cysteine uptake rates are significantly higher for CSG+solASG condition *vs.* CDG condition with a corresponding increase in albumin synthesis rate, whereas arginine, alanine, lysine, isoleucine, leucine, taurine, and cysteine are up-regulated for the CSG+ASG condition *vs.* the CDG condition with no change in albumin synthesis. Thus, we can correlate increased amino acid uptake rates and changes in glucose metabolism to hepatocellular function without the development of a comprehensive metabolic network.

DISCUSSION

Basement membranes are growth factor-enriched specialized ECM protein complexes providing structural and functional support to cell monolayers separating from connective tissue. The ECM is a complex mixture of matrix molecules, comprised of glycoproteins, fibronectin, collagens, laminins, proteoglycans, and nonmatrix proteins, including growth factors. The composition of the ECM, rather than simply the presence of an extracellular scaffold, is critical for regulating cell phenotype. These matrices include ubiquitously occurring BMs, which are 20- to 200-nm broad deposits of specific proteins in proximity to epithelial, muscle, fat, and nerve cells.

BM compositions are extremely diverse, tissue specific, and dynamic. BM provides structural support and organizes cell monolayers during tissue development. BM proteins possess multiple binding sites for cell adhesion molecules and motifs that serve as ligands for cell surface receptors. BM components guide cellular differentiation and inhibit or promote cell proliferation and migration. BM sequesters growth factors that influence cell behavior during BM remodeling.

Cell-matrix adhesions are essential for cell migration and tissue reorganization and differentiation, and as a result play central roles in embryonic development, tissue remodeling, and homeostasis of tissue and organ systems. These signals, in synergy with other pathways, regulate biological processes such as cell survival, cell proliferation, wound healing, and tumorigenesis. The elucidation of the structure and function of cell-matrix adhesions provides a critical vantage point for understanding regulation of eukaryotic cellular phenotypes *in vivo*. The profiles of proteins recruited to matrix adhesions specify the biochemical signals and biophysical properties of matrix adhesions. Principles such as roles of matrix composition, 3-dimensionality, and rigidity as well as existence of distinct types of cell-matrix adhesions and bidirectional signaling responses provide a rationale foundation for development of novel approaches to tissue repair and intervention in disease processes.

To develop a BM that can perform these functions, a novel *in vitro* cell culture system has to be established that has the ability to efficiently generate substantial amounts of natural ECM with defined growth factor and ECM protein composition. Although various cell lines can be utilized to produce natural BMEs, a majority of the cells do not produce sufficient ECM. We have developed a novel murine preadipocyte differentiation system that generates copious amounts of BME

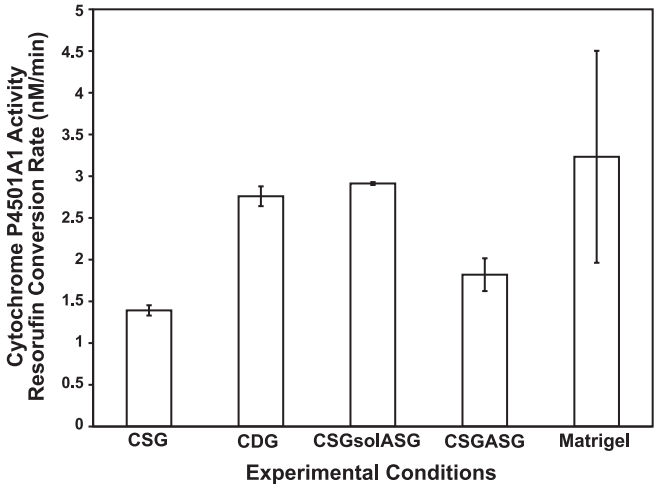


Figure 6. Cytochrome P4501A1 activity of rat hepatocytes *in vitro* using Adipogel. On d 10 of hepatocyte culture, cells were induced with 3MC for 48 h, followed by cytochrome P450 activity measurements using ethoxyresorufin as fluorogenic substrate. CSG, culture on single collagen gel; CDG, culture in collagen double-gel sandwich configuration; CSG + solASG, hepatocytes cultured on collagen single gel with soluble Adipogel in medium; CSG + ASG, culture on collagen single gel overlaid with Adipogel; Matrigel, single-gel culture on Matrigel. As shown, resorufin conversion rates are similar for the CDG (positive control), CSG+solASG, and Matrigel conditions.

(Fig. 1); this matrix, termed Adipogel, has some advantages over Matrigel (**Table 2**), the most widely used tissue-derived BME, derived from Engelbreth-Holm-Swarm (EHS) tumors. The synthesis and biochemical characterization of the BME has been performed with application to a hepatocyte culture system for functional augmentation.

Adipogel surface characteristics and composition

SEM provides rapid and easy evaluation of the morphological structure of materials. This technique can be utilized to determine the physical state and characteristics of the materials and obtain a qualitative analysis of the biomaterial surface texture and roughness with high resolution. In the current work, we have obtained SEM images of Adipogel in its native format, thus confirming the gel-like configuration (Fig. 2). This information provides us the basis for embedding cells into the gel in future work to evaluate the structure and interaction between the matrix composite and the cells.

The complex mixture of ECM molecules, 3-dimensional structural patterns, and distribution of ECM constituents depending on the tissue source mediate structural and biological properties of the biological material. Elucidating the biochemical composition of ECMs reveals its applicability in cell-biomaterial phenomena, such as embryonic development, tissue remodeling, and homeostasis of tissues and organ systems. As shown in Figs. 3 and 4, the ECM and protein composition have been determined for Adipogel. Collagen IV, the most abundant ECM in BN, is present in Adipogel. Typically, collagen IV accounts for 50% of

TABLE 1. *Effect of Adipogel substrate on amino acid and glucose metabolism of d 10 hepatocyte cultures*

Metabolite	CSG	CDG	CSG + solASG	CSG + ASG
Aspartate	−0.004 ± 0.001	−0.003 ± 0.0004	−0.002 ± 0.0004	−0.003 ± 0.0004
Serine	−0.02 ± 0.0172*	0.038 ± 0.0023	0.062 ± 0.0021*	0.03 ± 0.0017*
Glutamate	−0.043 ± 0.0102*	−0.142 ± 0.0034	−0.088 ± 0.0045*	−0.092 ± 0.012*
Glycine	−0.016 ± 0.043	0.035 ± 0.008	0.089 ± 0.0058*	0.006 ± 0.0216
Histidine	0.038 ± 0.0092*	0.091 ± 0.0004	0.091 ± 0.0005	0.086 ± 0.0001*
Ammonia	−0.422 ± 0.2897	0.005 ± 0.0036	0.043 ± 0.003*	−0.005 ± 0.0061
Arginine	0 ± 0.001*	−0.008 ± 0.0003	0.002 ± 0.0007*	0.001 ± 0.0012*
Threonine	0.053 ± 0.024*	0.188 ± 0.0013	0.205 ± 0.0015*	0.73 ± 0.5428
Alanine	−0.014 ± 0.0331	−0.011 ± 0.0047	0.099 ± 0.0038*	0.037 ± 0.0058*
Proline	−0.357 ± 0.1855	−0.021 ± 0.0035	−0.066 ± 0.0064*	−0.063 ± 0.0032*
Tyrosine	0.013 ± 0.0363*	−0.104 ± 0.0118	0.11 ± 0.0109*	−0.03 ± 0.027*
Valine	−0.029 ± 0.0522*	−0.203 ± 0.0079	0.272 ± 0.1765*	−0.007 ± 0.0326*
Methionine	0.001 ± 0.0168*	0.082 ± 0.0038	0.096 ± 0.0016*	0.049 ± 0.0037*
Lysine	−0.022 ± 0.0326	0.003 ± 0.0084	0.096 ± 0.0052*	0.041 ± 0.0097*
Isoleucine	−0.023 ± 0.0556*	−0.172 ± 0.0097	0.123 ± 0.0157*	0.019 ± 0.0327*
Leucine	−0.001 ± 0.0477*	−0.134 ± 0.0099	0.156 ± 0.0138*	0.045 ± 0.0295*
Phenylalanine	0.015 ± 0.0371*	0.198 ± 0.0063	0.249 ± 0.0043*	0.167 ± 0.0069*
Asparagine	−0.008 ± 0.0016*	−0.005 ± 0.0004	0 ± 0*	−0.004 ± 0.0002*
Glutamine	−12.662 ± 2.635*	−6.444 ± 0.4309	−0.387 ± 0.1458*	−2.496 ± 1.1015*
Taurine	−0.006 ± 0.0659	−0.064 ± 0.0031	0.172 ± 0.0124*	0.035 ± 0.0171*
Cysteine	0.016 ± 0.0299	−0.042 ± 0.0028	0.065 ± 0.0041*	0.008 ± 0.0155*
Ornithine	−0.04 ± 0.0063*	−0.082 ± 0.0019	−0.017 ± 0.007*	−0.066 ± 0.0066*
Glucose	0.07 ± 0.01*	−0.001 ± 0.02	0.06 ± 0.02*	0.07 ± 0.02*
Lactate	0.07 ± 0.01	0.07 ± 0.01	0.1 ± 0.01*	0.1 ± 0.02

Hepatocytes were cultured in 4 different configurations at a density of 5×10^5 cells/well in a 12-well plate. CSG, culture on single collagen gel; CDG, culture in collagen double-gel sandwich configuration; CSG + solAS, hepatocytes cultured on collagen single gel with soluble Adipogel in medium; CSG + ASG, culture on collagen single gel overlaid with Adipogel. Negative values correspond to metabolite synthesis rates; positive values correspond to metabolite consumption rates. Values are means ± SE (μmol/10^5 cells/d). *$P < 0.05$ *vs.* CDG.

TABLE 2. *Comparison of Matrigel and Adipogel*

Feature	Matrigel	Adipogel
Method of synthesis	Purified from extract of murine EHS tumor	Purified from mammalian preadipocyte secretions
Composition	Collagen IV, laminin, perlecan, nidogen, FGF, IGF, EGF [16]	Collagen IV, laminin, fibronectin, hyaluronan, HGF, VEGF, LIF
Applications	Angiogenesis, transplantation, tissue engineering	Primary cells and cell line culture, functional maintenance
Disadvantages	Batch-to-batch variability, animal derived, chemical digestion, not completely characterized, complex mixture	Not completely characterized, complex mixture, animal cell line derived
Advantages	Tested in multiple applications, basement membrane-like complex, gelation procedure easy	No chemical enzymatic procedures, less cumbersome, cheap basement membrane-like complex

BM proteins and is an integral component of BM structures and provides ligands for cell adhesion.

Although the heterogeneous molecular compositions and biochemical complexity of different organ BMs constitute biological function, the principal component comprising the BM composition is collagen IV. Collagen IV isolated from BMs has a size and amino acid composition similar in many ways to other collagen components. Collagen IV plays a very different role in histological structures, forming a sheet made by a meshwork of filaments rather than by linear fibrils. Fibronectin determination in Adipogel is also critical because this component is abundant in BMs. Fibronectin influences cell growth and differentiation through its effects on gene-encoding cell cycle components. Also, fibronectin is required for self-assembly of ECM molecules such as collagen I fibril formation. Laminin is an essential component of BMs and is associated with numerous biological activities, including promotion of cell adhesion, growth, proliferation, and differentiation.

The main growth factors identified for tissue-engineering applications are VEGF, HGF, and LIF. VEGF is a potent proangiogenic factor that promotes formation of new blood vessels at sites of injury and is also active in vasculogenesis and endothelial cell growth. VEGF also induces endothelial cell proliferation, promotes cell migration, inhibits apoptosis, and induces permeabilization of blood vessels. It binds to the VEGFR1/Flt-1 and VEGFR2/Kdr receptors, heparan sulfate, and heparin (by similarity).

HGF/scatter factor is a paracrine cellular growth, motility, and morphogenic factor. It is secreted by mesenchymal cells and targets and acts primarily on epithelial cells and endothelial cells but also on hemopoietic progenitor cells. It has been shown to have a major role in embryonic organ development, adult organ regeneration, and wound healing. HGF regulates cell growth, cell motility, and morphogenesis by activating a tyrosine kinase signaling cascade after binding to the proto-oncogenic c-Met receptor. Its ability to stimulate mitogenesis, cell motility, and matrix invasion gives it a central role in angiogenesis, tumorigenesis, and tissue regeneration. It is secreted as a single inactive polypeptide and is cleaved as serine proteases into a 69-kDa α chain and 34-kDa β chain.

LIF derives its name from its ability to induce the terminal differentiation of myeloid leukemic cells. Other properties attributed to the cytokine include the growth, promotion, and cell differentiation of different types of target cells, influence on bone metabolism, cachexia, neural development, embryogenesis, and inflammation.

Adipogel and hepatocyte culture assessment

Various methodologies to maintain hepatocytes *in vitro* include effect of ECM topology and function, cellular environment, and medium composition. The traditional technique for culturing rat hepatocytes *in vitro* is the collagen I sandwich configuration. Although this system has been extensively characterized with expression of basolateral and apical markers, up-regulation of differentiated function, and maintenance of cell polarity, there is also evidence for the role of ECM composition on maintenance of cell function. Variations in ECM composition include the addition of glycosaminoglycans and hepatic proteoglycans that promote the formation of gap junctions. EHS biomatrix Matrigel cultures have also been used as a substitute for collagen I that induces expression of cell adhesion molecules, *viz.,* connexins with up-regulation of differentiated function comparable to double-gel cultures. Matrigel, prepared from extract of murine EHS tumor, is comprised primarily of collagen IV, laminin, perlecan, nidogen, fibroblast growth factor, epidermal growth factor, and insulin-like growth factor (16). Although EHS tumor-derived matrix composition, *viz.,* collagen IV, laminin, and heparan sulfate proteoglycan, is prevalent in the space of Disse, utilizing a matrix that resembles the hepatic ECM will induce improved differentiated function similar to the *in vivo* microenvironment.

Adipogel laden on top of CSG hepatocyte cultures has been shown to induce increased albumin-differentiated function as compared to CDG cultures and Matrigel cultures in this work. Based on evidence in the literature (17, 18), a culture period of 10 d was utilized to investigate the effect of Adipogel on hepatocyte cultures. The reasons we did not perform 3–4 wk of cultures are that chemical induction of hepatotoxicity is generally assessed in week-long cultures (19, 20), and longer culture periods

require optimizing medium formulation and perhaps coculturing with either different cells that maintain stability of hepatocytes (21, 22) and/or perfused conditions in bioartificial liver devices (23, 24). This is part of our future work, in which we are studying the long-term maintenance of hepatocytes in optimized medium conditions.

We have not observed a significant difference in cell numbers across the different experimental conditions. There was also no difference in cell viability during the 10 d of culture in the different culture conditions (data not shown). This implies that the enhanced albumin secretion rate of hepatocytes in Adipogel cultures is primarily due to the effect of the natural biological material. Based on the comparison of the protein composition (Figs. 3 and 4) and the differentiated function (Fig. 5), we hypothesize that increased albumin synthesis in hepatocyte cultures as compared to CDG cultures may be due to increased levels of laminin and collagen IV in Adipogel as compared to absence of these factors in double-gel sandwich configurations. Literature evidence shows the effects of these components in augmenting albumin synthesis rates (25, 26); however, the combined effects of these components has not been thoroughly investigated. Nevertheless, because Adipogel BME has a complex composition, it is not trivial to identify the exact mechanism of the improvement of hepatic differentiated function. However, single ECM protein component blocking experiments can provide vital information about previously unknown factors implicated in improved differentiated function.

As shown in Fig. 6, Adipogel resulted in cytochrome P450 1A1 activity comparable to CDG or Matrigel conditions. Thus, the effect of Adipogel is more prominent in increasing albumin synthesis of hepatocytes, which is possibly due to the composition of Adipogel as mentioned previously and its effect on intermediary metabolism as discussed below.

Numerous researchers have investigated the intermediary metabolism of hepatocytes, the primary functional cells of the liver (27–30). Because hepatic metabolism is directly linked to cellular energetic and functions, the effect of hepatocyte culture configurations on the biochemical pathways inherent in mature cells is critical. Although mathematical programming tools have been used as an additional step to elucidate the effect of environmental perturbations on hepatic function (31–36), we have performed a metabolic analysis of the effect of the different culture configurations, *viz.,* CSG, CDG, CSG + ASG, and CSG + solASG. The analysis shows that the key amino acids implicated in increasing albumin synthesis rates for the CSG + solASG conditions are serine, glycine, threonine, alanine, tyrosine, methionine, lysine, isoleucine, leucine, phenylalanine, taurine, and cysteine. This is in agreement with literature evidence that indicates that amino acid availability is imperative for increased albumin synthesis in perfused livers and cultured rat hepatocytes (37–40). Also, supplementation of branched-chain amino acids, such as leucine and isoleucine, has been shown to increase albumin synthesis (41), consistent with our findings.

CONCLUSIONS

The development of a novel preadipocyte cell differentiation system has been utilized in this work for the generation of a natural BME termed adipogel with a unique ECM and growth factor composition. This methodology has some advantages over commonly used biomaterials such as Matrigel, *viz.,* presence of fibronectin, hyaluronan, HGF, VEGF, or LIF in Adipogel, and obviation of enzymatic and chemical processing methods to generate the extract and an animal-free extraction procedure that is not cumbersome and relatively inexpensive. We have shown that Adipogel can be utilized for augmenting hepatocyte differentiated function *in vitro* in combination with collagen over a 10-d period. In addition, the increase in amino acid and glucose metabolism in the presence of Adipogel shows promise in the integration of cellular metabolism with cell-ECM interactions.

One of the major goals in the comprehensive characterization of BM proteins is the understanding of principles that determine supramolecular organization. The self-assembly of individual collagen IV chains and other ECM proteins into superstructures and networks is important for BM stability and function. Also, complex biochemical and molecular pathways activated or modified by integrin-mediated adhesion provide insights into mechanisms that regulate adhesion-dependent cellular processes. The shifting progression of integrin receptor expression may influence endogenous or exogenous tension or facilitate cell survival and migration in multiple tissues with different matrix compositions.

Future work will entail the mechanical characterization and optimization of the BME for gels and scaffold formation. We will also evaluate cell-matrix adhesion molecules and their role in augmentation of hepatic function and effects on cell metabolism. The determination of subchains of the collagen IV family along with interactions with other ECM proteins in Adipogel also will be determined. To elucidate the effects of Adipogel on intracellular hepatic function, we will utilize metabolite measurements for development of a metabolic flux analysis model. These methodologies will provide a better understanding of the mechanistic implications of utilization of Adipogel for hepatocyte functional augmentation. FJ

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