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Cultivating liver cells on printed arrays of hepatocyte growth factor

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ABSTRACT

Growth factors are commonly present in soluble form during *in vitro* cell cultivation experiments in order to provide signals for cellular proliferation or differentiation. In contrast to these traditional experiments, we investigated solid-phase presentation of a hepatocyte growth factor (HGF), a protein important in liver development and regeneration, on microarrays of extracellular matrix (ECM) proteins. In our experiments, HGF was mixed in solution with ECM proteins (collagen (I), (IV) or laminin) and robotically printed onto silane-modified glass slides. Primary rat hepatocytes were seeded onto HGF/ECM protein microarrays and formed cellular clusters that corresponded in size to the dimensions of individual protein spots (500 μm diameter). Analysis of liver-specific products, albumin and α1-antitrypsin, revealed several fold higher levels of expression of these proteins in hepatocytes cultured on HGF/ECM microarrays compared to cells cultivated on ECM proteins alone. In addition, cultivation of hepatocytes on HGF/ECM protein spots led to spontaneous reorganization of cellular clusters from a monolayer into three-dimensional spheroids. We also investigated the effects of surface-tethered HGF on hepatocytes co-cultivated with stromal cells and observed a significantly higher level of albumin in co-cultures where hepatocytes were stimulated by HGF/ECM spots compared to co-cultures created on ECM protein islands without the growth factor. In summary, our study suggests that incorporation of HGF into ECM protein microarrays has a profound and long-lasting effect on the morphology and phenotype of primary hepatocytes. In the future, the number of growth factors printed on ECM microarrays will be expanded to enable multiplexed and combinatorial screening of inducers of cellular differentiation or proliferation.

1. Introduction

Growth factors play an important role in regulating cellular behavior, including stimulation of proliferation [1,2], migration [3,4] and differentiation [5,6]. In the liver, production and release of growth factors are part of a complex interplay between mature and immature hepatocytes, non-parenchymal cells and recruited inflammatory cells. Hepatocyte growth factor (HGF), a mesenchyme-derived protein, has been found to play a central role in liver development and regeneration after injury. HGF is a pleiotropic morphogen that has been shown to have mitogenic, motogenic, and antiapoptotic effects [6,7]. In addition, HGF is being explored as an anti-fibrotic agent and may have applications for treatment of liver fibrosis [8,9].

Current *in vitro* cell cultivation strategies commonly rely on providing growth factors in the soluble form. These traditional approaches require significant amounts of expensive growth factors (GFs) and are not optimal for stem cell differentiation or primary cell maintenance studies where frequent media changes are required. This presents a particularly challenging problem in stem cell differentiation studies where a formulation of GFs required for stem cell lineage selection is often unknown, requiring extensive and expensive experiments involving combinations of GFs [10–13].

In contrast to *in vitro* experiments where GF molecules are present in solution, *in vivo*, GFs bind to ECM matrix proteins and are dynamically released during matrix remodeling and protease secretion by the surrounding cells [14–16]. To mimic solid-phase presentation observed *in vivo* a number of reports have described strategies for surface immobilization of GF molecules via covalent tethering [17–20]. In addition, given that *in vivo* GFs form secondary bonds with either glycosaminoglycans [2,21–24] or with matrix proteins [15,25–27], non-covalent binding represents an alternative route for surface immobilization of these molecules.

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Association with ECM proteins may provide additional benefits of stabilizing GF molecules against proteolytic degradation and enhancing their activity [28].

Beyond offering a more physiological scenario, surface immobilization of GF molecules makes it possible to design strategies for high-throughput screening of cell function. Robotic microarraying, a technology originally designed for high-throughput screening of DNA hybridization events [29], is particularly amenable for printing arrays of biomolecules on cell culture surfaces. This robotic printing technology has been previously employed for high-throughput studies of cell–ECM [30], cell–biomaterial [31] and cell–small molecule interactions [32]. More recently Soen and Davis employed arrays of ECM proteins and morphogens to investigate differentiation of primary neuronal cells [33].

We have previously reported on the use of ECM protein microarrays for cultivation of hepatocytes in mono- and co-cultures [34]. These microarray-based cell cultures were complemented with methods for the analysis of gene expression and secreted product signatures within the local microenvironment [35,36]. In the present study, we describe GF presentation on printed ECM microarrays as a means to deliver stimuli to cultured hepatocytes. This study represents a step towards an integrated cell culture platform where signals are delivered and cell function is detected in a high-throughput and location-specific manner.

2. Materials and methods

2.1. Chemicals and materials

Glass slides (75×25 mm^2) were obtained from VWR (West Chester, PA). (3-Acryloxypropyl)trichlorosilane was purchased from Gelest, Inc. (Morrisville, PA). Sulfuric acid, hydrogen peroxide, ethanol, collagenase, collagen from rat tail (type I), collagen IV, laminin, hepatocyte growth factor (HGF), streptavidin-conjugated Alexa 546 and protease inhibitor cocktail were obtained from Sigma–Aldrich (St. Louis, MO). Concentrated phosphate-buffered saline (10× PBS) was purchased from Lonza (Walkersville, MD). Minimal essential medium (MEM), sodium pyruvate, nonessential amino acids, fetal bovine serum (FBS), Superscript III, RNaseOut (RNase inhibitor), dNTPs and biotinylated anti-HGF antibodies were purchased from Invitrogen (Carlsbad, CA). 384-well polypropylene microarray plates were obtained from Genetix (New Milton, Hampshire). Goat anti-rat cross-adsorbed albumin antibody, reference serum, and HGF ELISA Quantitation Kit were obtained from Bethyl Laboratories (Montgomery, TX). Goat anti-rat IgG Texas Red conjugate was purchased from Santa Cruz Biotechnologies, Inc. Formalin was purchased from Fisher (Pittsburgh, PA). Slide-A-Lyzer Mini Dialysis Units were purchased from Pierce (Rockford, IL).

2.2. Preparation of glass substrates

Glass slides were cleaned by immersion in "piranha" solution consisting of 3:1 ratio of aqueous solutions of 50% v/v of sulfuric acid and 30% w/v of hydrogen peroxide for 30 min (*caution*: *this mixture reacts violently with organic materials and must be handled with extreme care*). The glass slides were thoroughly rinsed with deionized water, dried under nitrogen, and kept in Class 10000 air prior to use. For silane modification, the glass slides were exposed to oxygen plasma for 5 min at 300 W (YES3, Yield Engineering Systems, Livermore, CA) and then placed for 10 min in a 2 mM solution of (3-acrylopropyl)trichlorosilane diluted in anhydrous toluene. The reaction was performed in a glove box under a nitrogen blanket to avoid exposure to atmospheric moisture. After silanization, the slides were rinsed with fresh toluene, dried under nitrogen, and cured at 100 °C for 4 h.

2.3. Printing ECM and growth factor microarrays

All ECM proteins employed for printing were dissolved in 1× PBS + 0.005% Tween-20 at 0.2 mg/mL concentration. HGF was mixed with the ECM solution to a final concentration of 500 ng/mL and allowed to bind to the ECM protein for 30 min at room temperature prior to printing. Protein microarrays were contact-printed under ambient conditions on silane-modified 75×25 mm^2 glass slides using a hand-held MicroCaster. The pins collected protein (0.2 mg/mL ECM + 500 ng/mL HGF in 1× PBS with 0.005% Tween) from a 382-well plate, dispensing 20–70 nL of solution onto the glass slide and forming circular spots ~500 μm in diameter. Protein arrays were kept in a refrigerator before use and were functional for at least one week.

2.4. Characterization of HGF retention on ECM microarrays

An array of ECM/HGF (6×6) was printed onto silane-modified glass slides as described before and incubated in 1× PBS at 37 °C for 2 h. HGF molecules were extracted from the silane surface using 50 μL of 4 M guanidine–HCl (pH 7.2) supplemented with 0.2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 10 mM EDTA, 0.05 M Tris and protease inhibitor cocktail. The guanidine solution was incubated with the HGF microarray for 30 min. The supernatant was buffer-exchanged with 6 M urea containing 0.05 M Tris (pH 7.4) using Slide-A-Lyzer Mini Dialysis Units with 3500 MWCO for 1 h. The concentration of HGF extracted from the printed microarray was determined according to the manufacturer's instructions using the HGF ELISA.

Immunofluorescent staining was used to determine retention of HGF on ECM microarrays after 1, 3 and 5 days in media at cell culture conditions. Protein solution containing 500 ng/mL HGF and 0.2 mg/mL collagen (I) was printed onto silanized glass slides and in cell culture media at 37 °C. At the desired time point, glass substrates were removed from media and incubated with 1 μg/ml (in 1× PBS) of anti-human HGF biotin conjugate at 37° for 2 h followed by incubation in 10 μg/ml of streptavidin, Alexa Fluor® 546 conjugate for 1 h at room temperature. Samples were washed between each staining step with 1× PBS + 0.05% Tween-20. In order to create a quantitative readout of fluorescence signal emanating from the array, the laser microarray scanner (Agilent G2565BA fluorescent scanner, Expression Analysis Facility, UC Davis Genome Center) was employed to scan the glass slides at a spot pixel resolution of 5 μm. The fluorescence intensity of each array element was determined using GenePix Pro 6.0 data analysis software (Molecular Devices, Downingtown, PA). Fluorescent intensity was converted to a percentage of the control array.

2.5. Cultivation of primary hepatocytes on HGF microarrays

Our studies employed primary rat hepatocytes. Cells were isolated from adult female Lewis rats (Charles River Laboratories, Boston, MA) weighing 125–200 g, using a two-step collagenase perfusion procedure as described previously [37]. Typically, 100–200 million hepatocytes were obtained with viability >90% as determined by trypan blue exclusion. Primary hepatocytes were maintained in DMEM supplemented with epidermal growth factor (EGF), glucagon, hydrocortisone sodium succinate, recombinant human insulin, 200 units/mL penicillin, 200 μg/mL streptomycin and 10% FBS.

For cell seeding experiments, a glass slide containing printed arrays of ECM + HGF was cut to fit into a 6-well plate. Hepatocytes were seeded to form cellular arrays using protocols described earlier [34]. In brief, glass slides containing printed ECM/HGF spots were first exposed to 3 mL of rat primary hepatocytes suspended in culture medium at a concentration of 1×10^6 cells/mL. After 1 h of incubation at 37 °C, hepatocytes became localized on ECM/HGF domains, but did not attach on the surrounding silane-modified surface. The samples were then washed twice in PBS to remove unbound hepatocytes and fresh media was added to the sample well.

Murine 3T3 fibroblasts were maintained in DMEM supplemented with 10% FBS, 200 units/mL penicillin, and 200 μg/mL streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. Cells were cultured until 90% confluence and then passaged. When performing co-culture experiments, hepatocytes were allowed to spread out on the protein spots overnight. The following day 3T3 fibroblasts were seeded on the sample at 0.25×10^6 cells/mL and were allowed to attach for 30 min. Unbound cells were washed away and fresh media was added as previously described. In our previous experiments [34,35] modification of glass substrates with acrylated silane was found to render these surfaces partially non-fouling. The silane layer prevented attachment of primary hepatocytes seeded first but supported adhesion of 3T3 fibroblasts that were seeded in the second step. Therefore, this micropatterning strategy led to hepatocytes residing on protein islands and fibroblasts adhering on the surrounding glass regions.

In our experiments, hepatocytes cultured on HGF/ECM microarrays were compared with cells cultured on ECM protein arrays without HGF. Another control experiment performed in parallel involved cultivation of hepatocytes on ECM protein arrays with HGF present in solution at a concentration of 10 ng/mL. Importantly, in contrast to HGF microarrays where no exchange or supplementation of GF molecules was possible, soluble GF was changed daily along with the culture media. In all three cases hepatic function was analyzed as described below.

2.6. Analysis of hepatic function

Expression of hepatic phenotype was assessed by intracellular staining of albumin, ELISA of albumin and real-time RT-PCR of albumin and α1-antitrypsin gene expression. For immunostaining, cells were fixed in 4% formalin in PBS for 20 min and then permeabilized with 0.1% Triton X-100 for 5 min. The cells were then incubated in blocking solution (1% bovine serum albumin (BSA) in 1X PBS) for 1 h at room temperature and exposed to 1:250 diluted anti-rat serum albumin antibody for 2 h at 37 °C. Finally, cells were incubated in 1:100 diluted anti-mouse IgG conjugated with Texas Red for visualization. Cells were washed between each step with 1X PBS three times for 5 min. All incubations were performed at room temperature if not specified. Stained cells were visualized and imaged using