Acta Biomaterialia 9 (2013) 7651–7661

Contents lists available at SciVerse ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

A self-assembling peptide matrix used to control stiffness and binding site density supports the formation of microvascular networks in three dimensions

M.D. Stevenson [a], H. Piristine [b,c], N.J. Hogrebe [a], T.M. Nocera [a], M.W. Boehm [d], R.K. Reen [a], K.W. Koelling [d], G. Agarwal [a], A.L. Sarang-Sieminski [b], K.J. Gooch [a,e,*]

[a] The Ohio State University, Department of Biomedical Engineering, 270 Bevis Hall 1080 Carmack Rd., Columbus, OH 43210, USA
[b] Franklin W. Olin College of Engineering, Olin Way Needham, MA 02492, USA
[c] Wellesley College, 106 Central Street Wellesley, MA 02481, USA
[d] The Ohio State University, Department of Chemical Engineering, 125A Koffolt Laboratories 140 West 19th Ave., Columbus, OH 43210, USA
[e] The Ohio State University, Davis Heart Lung Research Institute, 473 W 12th Ave., Columbus, OH 43210, USA

ARTICLE INFO

Article history:
Received 21 December 2012
Received in revised form 1 March 2013
Accepted 1 April 2013
Available online 17 April 2013

Keywords:
Three-dimensional cell culture
Self-assembling peptide
Microvascular networks
Stiffness
Binding site density

ABSTRACT

A three-dimensional (3-D) cell culture system that allows control of both substrate stiffness and integrin binding density was created and characterized. This system consisted of two self-assembling peptide (SAP) sequences that were mixed in different ratios to achieve the desired gel stiffness and adhesiveness. The specific peptides used were KFE ((acetyl)-FKFEFKFE-CONH2), which has previously been reported not to support cell adhesion or MVN formation, and KFE-RGD ((acetyl)-GRGDSP-GG-FKFEFKFE-CONH2), which is a similar sequence that incorporates the RGD integrin binding site. Storage modulus for these gels ranged from ~60 to 6000 Pa, depending on their composition and concentration. Atomic force microscopy revealed ECM-like fiber microarchitecture of gels consisting of both pure KFE and pure KFE-RGD as well as mixtures of the two peptides. This system was used to study the contributions of both matrix stiffness and adhesiveness on microvascular network (MVN) formation of endothelial cells and the morphology of human mesenchymal stem cells (hMSC). When endothelial cells were encapsulated within 3-D gel matrices without binding sites, little cell elongation and no network formation occurred, regardless of the stiffness. In contrast, matrices containing the RGD binding site facilitated robust MVN formation, and the extent of this MVN formation was inversely proportional to matrix stiffness. Compared with a matrix of the same stiffness with no binding sites, a matrix containing RGD-functionalized peptides resulted in a ~2.5-fold increase in the average length of network structure, which was used as a quantitative measure of MVN formation. Matrices with hMSC facilitated an increased number and length of cellular projections at higher stiffness when RGD was present, but induced a round morphology at every stiffness when RGD was absent. Taken together, these results demonstrate the ability to control both substrate stiffness and binding site density within 3-D cell-populated gels and reveal an important role for both stiffness and adhesion on cellular behavior that is cell-type specific.

1. Introduction

There is a growing body of literature demonstrating that cell function is affected by substrate stiffness [1]. For cultured fibroblasts, substrate stiffness affects the rate [2] and direction [3] of cell migration, focal adhesion [2] and stress fiber formation [4,5], responsiveness to exogenous growth factors [6] and proliferation [7]. The spreading of smooth muscle cells is dependent on both the density of binding ligands on a surface and the stiffness of the material [8]. Neurons have increased branching densities when cultured on soft substrates, while glial cells—which are normally co-cultured with these neurons—only survive on more rigid substrates [9]. Substrate stiffness influences the differentiation of mesenchymal stem cells, with soft, intermediate and stiff materials being neurogenic, myogenic and osteogenic, respectively [10].

The aforementioned studies cultured cells on top of protein-laminated polyacrylamide gels. Cells do not directly bind to the polyacrylamide, but instead interact with the laminating layer of protein. The concentration of this protein layer can be held constant while changing the stiffness of the polyacrylamide gel, and thus the cells can be exposed to a constant binding site density

* Corresponding author at: The Ohio State University, Department of Biomedical Engineering, 270 Bevis Hall, 1080 Carmack Rd., Columbus, OH 43210, USA. Tel.: +1 614 292 4665.
E-mail address: gooch.20@osu.edu (K.J. Gooch).

1742-7061/$ - see front matter © 2013 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.actbio.2013.04.002

while the stiffness is varied [8,9]. The most widely recognized limitation of the system, however, is that living cells cannot be embedded within polyacrylamide gels, but instead can only be cultured on top of them (i.e. two-dimensional (2-D) culture) [11,12]. While it is tempting to assume that cells cultured on top of a biomaterial represent a simpler but faithful 2-D analog of a three-dimensional (3-D) environment, this assumption is not well justified by the available literature. For example, relative to cells cultured on top of planar surfaces, cells cultured within 3-D gels exhibit altered morphology [13–15], morphogenesis [16–18], cytoskeletal organization [19,20], matrix adhesion complexes [21–23], proliferation [24], survival [25] and differentiation [20,26]. Thus, there is a growing consensus that a 3-D biomaterial environment is crucial to properly study many key cellular functions [27–29].

The behavior of cells cultured within 3-D extracellular matrix (ECM) gels changes as the hydrogel concentration is altered [30–32]. Since gel stiffness rises with increasing gel concentration, these changes in behavior are often attributed to matrix stiffness [33]. Other factors, however, such as cell–matrix binding site density, pore size, fiber diameter and diffusion rates, can also vary with gel concentration. Thus, decoupling stiffness from these other variables is essential to evaluate cell behavior properly as a function of gel concentration [34]. A number of approaches have been used to isolate the independent contribution of stiffness, such as altering mechanical boundary conditions [30,35,36], crosslinking [37–39] or composition [32,40–42]. While such approaches to control stiffness in natural polymers have many merits, they are often limited by the interdependence of fiber size, pore size and stiffness, as well as an inability to control both matrix stiffness and binding site density.

The self-assembling peptides (SAP) used in this study consist of alternating hydrophilic and hydrophobic amino acids that form a fibrous hydrogel structure when exposed to millimolar salt solutions [43–47]. Jung et al. [48] recently described the co-assembly of a SAP that lacked known binding sites with another SAP of the same base sequence that contained RGD or IKVAV integrin binding sites. Using this system, they demonstrated that endothelial cells adhere to, spread out on and proliferate more when seeded on top of a gel that included integrin binding sites, compared with a SAP lacking an integrin binding sequence. While this system has many merits, it is not useful for studying the effects of matrix stiffness within a 3-D environment because the endothelial cells were cultured as a 2-D monolayer on top of the SAP gels, in a similar way to what has been done with polyacrylamide systems. Herein, a co-assembly system that supports the culture of cells within 3-D gels is described. The present system consists of one SAP that lacks integrin binding sites and another SAP possessing the same base sequence that contains RGD, similar to that of Jung et al. The RGD sequence was chosen because it is the cell recognition site of a large number of ECM and platelet adhesion proteins, including vitronectin, type I collagen, fibrinogen, von Willebrand factor, osteopontin, thrombospondin, other collagens and laminin [49]. Endothelial cells express at least 11 different integrins [50], of which six recognize RGD ($\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_v\beta_8$). Furthermore, the significance of RGD to integrin attachment is demonstrated through the inhibition of cell adhesion to surfaces coated with various ECM proteins when soluble RGD containing peptides are present in the medium [51].

Rheology was used to determine the stiffness profile of the SAP system, and its fiber morphology was characterized using atomic force microscopy (AFM). Using this system to control both matrix stiffness and integrin binding site density, the present authors studied the response of both endothelial cells and human mesenchymal stem cells (hMSC) embedded within the 3-D gels to these parameters in terms of microvascular network (MVN) formation and cellular projections, respectively. MVN formation is a useful model system because of its importance in physiology, pathology and tissue engineering. In addition, previous studies that have explored the impact of only matrix stiffness on MVN formation served as comparisons in an effort to understand the contributions of both stiffness and binding site density [30,32,37,39–42,47]. The choice of HUVEC allowed for a more direct comparison with these studies since they are a popular cell type for such experiments [30,32,41,42,47].

2. Materials and methods

2.1. SAP gelation and cell culture

Peptide sequences (acetyl)-GRGDSP-GG-FKFEFKFE-CONH2 (KFE-RGD) and (acetyl)-FKFEFKFE-CONH2 (KFE) from BiomerTechnology (Pleasanton, CA) were dissolved in deionized water to a final concentration of 1% (10 mg ml^{-1}) and sonicated for 20–60 min. Mixtures of the two peptides were also sonicated before use in cell studies.

Human umbilical vein endothelial cells (HUVEC) (Lonza, Walkersville, MD) were cultured in EGM-2 (Lonza) on collagen-coated flasks and used between passages 4 and 7. Cells were suspended in peptide gels as previously reported [42]. Briefly, HUVEC were washed with PBS, trypsin and then Versene (all from Invitrogen, Carlsbad, CA). After they detached, the cells were pelleted, washed in 20% sucrose, pelleted and resuspended in 20% sucrose. The cell–sucrose mixture was mixed 1:1 with peptide (mixtures of KFE and KFE-RGD) to achieve a final concentration of 2.5×10^6 cells ml^{-1}. Cell-peptide mixtures (150 μl) were gently pipetted into 12 mm membrane inserts (PICM-012–50, Millipore, Billerica, MA) prewet from the bottom with 300 μl gelation media (complete EGM-2 with 10% serum added). After being allowed to solidify for ~5 min, 700 μl of gelation medium was added to the top of the gel and the outside of the insert. After 1 h, this medium was exchanged for regular medium supplemented with 50 ng ml^{-1} phorbol-myristate acetate (Sigma–Aldrich, St. Louis, MO) and 50 ng ml^{-1} vascular endothelial growth factor (R&D Systems, Minneapolis, MN). Supplemented media was replaced after 24 h, and the gels were fixed in 4% paraformaldehyde after 48 h.

The hMSC (Lonza) were expanded with MSC growth medium (Lonza) in T-75 flasks and used by passage 5. The hMSC were encapsulated within SAP gels in the same manner as HUVEC, with the exception that MSC growth medium was used for gelation and culture.

2.2. Morphology visualization and quantification

After fixation, HUVEC-containing gels were stained with Alexa 633 phalloidin (160 nM, Invitrogen) and DAPI (Sigma), mounted on glass slides and were subsequently imaged on a confocal microscope (405 and 633 lasers; Leica TCS SP5 AOBS confocal system, Bensheim, Germany). Quantification was performed as previously described [30] with slight modification. Briefly, 10 μm confocal stacks were taken (1 μm spacing) and then compressed into a single maximum projection image using ImageJ (NIH, Bethesda, MD). Using a custom-written ImageJ macro, images were thresholded, skeletonized and the average length of skeletonized objects (ALS) was recorded for five images for each gel. Data represent three independent experiments and five to seven gels for each condition.

After 24 h of culture within SAP gels, hMSC were imaged by differential interference contrast (DIC) microscopy (Olympus IX81, Center Valley, PA). The gels were taken directly from the incubator and imaged immediately, and thus fixatives were not used. All images were taken near the middle of the gels to ensure that the imaged cells were in a true 3-D environment rather than growing