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High-Throughput Screening of Vascular Endothelium-Destructive or Protective Microenvironments: Cooperative Actions of Extracellular Matrix Composition, Stiffness, and Structure

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Abstract

Pathological modification of the subendothelial extracellular matrix (ECM) has closely been associated with endothelial activation and subsequent cardiovascular disease progression. To understand regulatory mechanisms of these matrix modifications, the majority of previous efforts have focused on the modulation of either chemical composition or matrix stiffness on 2D smooth surfaces without simultaneously probing their cooperative effects on endothelium function on in vivo like 3D fibrous matrices. To this end, a high-throughput, combinatorial microarray platform on 2D and 3D hydrogel settings to resemble the compositions, stiffness, and structure of healthy and diseased subendothelial ECM has been established, and further their respective and combined effects on endothelial attachment, proliferation, inflammation, and junctional integrity have been investigated. For the first time, the results demonstrate that 3D fibrous structure resembling native ECM is a critical endothelium-protective microenvironmental factor by maintaining the stable, quiescent endothelium with strong resistance to proinflammatory stimuli. It is also revealed that matrix stiffening, in concert with chemical compositions resembling diseased ECM, particularly collagen III, could aggravate activation of nuclear factor kappa B, disruption of endothelium integrity, and susceptibility to proinflammatory stimuli. This study elucidates cooperative effects of various microenvironmental factors on endothelial activation and sheds light on new in vitro model for cardiovascular diseases.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

1. Introduction

Cardiovascular diseases, including atherosclerosis-related coronary heart disease and peripheral or carotid artery diseases, are the leading cause of morbidity and mortality in developed countries.^[1] A major event preceding most cardiovascular diseases is the inflammation of vascular endothelium. Atherosclerosis, for instance, is a chronic inflammatory condition that begins with endothelium activation.^[2] Upon exposure to cardiovascular risk factors, the vascular endothelium becomes proliferative, proinflammatory, and permeable, with increased leukocyte adhesion and transmigration.^[2c,d] Vascular endothelial cells (ECs) in vivo reside in a complex, 3D basement membrane consisting of a network of fibers in the sub-micrometer range,^[3] and are mainly comprised of collagen IV (C4) and laminin (LN).^[4] The progression of atherosclerosis or hypertension-related inflammation is closely associated with the deposition of transitional extracellular matrix (ECM) proteins such as collagen I (C1), collagen III (C3), and fibronectin (FN) into the subendothelial matrix.^[5] Moreover, excessive protein deposition and remodeling reduces ECM elasticity, resulting in vascular stiffening.^[6] For instance, the elastic modulus of the subendothelial matrix increases from less than 5 kPa in healthy blood vessels^[7] to 6–891 kPa when atherosclerosis occurs.^[8] Such ECM changes reveal the important roles of the matrix composition and stiffness in cardiovascular diseases.

Recently, it has become apparent that matrix composition and stiffness have significant impacts on endothelial functions in vitro.^[9] For instance, ECM proteins coated on polystyrene culture plates affect the ability of endothelial monolayer formation, expression of tissue factors, and endothelial thrombogenicity.^[9c,d] Further, accumulating reports demonstrate that matrix stiffening promotes an athero-prone EC phenotype by inducing increased endothelium permeability and leukocyte transmigration, both of which are hallmarks of atherosclerosis progression.^[10] However, while these reports have informed tremendously on the individual roles of matrix stiffness and composition in regulating endothelial function, the combined effects of such factors remain elusive. Additionally, performing an unbiased systematic evaluation of complex ECM components in terms of their effects on cell functions can be challenging. Our group, as well as others, have reported the use of robotic spotting technology to generate combinatorial ECM proteins for the investigation of stem cell differentiation.^[11] Such combinatorial ECM microarray platforms have also been applied to increase our understanding of diseases, including identifying the roles of ECM proteins in hepatic fibrosis^[12] or tumorigenesis stages.^[13] However, previous disease-related studies using ECM protein arrays have not considered the impacts of matrix stiffness, whereas the progression of cardio-vascular diseases such as atherosclerosis is accompanied by not only ECM composition changes but also matrix stiffening.

Besides the interplay between matrix composition and stiffness, the structure or dimensionality of culture would further add significant implications in correlating in vitro results to in vivo responses. Conventional 2D in vitro cell culture models have been widely employed for drug development and fundamental studies of cellular functions, although 2D matrices fail to replicate the 3D fibrous structure of native ECM where ECs reside in vivo; consequently, the findings on stiff, flat 2D matrices are less predictive by comparison to soft, fibrous 3D matrices. Interestingly, both our group^[11d] and Chen's group^[14] recently

demonstrated an apparent departure of the relationship between matrix stiffness and cell spreading on 3D fibrous hydrogels when compared to that on well-established 2D hydrogel systems, suggesting that 3D fibrous hydrogels may represent a more favorable environment for studying in vivo like cell-matrix interactions.

Understanding how ECM microenvironments affect endothelial activation and inflammation is critical to evaluate the effects of ECM factors (stiffness, composition, geometry) of the vascular intima or neointima during cardiovascular disease progression; the product of which can inform toward the discovery of new therapies in a disease-relevant physiochemical context. Herein, we utilized a high-throughput ECM protein array platform in combination with tunable matrix stiffness and structure to investigate the cooperative effects of these microenvironmental cues on endothelial functions, with focused analyses on endothelial proliferation, proinflammatory response, and junctional integrity. The entire process flow is illustrated in Figure 1A.

2. Results

2.1. Material Characterization of 2D and 3D Hydrogels

The surface topographies of 2D and 3D hydrogels were revealed by atomic force microscope (AFM) (Figure 2A) and scanning electron microscopy (SEM) (Figure S1, Supporting Information). For the 2D hydrogels, no significant differences in surface morphology were found between soft and stiff matrices. Both surfaces were smooth with root-mean-square (rms) surface roughness $\approx 5.5 \pm 0.6$ nm for 2D soft hydrogel and $\approx 6.2 \pm 0.5$ nm for 2D stiff hydrogel. Only nanoscale surface features were observed for 2D hydrogels, which is consistent with a previous study showing a pore size of ≈ 7 nm for poly(ethylene glycol) dimethacrylate (PEGDM) ($M_n \approx 8000$).^[15] The 3D hydrogels in this study contain meshwork of fibers with diameters of 322 ± 60 nm before hydration and 622 ± 147 nm after hydration (Figure 2A,B, and Figure S1, Supporting Information).

The shear storage moduli of the swollen hydrogels were measured using oscillatory bulk rheometry (Figure 2C). By modulating the initial concentration of PEGDM and UV time, the storage modulus at 1 Hz can be tuned from ≈ 0.5 to ≈ 5 kPa. For a Poisson's ratio of 0.5, this corresponds to Young's moduli of ≈ 1.5 kPa (soft) and ≈ 15 kPa (stiff), reflecting the stiffness of the healthy and the diseased subendothelial matrices.^[7,8]

2.2. Design and Characterization of ECM Protein Arrays

To mimic the major ECM compositions of healthy and diseased subendothelial matrices, ECM protein arrays were fabricated on three distinct hydrogels containing single and two-factor combinations of gelatin (G), collagen I (C1), collagen III (C3), collagen IV (C4), laminin (LN), and fibronectin (FN) (Figure 1B). Gelatin was used as a control since it is a well-established inert substrate for routine in vitro maintenance of vascular endothelial cells. The protein adsorption on various hydrogels was illustrated by immunostaining of C1, C4, and LN (Figure S2, Supporting Information). Intriguingly, the immunofluorescent signals of LN showed dotted structure and scattered distribution on 3D fibrous hydrogels in contrast to the continuous, uniform distribution on 2D hydrogels (Figure S2A, Supporting Information).

The immunofluorescent intensity of C1, C4, and LN on 3D fibrous hydrogels was lower than 2D hydrogels while no significant difference was observed between 2D soft and 2D stiff hydrogels (Figure S2B, Supporting Information). The endothelium tissue arrays were constructed by seeding ECs on the ECM protein arrays with cells preferentially attaching to the protein dots (Figure 1C).

2.3. Matrix Effects on Cell Attachment

In this study, we evaluated the individual and combined effects of matrix composition, stiffness, and structure on EC cell attachment (Figure 3). It was found that the number of attached cells varied not only with protein composition but also with hydrogel structure; remarkable differences were observed in the cell attachment for the same protein composition on 2D smooth versus 3D fibrous hydrogels. Notably, LN or LN-containing combinations (e.g., C1+LN, C4+LN) showed considerable cell attachment on 2D smooth hydrogels (Figure 3A). In sharp contrast, all LN-containing combinations showed quite limited cell attachment on 3D fibrous hydrogels (Figure 3B). Interestingly, such difference induced by the matrix structure was largely protein-dependent. For instance, similar cell attachments were found with the C4 matrices on 2D smooth and 3D fibrous hydrogels. This is further illustrated with corresponding images that depict adhesion profiles for some representative protein combinations on 2D smooth and 3D soft hydrogels (Figure 3C, and Figure S3, Supporting Information). These results highlight the combined effect of matrix protein composition and structure on cell attachment to the matrix. In addition, no statistically significant differences in the cell attachment were found with the same protein condition between 2D soft and 2D stiff hydrogels (Figure S4, Supporting Information), indicating that the matrix stiffness in our experimental range ($\approx 1\text{--}15$ kPa) does not significantly affect cell attachment.

It is worth noting that cell confluency varying with the protein composition may result in distinct cell-cell interactions, which further affects EC proliferation and function.^[16] To eliminate this confounding factor in our study, we chose to only evaluate representative protein combinations with acceptable cell confluency from both 2D and 3D hydrogels for our study of matrix effects on the proliferation, activation, and integrity of the endothelium. Furthermore, cell seeding density and time were optimized such that similar cell confluency was obtained among different protein combinations studied here (Figure 1C).

2.4. Matrix Effects on Endothelial Proliferation

Normal endothelium exists in a quiescent phenotype with a low level of proliferation in vivo, while some diseased risk factors, such as endothelial injury, stimulate the endothelial proliferation and turnover.^[17] Our immunostaining results on Ki-67 indicated that the EC proliferation was sensitive to both matrix composition and stiffness (Figure 4A,C, and Figure S5, Supporting Information). In particular, the C3 component induced the highest levels of cell proliferation among all protein combinations on both 2D soft and 2D stiff hydrogels, while several C4-containing protein combinations, for example, C1+C4, C3+C4, and C4+FN, inhibited the cell proliferation compared to others. The dependence of cell proliferation on the matrix composition showed similar trend on 2D soft and 2D stiff

hydrogels. However, higher matrix stiffness induced much higher levels of cell proliferation for a majority of the protein combinations.

In contrast, EC proliferation was remarkably inhibited by 3D fibrous structure (Figure 4B,C, and Figure S4, Supporting Information). Cells on 3D soft hydrogels showed significantly lower levels of cell proliferation than those on 2D soft hydrogels for all the protein combinations studied here. Notably, compared to the ECs on 2D soft hydrogels, there was an 80% and a 74% decrease in cell proliferation rates for the cells on the 3D soft hydrogels with C3 and C4, respectively. It is also interesting to note that the dependence of cell proliferation on the matrix composition of 2D soft hydrogels diminished in the case of 3D soft hydrogels, indicating that the 3D fibrous structure may play a dominant role in maintaining low levels of cell proliferation.

2.5. Matrix Effects on Proinflammatory Response of Endothelium: NF- κ B Activation

The activation of nuclear factor kappa B (NF- κ B; translocation from cytoplasm to nucleus) is known to play a significant role in upregulating inflammatory genes of ECs and subsequent recruitment of inflammatory cells, which are the hallmarks of many cardiovascular diseases such as atherosclerosis.^[18] We thereby studied the respective and combined effects of matrix composition, stiffness, and structure on the NF- κ B activation (nuclear localized) in ECs (Figure 5). For cells on 2D soft hydrogels, the NF- κ B activation index was low (≈ 0.5 for most protein combinations), while some C1- or FN-containing protein combinations exhibited relatively high levels of NF- κ B activation (Figure 5A). Compared to 2D soft hydrogels, 2D stiff hydrogels significantly increased the NF- κ B activation, with a strong dependence on the protein composition. Notably, cells on the C3 with 2D stiff hydrogels showed ≈ 2 -fold increase in the NF- κ B activation index when compared to those on the C3 with 2D soft hydrogels, indicating that the C3 protein augmented the inflammatory signals induced by matrix stiffening. Further, no statistically significant differences in the NF- κ B activation of ECs were found on 2D soft versus 3D soft hydrogels (Figure 5B), suggesting that the matrix structure might play less important roles in basal NF- κ B activation than the matrix composition or stiffness. Representative fluorescent images showed that NF- κ B signals were mainly located in the cytoplasm rather than the cell nuclei for the cells on 2D soft and 3D soft hydrogels (Figure 5C). In contrast, for the cells on 2D stiff hydrogels, portions of NF- κ B signals began to translocate from the cytoplasm to nucleus, especially for those on certain protein composition like C3.

Meanwhile, the respective and combined effects of the matrix composition, stiffness, and structure on the inflammatory responsiveness of ECs to tumor necrosis factor alpha (TNF- α) stimulation were investigated (Figure 6). Results showed that TNF- α induced a remarkable increase in the NF- κ B activation for all matrix conditions, and such increases were largely dependent on matrix composition, stiffness, and structure. For example, C4 yielded ≈ 2 -fold increases in the NF- κ B activation uniformly for ECs on 2D soft, 2D stiff, and 3D soft hydrogels, whereas C3 yielded ≈ 4 -fold, ≈ 3.5 -fold, and ≈ 1.5 -fold increases for ECs on 2D soft, 2D stiff, and 3D soft hydrogels, respectively. These data suggest that physiologically relevant healthy subendothelial matrix conditions, that is, C4-rich, 3D fibrous hydrogel, resulted in ECs more resistant to TNF- α stimulation, while disease-related subendothelial

matrix conditions such as C3-rich matrices aggravated the cell responsiveness to TNF- α stimulation. It should also be noted that TNF- α -treated ECs on 2D soft hydrogels in general exhibited lower NF- κ B activation than those on 2D stiff hydrogels regardless of matrix composition, whereas they showed higher or similar levels of NF- κ B activation when compared to those on 3D soft hydrogels (Figure 6B), which was composition-dependent. For instance, C3 induced higher levels of NF- κ B activation on 2D soft hydrogels versus 3D soft hydrogels, while C4 induced similar NF- κ B activation on these two types of hydrogels. The representative fluorescent images are shown in Figure 6C which illustrates NF- κ B activation in ECs with the translocation of NF- κ B signal into the cell nucleus. For example, TNF- α induced a significant NF- κ B translocation in ECs on C3 with 2D stiff hydrogels, demonstrating perfect colocalization of NF- κ B signal and nuclear signal, at the highest levels of NF- κ B activation among all the matrix conditions. In contrast, TNF- α -treated ECs on C4 with 2D soft or 3D soft hydrogels only displayed partial NF- κ B translocation, at the lowest levels of NF- κ B activation among all the matrix conditions.

Collectively, C3 component and matrix stiffening synergistically increased the basal and TNF- α -stimulated inflammatory signals, whereas C4 in concert with elastic matrix maintained low levels of inflammatory signals. Furthermore, the 3D fibrous matrix structure is important to lower the cell susceptibility to TNF- α stimulation, especially for matrix conditions containing C3.

2.6. Matrix Effects of Endothelium Integrity: VE-Cadherin Junction

The integrity of the endothelium is critical for healthy endothelial function. The disruption of EC junctions, as evident in many cardiovascular disease conditions, increases the endothelium permeability followed by infiltration of leukocytes through the exposed subendothelial matrix^[2a,c] As VE-cadherin is the major adherens junction molecule that maintains endothelium integrity,^[16] we examined its expression to investigate the endothelium integrity as a function of matrix composition, stiff, and structure (Figure 7). The ECs on all matrix conditions formed a confluent monolayer with a junctionally located, highly continuous pattern of VE-cadherin (Figure 7A). Interestingly, apart from the junctional VE-cadherin signals, a significant amount of cytoplasmic and nuclear signals of VE-cadherin were observed for ECs on both 2D soft and 2D stiff hydrogels while not the case for ECs on 3D soft hydrogels. Such strong internalization of VE-cadherin may be linked to the leakiness phenotype of ECs.^[19] Moreover, the measurement of the separation width of intercellular junctions revealed an increased distance between cells on C3 matrices when compared to C4 (Figure 7B). Notably, 3D soft hydrogels significantly decreased cell junction width when compared to 2D soft hydrogels regardless of matrix composition, suggesting that the 3D fibrous structure is favored during the formation of endothelial tight junction barrier.

Furthermore, we investigated the combined effects of the matrix composition, stiffness, and structure on the EC responsiveness to TNF- α stimulation (Figure 7). In response to TNF- α stimulation, the VE-cadherin pattern was markedly fragmented and with apparent disorganization (Figure 7A). Remarkably, for ECs on C3 with 2D stiff hydrogels, VE-cadherin junctions were completely disrupted in most cell-cell interactions forming

considerable intercellular gaps (arrowheads), and only diffuse VE-cadherin was found inside cytoplasm. In contrast, for cells on C4 with 2D soft or 3D soft hydrogels, VE-cadherin junctions were only partially fragmented. Further, with TNF- α stimulation, cells appeared more elongated and mobile with more cells migrating and escaping from the cellular islands (data not shown), which was more prevalent for those on 2D stiff hydrogels. Additionally, the cell-cell junction width significantly increased under TNF- α stimulation (Figure 7C). It was found that cells on 3D soft hydrogels exhibited less increase in the junction width in response to TNF- α stimulation when compared to 2D soft or 2D stiff hydrogels.

Taken together, C3 component and matrix stiffening synergistically aggravated the disruption of endothelium integrity induced by TNF- α stimulation. The high matrix elasticity and 3D fibrous structure jointly empowered the capability of maintaining endothelium barrier.

3. Discussion

Endothelial dysfunction or the loss of proper endothelial function, including EC overgrowth, proinflammation, and loss of barrier function, is a hallmark for cardiovascular diseases such as pulmonary hypertension and atherosclerosis. The subendothelial matrix, called basement membrane, is a thin, 3D fibrous ECM network underlying endothelium, providing signaling cues to the ECs through its biochemical constituents and biophysical properties. The biophysical cues are provided to the overlying ECs through its intrinsic topography (e.g., submicrometer 3D fibers and pores) as well as through its local stiffness, both of which can significantly influence EC functions.^[20] Dynamic basement membrane deposition and modification instructs coordinated endothelial behavior. Despite extensive prior investigations correlating select subendothelial ECM compositions with EC dysfunction, [9c,d,21] disease-relevant ECM remodeling involves complicated changes in multiple matrix proteins as well as their biophysical properties like stiffness.^[5] New exploratory tools which better recapitulate such matrix complexity may provide novel insights and comprehensive perspectives into the roles of matrix proteins in a sophisticated physiologically or pathologically relevant ECM context. To this end, we described a high-throughput, ECM microarray platform with highly tunable matrix stiffness and 3D structure, which is capable of recapitulating compositional complexity in healthy and diseased subendothelial matrices. With that, we systematically evaluated in a high-throughput manner the influence of matrix microenvironments including composition, stiffness, and/or structure, independently or cooperatively, on EC attachment, proliferation, NF- κ B activation, formation of VE-cadherin junction, and their responsiveness to proinflammatory stimuli (TNF- α). Our parametric studies of matrix effects reveal the respective and combined contributions of the matrix composition and biophysical properties to endothelial activation, which correlate well with healthy and diseased sub-endothelial microenvironments in vivo.

Strikingly, to the best of our knowledge, our study is the first time to demonstrate that 3D fibrous structure of matrices plays dominant roles in maintaining the stable, quiescent endothelium with strong resistance to inflammatory stimulation. Although it has been demonstrated that the electrospun, 3D fibrous structures mimic the natural ECM structure and supports the adhesion and growth of vascular ECs,^[22] few studies have explored how

such architectural feature impacts endothelial activities in relation to cardiovascular diseases. Interestingly, Han et al.^[9c] demonstrated that ECs cultured on 3D fibrous materials were less active in terms of thrombogenic-or inflammatory-related gene expression compared to those on 2D smooth films, though matrix stiffness as well as decoupled effects of matrix composition and structure were not taken into account. Recently, Chen's group demonstrated that the cells sense stiffness in 3D fibrous hydrogels in a way that is substantially different from the cells in 2D hydrogels.^[14] In line with our study here, these findings highlight the importance of investigating cell behavior in settings structurally similar to native ECM. Many efforts to circumvent the high cost of pharmaceuticals focus on development of in vitro cell culture models that can replace costly and time-consuming animal studies. However, cultured cells commonly fail to maintain differentiation and expression of tissue-specific functions, which may further contribute to the high attrition rate that we observed when drugs enter clinical trials.^[23] Our findings suggested that failing to reconstruct the 3D fibrous structure may be one of major obstacles to reconstitute the in vivo like endothelial functions and to achieve accurate prediction to clinical outcomes of vascular drugs with conventional 2D, stiff cell culture models.

Using a physiological-relevant matrix system, we found strong matrix composition dependence for EC activation. As a major component of diseased ECM, C3 significantly aggravated endothelial activation by increasing endothelial proliferation, NF- κ B activation, and disruption of endothelium integrity. In contrast, the compositions containing C4, a protein found in abundance in healthy subendothelial ECM, remarkably inhibited endothelial activation. However, it was nonintuitive to find some LN-containing conditions, especially together with stiffer matrix, displayed higher NF- κ B translocation on 2D hydrogels, which might be due to its departure from the in vivo setting regarding matrix protein density, unfolding, as suggested by recent findings.^[24] The complexity of endothelium-ECM interactions revealed in this study highlights the importance of using such cellular array platforms capable of measuring responses to more than individual proteins. We note that one limitation of our cellular array platform is that it cannot isolate cellular cross talk events through the medium (neighboring effects) since the entire array is exposed to the same medium chamber. But such cellular arrays are good for initial high-throughput screening of complex ECM microenvironments. Based on the screening results, in the future, we propose to scale-up selected individual conditions, such as the C3 and C4, to entire PEGDM hydrogels to further study the protein and gene expression without neighboring effects.

Our finding that matrix stiffening alone leads to a highly activated endothelium, characteristic of athero-prone phenotype, is in line with previous findings showing matrix stiffening increased EC monolayer disruption,^[10a] endothelial permeability,^[10b] and neutrophil transmigration.^[10c] These previous studies mostly applied an adhesive protein coating; however, little is known about effects of protein coating on endothelial responses to matrix stiffening. The interplay between matrix composition and stiffness was revealed by a recent study, where C4 coating coupled with physiologically relevant substrate elasticity was shown to improve the in vitro culture system for corneal EC expansion.^[9d] However, our study utilizes a more systemic approach toward parametric studies of matrix effects, through a large set of protein conditions and mimetic 2D and 3D hydrogel systems which more

closely resemble the complex compositions in healthy and diseased subendothelial matrices in vivo.^[4,5] Our data reveals that different matrix compositions can augment or mitigate the effects of matrix stiffening on ECs, providing new insights into pathologically relevant conditions. For instance, NF- κ B activation and disruption of VE-cadherin junctions induced by matrix stiffening of 2D hydrogels was promoted on C3 matrices, while appearing less on C4 matrices. Matrix stiffening has been shown to increase endothelial monolayer disruption and permeability through enhanced RhoA-mediated cell contractility.^[10a,b] We therefore speculated that the dependence of stiffness-induced endothelial activation on the matrix composition might be closely related to diverse receptor-ligand interactions presented by different matrix compositions. For instance, as two major collagen receptors, integrin $\alpha_1\beta_1$ prefers network-forming collagen type C4, whereas $\alpha_1\beta_1$ binds fibrillary collagen, such as C1 and C3, more strongly than C4.^[25] Such distinct receptor-ligand interactions would mediate cellular mechano-transduction,^[26] leading to altered interpretation of matrix stiffening, that is, altered RhoA-dependent cellular contractile response to matrix stiffening. Further investigation of the interplay between matrix composition and stiffness in molecular levels is necessary to uncover the underlying mechanism.

In addition, our findings reveal a significant interplay between matrix composition and structure in regulating cell attachment and confluency. For instance, in sharp contrast to the enhanced cell attachment to LN on 2D smooth hydrogels, few cells attached to all the LN-containing conditions on 3D fibrous hydrogels (Figure 3), which might result from different protein adsorptions, that is, density and distribution, on 3D fibrous versus 2D smooth hydrogels (Figure S2, Supporting Information). In line with this observation, Watt and Huck also demonstrated the strong interactions between protein tethering and underlying matrix structure, which further affect stem cell differentiation by altering the cellular mechanosensing.^[24a] Therefore, we propose two possible explanations for impaired cell attachment by the LN on 3D fibrous hydrogels. First, the reduced cell attachment seems associated with lower density of LN adsorbed on 3D fibrous hydrogels compared to 2D smooth hydrogels, as well as low integrin-ligand binding efficiency to LN. In agreement with our data, previous studies have shown that EC attachment is less effective upon LN compared to other ECM proteins, such as C1, C4, and FN.^[21c,27] Second, tethering profile of LN on 3D fibrous structures might be unfavorable to cell attachment. The dotted structure and scattered distribution of LN on 3D fibrous hydrogels revealed that the distance between anchoring points on 3D fibrous hydrogels is likely longer, when compared to the continuous, uniform presentation of LN on 2D smooth hydrogels. Such matrix structure-induced difference in tethering profiles was largely dependent on the protein type since both C1 and C4 displayed similar tethering profiles between 3D fibrous and 2D smooth hydrogels. Nevertheless, further investigation into the interplay between matrix structure and the superimposed meshwork of ECM proteins in micro/nanoscales is needed to better understand this observation.

4. Conclusions

Here, we demonstrate that complex subendothelial microenvironments may be captured by combining 2D and 3D hydrogel settings with combinatorial ECM microarray, which provides a high-throughput platform to investigate how matrix composition, stiffness, and

structure affect endothelium functions, both independently and cooperatively. Our findings, for the first time, demonstrate that 3D fibrous structure resembling native subendothelial ECM plays critical roles in maintaining healthy endothelium with strong resistance to proinflammatory stimuli. Our results also provide direct evidence that the diseased protein compositions, particularly the presence of the C3, in concert with matrix stiffening serve as a potent endothelium-destructive factor by initiating EC proliferation, NF- κ B activation, and disruption of endothelium integrity as well as the susceptibility to proinflammatory stimuli. Our data suggests a significant, as well as complex, interplay between matrix composition, stiffness, and structure exists in the regulation of both healthy and pathological endothelium. Furthermore, this work highlights the importance of the ECM microenvironments when creating more physiologically relevant models in vitro toward improved drug discovery in cardiovascular related diseases.

5. Experimental Section

Fabrication and Characterization of 2D and 3D Hydrogels:

For 2D hydrogels, the macromer solution was prepared by dissolving 10% (w/w) PEGDM (MW 750 Da; Sigma, St. Louis, MO) for soft gel or 15% (w/w) PEGDM for stiff gel, and 0.05% (w/w) photoinitiator solution I2959 (Ciba Specialty Chemicals Corp., Tarrytown, NY) in deionized (DI) water. A 120 μ L drop of macromer solution was pipetted onto the 25 $\times$ 75 mm standard glass slide pretreated with (3-trimethoxysilyl) propyl methacrylate (TMPMA; Sigma), and an untreated 24 $\times$ 60 mm coverslip was carefully placed on top of the liquid to form a thin layer estimated to be 100 μ m thick. The solution was polymerized with UV light ($\approx$ 5 mW cm⁻²) for 5 min (soft gel) or 10 min (stiff gel), and the 24 $\times$ 60 mm coverslip was removed after incubation in DI water overnight. Gel-coated slides were dried on a hot plate at 40 $^{\circ}$ C for 30 min.

For 3D soft hydrogels, an electrospinning technique was used to prepare the soft fibrous PEG DM hydrogel network, as previously described.^[11d] Briefly, an electrospinning solution composed of 2.6% (w/w) PEGDM, 3.4% (w/w) polyethylene oxide (PEO; MW 400 kDa; Sigma), and 0.03% (w/w) I2959 (6 mg mL⁻¹ in DI water) in DI water was prepared. The solution was electrospun ($\approx$ 100 μ m thick after swelling) onto TMPMA pretreated 25 $\times$ 75 mm standard glass slide and exposed (in the dry state) to UV light ($\approx$ 5 mW cm⁻²) for 30 min under inert atmosphere (Ar).

To examine the surface topography, an AFM (EasyScan 2, Nanosurf AG, Switzerland) was used. The AFM images were obtained in tapping mode, and the rms was used to evaluate the surface roughness of the 2D hydrogels on the basis of a 20 μ m $\times$ 20 μ m scan area. A SEM (JEOL JSM-6480LV) was used to examine the microstructure of the eletrospun fibrous network before (dry) and after (hydrated and then lyophilized) swelling. ImageJ was used to analyze changes in fiber diameter. To measure the elasticity of the hydrogel matrix, the elastic modulus (G') was measured on an ARES TA rheometer (TA instruments, New Castle, DE) and data were collected from the linear viscoelastic region in the strain sweep (at a frequency of 1 rad s⁻¹). The Young modulus (E) was calculated using the formula $E = 2G(1+\nu)$ with $\nu = 0.5$.^[28] The loss modulus is much lower than the storage modulus ($G'' \ll$

G') for the hydrogels, indicating that these gels primarily exhibit elastic behavior (data not shown).

ECM Protein Array Preparation:

A printing buffer consisting of 0.1 M acidic acid (Sigma), 1% glycerol (Sigma), and 0.05% Triton X-100 (J.T. Baker, Phillipsburg, NJ) was prepared. The pH was adjusted ($\approx$ pH 5.0) to inhibit protein polymerization. For ECM arrays, stock solutions of gelatin extracted from porcine skin (Sigma), collagen I extracted from rat tail (Sigma), collagen III extracted from human placenta (Sigma), collagen IV extracted from human placenta (Sigma), fibronectin purified from human plasma (Corning, NY), and laminin extracted from Engelbreth-Holm-Swarm mouse tumor (Corning) were suspended at $500 \mu\text{g mL}^{-1}$ in the printing buffer. ECM protein solutions were then mixed in 21 combinations into a 384-well plate. Three individual spots of each protein combination were deposited with a $500 \mu\text{m}$ pitch on both 2D and 3D hydrogels using an Aushon 2470 arrayer equipped with 185 micro pins (Aushon BioSystems, Billerica, MA). Between different protein depositions, the print needles were cleaned by sonication in cleaning solution before use.

Cell Culture:

Bovine pulmonary artery endothelial cells (BPAECs; American Type Culture Collection CCL-209; Rockville, MD) were grown in Eagle's minimum essential medium (EMEM; ATCC 30-2003) supplemented with 20% fetal bovine serum (FBS; 35-0101-CV; Corning). Cells at passages 19–23 were used for all experiments.

Cell Seeding and TNF- α Treatment:

Prior to cell seeding, hydrogel-coated slides were equipped with eight-well ProPlate slide module (Grace Bio-Labs, Bend, OR) to partition individual ECM protein array replicates. The hydrogel-coated slides were washed in PBS and sterilized in 70% ethanol for 15 min to reduce potential contamination, followed by rinsing in sterile serum-free culture media. Cell suspensions of 15×10^4 cells in 0.3 mL serum-free media were seeded into each silicone well and incubated for 2 hours to allow for cell attachment (shaking the plates every 15 min to redistribute the cells). After cell attachment, the media was gently aspirated to remove unattached cells and fresh culture media with 10% FBS was added. The culture media was changed every second day. After culturing the cells on the ECM arrays for 3 days, cells were treated with 20 ng mL^{-1} recombinant bovine TNF- α protein (R&D Systems, Minneapolis, MN) in serum-free media for 6 hours.

Immunofluorescence:

Protein arrays or endothelium arrays were fixed with 4% formaldehyde (Fisher Scientific, Fair Lawn, NJ) for 10 min, permeated with 0.1% Triton X-100 for 15 min (note: permeabilization was not necessary for protein array), and blocked with 10% goat serum (Millipore, Billerica, MA) for 30 min at room temperature. Following that, the protein arrays or endothelium arrays were treated with primary antibodies overnight at 4°C , incubated with secondary antibodies for 60 min at room temperature, and finally counter-stained with 4',6-diamidino-2-phenylindole (DAPI; $1 \mu\text{g mL}^{-1}$; D9542; Sigma). Antibodies were used at

the following dilutions: 1:50 for polyclonal rabbit anti-human collagen IV primary antibody (AB748; Millipore), 1:50 polyclonal rabbit anti-mouse laminin primary antibody (PA1-16730; ThermoFisher Scientific, Fair Lawn, NJ), 1:200 for monoclonal rabbit anti-bovine Ki-67 primary antibody (RM-9106-S; ThermoFisher Scientific), 1:100 for polyclonal rabbit anti-bovine NF- κ B p65 primary antibody (sc-109; Santa Cruz Biotechnology Inc., Santa Cruz, CA), 1:100 for polyclonal rabbit anti-bovine VE-cadherin primary antibody (sc-28644; Santa Cruz Biotechnology Inc.), and 1:500 for goat anti-rabbit IgG (H+L) secondary antibody, Alexa Fluor 555 conjugate (A-21428; ThermoFisher Scientific). All samples were finally mounted with Vectashield HardSet mounting media (Vector Laboratories, Burlingame, CA) and confocal images were acquired using a Nikon Spinning Disc Confocal.

Image Analysis:

All image processing and analysis were performed in ImageJ (U.S. National Institutes of Health, Bethesda, MD). The same sized regions of interest (ROIs) were traced around the perimeter of each individual microtissue dot in the array to evaluate the fluorescent signals of each microtissue dot. For cell counts, the DAPI and Ki-67 images were converted into binary images and the segmentation was achieved by “Watershed.” The cell number was then determined with the “Analyze particles” tool. The fluorescent intensity of the cell nuclei signals was also quantified. To determine the NF- κ B activation index (or the NF- κ B nuclear-to-cytosolic ratio), the binary masks of the nuclei (0/1) were generated using the “Math” tool. Fluorescent NF- κ B images were then multiplied by the binary masks of the nuclei (0/1) using the “Image calculator” tool to isolate NF- κ B signal in the nuclei only. After that, the cytoplasmic NF- κ B signal was determined by deducting the intranuclear NF- κ B signals from the total NF- κ B signals. The VE-cadherin junction width was quantified using previously reported method,^[29] a line was drawn perpendicular to the cell junction in ImageJ to obtain a pixel intensity profile across each junction. After that, the intensity profile was fit with a two-Gaussian curve in MATLAB and junction widths were defined as the width of the curve 20% above the baseline pixel intensity.

Statistical Analysis:

Unless otherwise specified, data presented as mean $\pm$ s.e.m. All the cell data were quantified from at least three independent experiments. For each experiment, at least three printed slides (eight array replicates per slide, three ECM protein replicates per array) of each hydrogel were analyzed. Two-way ANOVA with Tukey’s multiple comparison test was used to analyze statistical significance. A p -value < 0.05 was considered statistically significant. Within the figures, the significance is denoted by the following marks: * or # for $p < 0.05$; ** or ## for $p < 0.01$; and *** or ### for $p < 0.001$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Extracellular matrix (ECM) microarray-based approach for investigation of endothelium function. A) Schematic of fabrication and utility of ECM microarray platform. B) Design of the ECM microarray layout consisting of 21 ECM combinations with three replicates for each combination. C) Representative confocal image of the endothelium array after 24 h cell culture with uniform cell confluency over most of the cellular islands (green: F-actin; blue: nuclei; scale bar: 500 μm).

Figure 2.

2D and 3D PEGDM hydrogels with tunable matrix structure and stiffness. A) Topographic AFM images of 2D soft, 2D stiff, and 3D soft hydrogels (left: 2D topographic mapping; right: 3D topographic mapping). B) Quantification of fiber diameters before (dry) and after swelling (hydrated) from SEM images of 3D soft hydrogels. C) Young's modulus E of 2D soft, 2D stiff, and 3D soft hydrogels. Significant difference is marked as * versus dry in (B) and * versus 2D soft in (C). $n = 3$, mean $\pm$ s.d.

Figure 3.

Cooperative actions of matrix composition and structure on EC attachment. Quantification of cell number attached to various matrix compositions on A) 2D soft hydrogel and B) 3D soft hydrogel; $n = 3$, mean $\pm$ s.e.m. C) Fluorescent images showing cell nuclei staining of representative 2D and 3D microtissue dots. Scale bars: 200 μ m.

Figure 4.

Cooperative actions of matrix composition, stiffness, and structure on EC proliferation. A,B) Quantification of Ki-67 proliferation index (% of Ki-67 positive cells; $n = 3$, mean $\pm$ s.e.m.). C) Representative fluorescent images showing Ki-67 (red) and nuclei (blue) staining of cells on various matrix combinations. Significant difference is marked as * versus G, # 2D soft versus 3D soft. All groups show statistically significant difference 2D soft versus 2D stiff except the groups marked with no significant difference (ns). Scale bar: 200 μ m.

Figure 5. Cooperative actions of matrix composition, stiffness, and structure on EC proinflammatory response (NF-κB activation). A,B) Quantification of NF-κB (p65) activation index (relative localization of NF-κB: nucleus/cytoplasm; $n = 3$, mean $\pm$ s.e.m.). C) Representative fluorescent images showing NF-κB (red) and nuclei (blue) staining of cells on various matrix combinations. Significant difference is marked as * versus G. Scale bar: 100 μ m. The insets show the magnified images highlighting the translocation of NF-κB (red) into nuclei (blue), under inflammatory condition.

Figure 6.

Cooperative actions of matrix composition, stiffness, and structure on EC proinflammatory response (NF- κ B activation) to TNF- α stimulation. A,B) Quantification of NF- κ B (p65) activation index (relative localization of NF- κ B: nucleus/cytoplasm; $n = 3$, mean $\pm$ s.e.m.). C) Representative fluorescent images showing NF- κ B (red) and nuclei (blue) staining of cells on various matrix combinations. Significant difference is marked as * versus C; # 3D soft versus 2D soft. Scale bar: 100 μ m. The insets show the magnified images highlighting the NF- κ B translocation into nuclei.

Figure 7.

Cooperative actions of matrix composition, stiffness, and structure on endothelium integrity with (+) and without (–) TNF- α treatment. A) Representative fluorescent images showing VE-cadherin staining of cells on various matrix combinations. Higher magnification images showed leakiness between cells. B,C) Tukey's box plot quantification of cell junction width. No data for C3 at 2D stiff hydrogels were measured, because all cell junctions were disrupted. Significant difference is marked as * versus C3; # versus 2D soft. Scale bar: 100 μ m.