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The Role of ERK Signaling in Protein Hydrogel Remodeling by Vascular Smooth Muscle Cells

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Abstract

Collagen Type I and fibrin hydrogels have been used for cell-based therapies and tissue engineering. These matrices can be broken down and remodeled by cells, but the effects that these proteins have on cell function are not completely understood. We examined activation of the extracellular-signal regulated kinase (ERK) signaling pathway by vascular smooth muscle cells (VSMC) in response to 2D and 3D matrices of Type I collagen, fibrin, or a 1:1 composite mixture of these proteins. After 3 days of culture, ERK phosphorylation, osteopontin secretion, and MMP-2 activation were all markedly increased in 3D matrices, compared with 2D substrates. A strong positive correlation existed between these protein markers of the synthetic phenotype and phosphorylated ERK levels, and this relationship persisted across matrix geometries and compositions. Cell proliferation in 3D matrices was inversely correlated to ERK activation, while on 2D substrates a modest positive correlation was observed. Pharmacologic inhibition of ERK signaling confirmed that this pathway was involved in the observed phenotype shifts. This study suggests that contextual activation of the ERK pathway results in different effects on cell phenotype, depending on the geometry and composition of the ECM. These findings add to our understanding of cell function and remodeling in protein-based hydrogel biomaterials.

Keywords

collagen; fibrin; smooth muscle cells; ERK; osteopontin; MMP-2

1. Introduction

Protein-based hydrogels are used in a variety of biomaterials applications. In particular, our laboratory has examined hydrogels of collagen Type I and fibrin, as well as mixed composites of these two proteins (collagen-fibrin), as biomaterials for use in tissue engineering. These naturally derived polymers were selected as 3D scaffolds due to their structural and bioactive roles in tissues. Collagen type I is a major component of the vascular media and most other tissues, and has been used widely as a biomaterial in vascular tissue engineering due to its known function as a structural protein, as well as its cell binding and signaling properties [1, 2,3]. Similarly, the blood clotting protein fibrin has been used for 3D culture and as a scaffold for vascular tissue engineering [4,5]. Composite biomaterials of collagen and fibrin also are of interest as scaffolds in tissue engineering, since the possibility exists of combining the structural properties of collagen and the bioactive properties of fibrin to create improved matrices for engineered tissues [6,7].

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In the present study we have used controllable two- and three-dimensional (2D, 3D) protein matrices of collagen Type I and fibrin to understand how varying extracellular matrix (ECM) composition and geometry (i.e. 2D versus 3D) affect ERK signaling and the phenotypic state of vascular smooth muscle cells (VSMC). The sequence of events that leads from an altered ECM environment to altered VSMC function has not yet been fully elucidated, but the extracellular signal-regulated kinase (ERK) branch of the mitogen-activated protein kinase (MAPK) pathways has been implicated in phenotype shifts caused by varying ECM compositions [8]. Previous studies on 2D protein substrates have shown that collagen Type I and fibronectin tend to induce a more synthetic phenotype [9,10], whereas cells cultured on basement membrane proteins such as collagen Type IV and laminin tend to retain a more contractile state [11,12]. The effect of 3D protein matrices has been studied using fibroblast-populated collagen gels (for a review, see e.g. [13]), but the effects on VSMC are less well characterized. Importantly, it has been shown that there are important differences between monolayer studies and corresponding 3D experiments [14]. In the case of VSMC, both the composition and structural nature (2D or 3D) of the matrix have been shown to affect cell proliferation and function [15], and a genomic analysis of VSMC cultured on 2D collagen substrates showed different gene expression patterns, compared with cells in 3D collagen lattices [16]. Such differences between 2D and 3D environments have implications on the study of vascular biology, as well as to our understanding of the pathogenesis of cardiovascular disease; however, the mechanisms by which ECM geometry and composition affect cell phenotype and function are not well defined.

Communication between the ECM and cells is crucial to tissue remodeling and repair. Specific cell-matrix interactions mediate cell functions, including adhesion, proliferation, migration, and differentiation. Cell attachment to the ECM can be mediated by the matricellular glycoprotein osteopontin via $\alpha v \beta 3$ and other integrins [17]. While most studies have focused on the role of osteopontin in mineralized tissue, it is now clear that it is involved in vascular injury [18]. For example, levels of osteopontin expression are increased in injured arteries [19,20] and osteopontin mRNA is strongly upregulated during the shift from contractile to synthetic phenotypes [21]. In addition, it has been shown that stimulation of SMCs with osteopontin leads to increased MMP levels [22]. These results suggest that osteopontin and MMP-2 may act in coordination to guide remodeling of the ECM by VSMC, a process that is important in the development of vascular disease as well as in the creation of engineered vascular tissues.

In the study reported here, we have characterized the expression of osteopontin and activation of MMP-2 by VSMC cultured as monolayers and in 3D matrices of collagen Type I, fibrin, or 1:1 mixtures of the two (collagen-fibrin). We also examined the role of ERK signaling in the expression of these proteins, and verified this role through ERK inhibition studies. Our results show that VSMC phenotype and ERK signaling are markedly altered in 3D matrices, relative to 2D ECM cultures, and further suggest a causative link between ERK signaling and phenotype shifts in 3D matrices. Understanding the mechanisms that govern cell behavior in the 3D cellular environment could lead to improved therapies for a variety of diseases in which phenotype shifts are implicated, including atherosclerosis and hypertension. In addition, this knowledge would be of benefit in the field of vascular tissue engineering, by enabling the control of cell function in order to tailor the properties of tissues grown *in vitro*.

2. Materials and Methods

Cell Culture

Rat aortic smooth muscle cells (RASMC) were isolated from normal adult male Sprague-Dawley rats using a collagenase digestion method [15], and were used for experiments between passages 5 and 8. Cells and ECM constructs were maintained in Dulbecco's Modified Eagle

Medium (DMEM, Mediatech Inc, Hendon VA) supplemented with 10% fetal bovine serum (FBS, Mediatech), 2 mM L-glutamine (Mediatech), 100 U/ml penicillin G sodium (Mediatech), and 100 µg/ml streptomycin sulfate (Mediatech). The medium was changed every 2-3 days and plated cells were enzymatically removed from the flask using 0.05% trypsin (Mediatech).

Preparation of 2D Collagen, Fibrin, and Collagen-Fibrin Gels

Pure collagen substrates were made at 2.0 mg/ml protein content using bovine Type I collagen (MP Biomedicals, Solon OH). The lyophilized protein was dissolved in 0.02 N acetic acid to obtain a 2X collagen solution (4.0 mg/ml). The ingredients for the final collagen substrates were combined: 2X collagen solution (50%), FBS (10%), 0.1 N NaOH (10%), 5X DMEM (14%), and DMEM (16%). This combination of constituents maintained the appropriate pH and osmolarity during gel formation, so that cell viability was preserved.

Pure fibrin gels were also made at 2.0 mg/ml protein content using bovine plasma Type IV fibrinogen (Sigma Chemical Co., St. Louis MO). The lyophilized protein was dissolved in cold DMEM supplemented with 4.0 mg/ml ε-amino caproic acid (ACA, Sigma), 2.0 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin to obtain a 2X fibrinogen solution. ACA is an inhibitor of plasmin activity. The ingredients for the final fibrin solution were mixed: 2X fibrinogen solution (50%), FBS (10%), and DMEM containing 0.1 U thrombin per mg fibrinogen (40%).

Collagen-fibrin gels were made using a 1:1 ratio of collagen to fibrin to obtain a final protein content of 2.0 mg/ml. ECM solutions and other reagents were prepared as described above.

Two-dimensional ECM substrates were formed by coating tissue culture plastic flasks with ECM solutions (2.0 mg/ml), and incubating them for 1 hour at 37°C. Each flask was rinsed with PBS before RASMC were plated at a cell density of 2.0×10^4 cells/cm² of the culture surface. Cells were allowed to adhere undisturbed for at least 24 h, and culture medium was changed after 1, 2, and 6 days in culture.

Preparation of 3D Collagen, Fibrin, and Collagen-Fibrin Gels

Three-dimensional constructs using pure collagen, pure fibrin, and collagen-fibrin mixtures were prepared by mixing the ECM solution with cells at a final concentration of 1.0×10^6 cells/ml. The cell-ECM suspension was then gently pipetted into a 6-well plate, which was subsequently incubated at 37.0 °C for 30 minutes to allow for gelation. After gelation, the edges of constructs were freed using the tip of a Pasteur pipette to allow for unconstrained compaction.

Inhibition of ERK Phosphorylation

The MEK inhibitor PD98059 (Alexis Biochemicals Inc., San Diego CA) was used to pharmacologically inhibit the phosphorylation of ERK. In a microfuge tube, 340 µl of DMSO were added to 5.0 mg of PD98059 to obtain a 1000 × stock concentration of 5.0 mM. For ECM samples, the ingredients of each matrix were supplemented with the addition of 1.0 µl of PD98059 stock solution per 1.0 ml of total volume to obtain a final PD98059 concentration of 5.0 µM. The culture medium also was supplemented to obtain a final inhibitor concentration of 5.0 µM.

Sample Collection

After 1, 3, and 7 days in culture, samples were collected and frozen. Media samples were collected and supplemented with a protease inhibitor solution (Roche Applied Science Inc., Indianapolis IN) at a 9:1 ratio. Constructs were rinsed with PBS and rapidly frozen using liquid nitrogen and stored at -80 °C.

Cell Number Quantification

The number of cells per construct was determined using a Hoechst dye-based assay. Frozen constructs were thawed, dehydrated using a vacuum oven at 37 °C for 3 hours, and digested in a Proteinase K (Sigma) lysis buffer at 55 °C for 16 hours. After constructs were completely dissolved, they were vortexed thoroughly, diluted appropriately, and loaded into a black 96-well plate in duplicate. Blanks, cell standards, and DNA standards (Bio-Rad Laboratories Inc., Hercules CA) were also loaded in duplicate to generate standard curves to determine cell number from fluorescence readings. Hoechst 33258 dye (Sigma) solution was added to each well. The plate was incubated in the dark at room temperature for 15 minutes. Fluorescence was measured using a microplate reader (BioTek, Winooski VT) at 25°C using an excitation wavelength of 365 nm and an emission wavelength of 458 nm. The blank readings were subtracted from all samples and standards. The standard curve was used to interpolate the number of cells per construct.

Total Protein Extraction from Cells and Constructs

All samples were lysed using ice cold RIPA buffer supplemented with a protease inhibitor cocktail. Two-dimensional samples were scraped off the flask bottom using a plastic scraper. Frozen gel constructs were homogenized by hand using a tissue grinder. To remove cellular debris and insoluble ECM, the lysates were then clarified at 4,500 g at 4 °C for 30 minutes.

Enzyme Immunometric Assay for Osteopontin

Osteopontin was quantified in the reserved media samples using a commercially available rat osteopontin EIA kit (Assay Designs Inc., Ann Arbor MI) following the manufacturer's protocols. Briefly, the media samples, blanks, and standards were appropriately diluted using the provided assay buffer. They were then pipetted into the provided 96-well plate and incubated for 1 hour at 37 °C. Following several washes, the labeled antibody was pipetted into the wells and incubated for 30 minutes at 4 °C. After extensive washing, the substrate solution was pipetted into the wells where the solution was allowed to develop in the dark for 30 minutes at room temperature. Upon the completion of development, a stop solution was added. The absorbance of the blanks, samples, and standards were read on a microplate reader at a wavelength of 450 nm.

Enzyme Immunometric Assay for pERK

To quantify the concentration of pERK in cell lysates, a commercially available pERK EIA kit (Assay Designs) was used following the manufacturer's protocols. In brief, cell lysates were diluted appropriately with the provided assay buffer and were pipetted into the 96-well plate along with blanks and standards. The plate was incubated at room temperature for 1 hour, and was then washed several times. The primary antibody was added to the plate and allowed to incubate for one hour at room temperature, after which it was washed thoroughly. Next, a conjugated secondary antibody was added to the wells, and allowed to incubate for 30 minutes at room temperature. The wells were carefully washed again. The substrate solution was added to the well and the plate was allowed to incubate for 30 minutes at room temperature. A stop solution was added to terminate the development of the plate, whose absorbance was then read at 450 nm.

Gelatin Zymography

To determine the relative quantity of MMP-2 in the media supernatant, we used commercially available 10% Zymogram Ready Gels containing 1% gelatin (BioRad). Media was diluted appropriately using a zymogram sample buffer (BioRad). After running the gel at 150 V for 55 minutes, gels were incubated in renaturation buffer (BioRad) for 30 minutes at RT. Following three washes with distilled water, the gel was incubated in Zymogram Development

Buffer (BioRad). The gel was then stained using Simply Blue Safe Stain (Invitrogen Corp., Carlsbad CA), and digitally imaged. Graphics software measured relative band densities. There were three bands representing the different fractions MMP-2. The high-molecular weight band contained the proenzyme; the intermediate band contained the intermediate enzyme; the low-molecular weight band represented the activated fraction.

Data Analysis

Assays were normalized as appropriate. Proliferation is expressed here in units of fold, where 1-fold means there was no change in the population and 2-fold means the population doubled; this is a change relative to original seeding density. ERK phosphorylation, OPN secretion, and MMP-2 activation were normalized to cell number present under each culture condition as determined by the cell number assay.

For all time points (days 1, 3, and 7), ECM geometries (2D and 3D), and ECM compositions (collagen, collagen-fibrin, and fibrin), the normalized values from four independent experiments were averaged and expressed as mean $\pm$ standard error (SE). Data sets were statistically analyzed for significance using unpaired, two-tail Student's t-test. Significance of correlations was determined using the Fisher Transformation.

3. Results

Effect of three-dimensional culture and ECM composition

Images of formed 3D protein matrices are shown in Figure 1 at both the macroscopic (panels A-C) and microscopic (panels D-G) length scales. Cells were embedded directly into these hydrogels during gel formation, such that they were surrounded by matrix. The original volume of the protein constructs was 3.0 mL, but the gels underwent cell-mediated compaction and by day 3 the volume was reduced to 1.2 to 1.8 mL, depending on the matrix composition. The corresponding decrease in gel diameter is shown in panels A-C of Figure 1, from an initial diameter of 35 mm to a final diameter of 11-15 mm at day 3 in culture. Scanning electron microscope analysis of the matrices showed collagen (panel D) and fibrin (panel F) fibrils. Mixed collagen-fibrin (panel E) constructs showed the larger collagen fibrils surrounded by a mesh-like network of fibrin. Panel G of Figure 1 shows a scanning electron micrograph of cells at the surface of a collagen-fibrin composite construct. Note that the cells were well spread and clearly were interacting with the protein fibers, with pseudopodia extending into the matrix.

Figure 2 shows that the geometry (i.e. 2D vs 3D) of the ECM environment markedly affected osteopontin secretion, MMP-2 activation, cell proliferation, and ERK activation. In Figure 2A it is evident that cells embedded within 3D ECM produced more osteopontin than their 2D counterparts. Figure 2B shows MMP-2 activation, and the same trend is observed. Both total MMP-2 and activated MMP-2 (low molecular weight band) demonstrated the same trend, but activated MMP-2 is reported here because it was the predominant fraction of total MMP-2 in our experiments. In panels A and B it can be seen that the presence of the ECM proteins in both 2D and 3D led to an increase in osteopontin and activated MMP-2, relative to culture on tissue culture plastic. In contrast, cell proliferation, which is typically a hallmark of the synthetic phenotype in VSMC, was markedly lower in 3D than in 2D, and much lower than on tissue culture plastic, as shown in Figure 2C. In fact the population of cells in 3D matrices declined slightly during the period of culture, while cells on 2D substrates actively proliferated. ERK activation (Figure 2D) mirrored the trends seen with osteopontin and MMP-2, in that 3D matrices increased levels relative to 2D. Although the levels of activated ERK varied between experimental conditions, there was no significant difference in total ERK protein expression. We also examined two other branches of the MAPK family, the JNK pathway and the p38

pathway, but found no correlation with osteopontin expression or MMP-2 activation (data not shown).

Figure 2 also shows that the composition of the matrix (*i.e.* collagen, fibrin, or mixtures) influenced the parameters of interest. In 3D constructs, the lowest levels of osteopontin and activated MMP-2 were in pure fibrin constructs and the highest were in pure collagen matrices, as shown in Figures 2A and 2B. By mixing collagen and fibrin, a modulated response was seen in osteopontin secretion, but there was no statistical difference in MMP-2 activation between 3D collagen and 3D collagen-fibrin. In 2D culture, the specific responses elicited by the ECM were different than those seen in 3D. Osteopontin secretion was highest in 2D fibrin compared with collagen-containing 2D ECM, while 2D collagen and collagen-fibrin had comparable levels of osteopontin secretion. MMP-2 activation was highest in 2D collagen-fibrin compared with other monolayer cultures. Whereas the presence of ECM lowered proliferation as shown in Figure 2C, specific composition had no significant effect in both 2D and 3D cultures. Like osteopontin, ERK phosphorylation in 3D cultures was highest in pure collagen and lowest in pure fibrin, as shown in Figure 2D. In monolayers, the presence of ECM led to elevated levels of ERK phosphorylation. Collagen-containing monolayer cultures had comparable levels of ERK phosphorylation, which were higher than 2D fibrin.

Effects of geometry on temporal expression of phenotype markers

The temporal pattern of the parameters studied varied depending upon the geometry of the matrix, as shown in Figure 3. Osteopontin secretion (Figure 3A) and MMP-2 activation (Figure 3B) decreased over the course of 7 days in both tissue culture plastic and 2D collagen. This trend was not observed in 3D collagen matrices nor did osteopontin secretion directly mirror MMP-2 activation. In 3D collagen, osteopontin secretion nearly doubled between days 1 and 3, and reached a plateau by day 7. The reverse was observed with MMP-2 activation: activated MMP-2 was consistent between days 1 and 3, but dropped sharply on day 7. Cell number (Figure 3C) increased over the course of 7 days on tissue culture plastic and 2D collagen, however it decreased in 3D culture. The same temporal trends were observed for collagen-fibrin and pure fibrin matrices (data not shown). Unlike the other three parameters, activation of ERK (Figure 3D) over varying geometry led to differing temporal patterns between the three culture environments. In the 2D TCP case there was no statistical difference in pERK between the different time points. The presence of a 2D matrix resulted in a decrease in pERK over the course of 7 days. In 3D collagen matrices, pERK peaked on day 3 and fell to nearly half its maximum by day 7.

Correlations between cell function and ERK Signaling

Taken together, the data presented in Figure 2 demonstrate that the presence and composition of ECM proteins modulates osteopontin secretion and MMP-2 expression, and furthermore that the effects of these proteins are dependent on whether they are presented as a 2D or 3D matrix. In addition, cell proliferation tended to be decreased in the presence of ECM proteins. Figures 4 and 5 show plots of osteopontin secretion, MMP-2 activation, and cell proliferation versus ERK activation. Correlation coefficients were determined for these three parameters over varying matrix geometries (Figure 4) and compositions (Figure 5) by combining all data points. The correlation coefficient (R) between pERK and osteopontin over varying ECM geometry was $R = 0.98$ ($P < 0.05$), indicating a strong positive correlation (Figure 4A). MMP-2 activation and pERK were also strongly correlated as shown in Figure 4B, with $R = 0.92$ ($P < 0.1$). The relationship between pERK and proliferation was not linear, but was fit using a logarithmic relationship with $R = -0.99$ ($P < 0.05$), indicating a negative correlation in this case (Figure 4C). Similar correlations were obtained when the data was analyzed across matrix compositions, as shown in Figure 5. The correlation between pERK and osteopontin was strongly positive, with $R = 0.99$ ($P < 0.05$) (Figure 5A). MMP-2 activation and pERK also were

strongly correlated, with $R = 0.90$ ($P < 0.1$) (Figure 5B). The correlation between ERK activation and proliferation was not as strong across matrix compositions ($R = 0.78$, $P < 0.15$), as shown in Figure 5C. The correlations revealed by this analysis suggest that ERK activation is involved in modulating osteopontin secretion and MMP-2 activation in VSMC. To determine whether these relationships were causative, ERK was pharmacologically inhibited and the effect on the parameters of interest was investigated.

Effects of ERK Pathway Inhibition

The small molecule PD98059 was used to inhibit ERK's upstream kinase MEK-1, rendering phosphorylated ERK levels undetectable following inhibition for the duration of the culture period (data not shown). ERK pathway inhibition greatly diminished osteopontin secretion and MMP-2 activation in both 2D and 3D cultures, as shown in Figures 6A and 6B. Osteopontin secretion in 3D matrices was affected more strongly than in monolayers. MMP-2 activation in TCP cultures was most affected (Figure 6B), but again 3D matrices were more affected than their 2D counterparts. The effect of ERK inhibition on proliferation depended upon matrix geometry, as seen in Figure 6C. In 2D cultures, ERK inhibition resulted in a marked drop in proliferation across all matrix compositions. In 3D matrices, ERK inhibition counteracted the decrease in cell number seen in 3D constructs, resulting in a higher cell number. It should be noted that in this case the cell population did not increase; instead, the positive change shown in Figure 6C is due to a decrease in cell loss in 3D constructs over the culture period. In general, the differences in response to ERK inhibition were not as pronounced across different matrix compositions, relative to across matrix geometry. However, these results suggest a causative link between the ERK pathway and the phenotype shifts seen in VSMC in response to different matrices.

4. Discussion

We have shown that both the composition and the physical structure (i.e. 2D versus 3D) of the protein matrix that surrounds cells can affect the expression of specific markers commonly associated with remodeling by VSMC. These findings are important in the fields of biomaterials and tissue engineering because they contribute to our understanding of how cell function can be controlled in 3D protein scaffolds. In this study, we focused on two molecules that are involved in vascular remodeling and that are often found at the site of vascular healing: osteopontin and MMP-2. Both of these markers were markedly increased in 3D protein matrices, relative to 2D. However cell proliferation, also commonly associated with synthetic VSMC, was greatly decreased in 3D compared to 2D culture, indicating that changes in cell function in response to the environment is under complex control. Characterization of ERK phosphorylation suggested that this signaling pathway was involved in VSMC phenotype shifts, and it was strongly correlated to protein markers of the synthetic phenotype. This relationship persisted across matrix geometries and compositions. In contrast, ERK signaling was inversely correlated to VSMC proliferation in 3D, while in 2D there was a modest positive correlation. Investigation of other MAPK pathways, specifically the JNK and p38 pathways, did not reveal a close relationship between signal molecule activation and VSMC phenotype shifts in this system. ERK inhibition studies confirmed that this pathway was involved in the observed phenotype shifts, though the effect of signaling inhibition was more strongly dependent on matrix geometry than on composition.

As a matricellular protein, osteopontin is involved in mediating attachment and migration of cells in the ECM. Osteopontin is expressed only at low levels in uninjured arteries but abundantly in injured arteries [19,20], suggesting a role in the vascular remodeling process. Cells interact with osteopontin through a variety of integrins, and osteopontin-deficient cells are unable to adhere to collagen and are more prone to apoptosis [23]. Osteopontin also can

modulate the expression of MMPs required for movement through the ECM [24], and it has been shown that ERK activates osteopontin expression in SMC in response to a variety of soluble stimuli [25,26,27]. Our results indicate that ECM alone can also be a potent and enduring stimulus for osteopontin production. More specifically, we observed that 3D matrices are particularly effective in raising and sustaining osteopontin levels in culture, and that the degree of increased expression was dependent on matrix composition. We also observed that osteopontin secretion remained high throughout the entire culture period. Our ERK inhibition studies supported the idea that ECM mediates osteopontin secretion by suggesting a causal relationship. The effect of ERK inhibition on osteopontin secretion was more pronounced in the presence of ECM proteins, compared to monolayers on tissue culture plastic, and 3D matrices were particularly affected.

MMP-2 is another important enzyme in tissue remodeling and repair. A number of studies have suggested, however, that MMP-2 expression is not directly stimulated by ERK signaling. A study using oncostatin M to regulate ECM synthesis and degradation found that while ERK strongly regulated MMP-9, its effects on MMP-2 were more modest [28]. Another study showed that nerve growth factor activates ERK and leads to selective upregulation of MMP-9, but not MMP-2 [29]. Instead, it has been suggested that osteopontin serves as the mediator between the ERK pathway and MMP-2 by inducing NF κ B [30]. Osteopontin and MMP-2 form a (small, integrin-binding ligand, N-linked glycoprotein) SIBLING-MMP pair, which has been shown to be important for MMP-2 activation [24]. In our studies, ERK signaling was strongly correlated with both osteopontin expression and MMP-2 activation, but this relationship depended on the geometry and composition of the matrix. We also observed that activated MMP-2 sharply declined after 3 days in culture, probably because by that time point the matrix had completed most of its final compaction and secretion of MMP-2 to aid in remodeling was no longer required. Our findings suggest that these molecules can be specifically modulated by providing the appropriate matrix stimulation.

The ERK pathway has a well established role in promoting SMC proliferation in response to a variety of biochemical and mechanical stimuli [31,32]. In the present study, increasing levels of ERK activation correlated with higher cell proliferation in 2D culture, and inhibition studies showed that without ERK activation cells in monolayers were capable of binding to the substrate but did not proliferate. In contrast, in 3D culture there was a clear negative correlation between presence of the ECM and VSMC number. Furthermore, in 3D cultures ERK activation led to a decrease in the cell population, but ERK inhibition resulted in a modest population increase. These results suggest that ECM geometry determines how the ERK signal is processed. Although the phenomenon of ECM geometry as a contextual cue has not been fully explored, contextual regulation of ERK is well documented. For example, an *in vitro* study using phenotypically distinct SMC populations demonstrated that ERK had greater regulatory effects on proliferation in synthetic cells relative to contractile cells [33]. In addition, our results showed contextual regulation in the temporal pattern of ERK activation. Like MMP-2, the peak at day 3 and subsequent decrease in ERK activation may be related to the remodeling state of the constructs. As gel compaction reaches its maximum after day 3, pERK may be downregulated. Our results support the concept that ERK activation can have markedly different effects depending on the cellular environment, in this case the presence of ECM proteins as a 3D matrix.

Collagen and fibrin hydrogel matrices have distinct biochemical and mechanical characteristics, and our laboratory is interested combining these materials in ways that harness the desirable properties of each component to produce robust and biologically relevant engineered tissues [34,35]. In this study, we sought to determine how these materials modulate vascular smooth muscle cell function and remodeling. The matrices we provided surrounded the cells, as in native tissues, but it was also very loose and disorganized relative to native

tissue. VSMC actively remodeled and compacted the 3D matrices, and it is likely that this process recapitulates elements of the response to vascular injury, in which osteopontin and MMP-2 play important roles. By secreting osteopontin and activating MMP-2, cells mediate their own adhesion and migration, and subsequent remodeling of the matrix. Collagen had the most stimulatory effect, while fibrin had the smallest effect. This finding is in agreement with the fact that both osteopontin and MMP-2 primarily interact with collagen. Mixed composites exhibited an intermediate level of stimulation, suggesting that the strong response of collagen is modulated by the presence of fibrin.

5. Conclusions

This study highlights the differences between conventional 2D monolayer culture and more complex 3D culture systems, and therefore has implications for tissue engineering applications in which cells are embedded in protein hydrogels. In 3D matrices, osteopontin secretion, MMP-2 activation, and ERK phosphorylation were clearly increased, relative to 2D ECM substrates and tissue culture plastic. Our results provide evidence for the importance of contextual activation of ERK, and the effects ECM composition and geometry in controlling cell function in 3D protein biomaterials. While the 3D protein matrices we used in this study do not recreate the biochemical complexity of the native tissue, they do represent a more tissue-like environment in which to study VSMC function. Such simplified tissue models are useful in adding to our understanding of how cells sense and respond to their environment, and therefore have application in vascular research. In addition, 3D protein matrices can be used as scaffolds in creating engineered tissues, with the goal of creating fully biological cardiovascular tissue substitutes.

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Figure 1.

Macro- and micro- scale images of 3D ECM constructs. Panels A-C show images of collagen (A), collagen-fibrin (B) and fibrin (C) constructs floating in culture medium after 3 days in culture. Panels D-G show SEM images of collagen (D), collagen-fibrin (E) and fibrin (F) matrices. Panel G shows an SEM image of VSMC on the surface of a collagen-fibrin scaffold. Collagen matrices were characterized by thin filaments, whereas fibrin matrices had a fine mesh-like morphology. Collagen-fibrin gels exhibited both morphologies forming an interpenetrating polymer network.

Figure 2.

Effects of matrix composition and geometry on osteopontin (A), MMP-2 (B), proliferation (C), and ERK phosphorylation (D). The matrices used were tissue culture plastic (TCP), collagen (C), collagen-fibrin (CF), and fibrin (F). The three matrix proteins were used in 2D (white bars) and 3D (gray bars) cultures. Osteopontin and pERK were measured using ELISA, MMP-2 activity was measured using zymography, cell number was assessed using a Hoechst-dye based DNA quantification assay (1-fold indicates there was no change in population). Osteopontin, MMP-2, and pERK were higher for 3D samples relative to 2D. Conversely, proliferation was markedly lower in 3D than in 2D. Panel E shows a representative zymogram, loaded based on

cell number, for the aggregate data shown in Panel B. Data in bar charts are expressed as mean $\pm$ SE for four experiments. *P<0.1 compared to 2D ECM control (student t-test).

Figure 3.

Effects of matrix geometry over time on osteopontin secretion (A), MMP-2 activation (B), cell proliferation (C), and ERK phosphorylation (D) on TCP, 2D collagen, and in 3D collagen gels. Samples were taken after 1 (black), 3 (gray), and 7 (white) days in culture. Both osteopontin and MMP-2 decreased with time in monolayer cultures. Osteopontin in 3D cultures increased between days 1 and 3, and did not change at day 7. MMP-2 decreased sharply after day 3. Proliferation increased in monolayers, but decreased in 3D. pERK did not change in TCP, decreased with time in 2D ECM, and peaked on day 3 in 3D.

Figure 4.

Correlations between phenotypic markers and pERK with varying geometry. Osteopontin secretion (A) and MMP-2 activation (B) demonstrated a strong positive correlation with pERK, whereas cell number and pERK were negatively correlated when fit with a logarithmic curve (C).

Figure 5. Correlations between phenotypic markers and pERK with varying 3D ECM composition. Osteopontin secretion (A), MMP-2 activation (B), and cell number (C) demonstrated a positive correlation with pERK.

Figure 6.

Effect of ERK pathway inhibition using the ERK pathway inhibitor PD98059 on osteopontin, MMP-2, and proliferation on TCP, collagen (C), collagen-fibrin (CF), and fibrin (F) both on 2D (white) and in 3D (gray) matrices. Effects of inhibition are shown in % change, where 0 indicates that there was no change relative to control in the measured parameter (i.e. - 100 indicates a parameter was completely suppressed relative to control, and 100 indicates a parameter is twice the control value). Osteopontin secretion (A) and MMP-2 activation (B) decreased more in 3D ECM relative to 2D ECM, whereas cell number (C) decreased in monolayer cultures, but increased in 3D cultures. Upon inhibition of the ERK pathway, there

was no detectable amount of pERK. Data are expressed as mean $\pm$ SE for four experiments.
*P<0.1 compared to 2D ECM control (student t-test).