

## Vascular smooth muscle cell response on thin films of collagen

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### Abstract

Vascular smooth muscle cells (vSMC) cultured on gels of fibrillar type I collagen or denatured collagen (gelatin) comprise a model system that has been widely used for studying the role of the extracellular matrix in vascular diseases such as hypertension, restenosis and atherosclerosis. Despite the wide use of this model system, there are several disadvantages to using collagen gels for cellular studies. These include poor optical characteristics for microscopy, difficulty in verifying that the properties of the preparations are identical from experiment to experiment, heterogeneity within the gels, and difficulty in handling the gels because they are fragile. Previously, we developed an alternative collagen matrix by forming thin films of native fibrillar collagen or denatured collagen on self-assembled monolayers of alkanethiols [Elliott, J.T., Tona, A., Woodward, J., Jones, P., Plant, A., 2003a. Thin films of collagen affect smooth muscle cell morphology. *Langmuir* 19, 1506–1514.]. These substrates are robust and can be characterized by surface analytical techniques that allow both verification of the reproducibility of the preparation and high-resolution analysis of collagen structure. In addition, they have excellent optical properties that allow more details of the cell-matrix interactions to be observed by microscopy. In this study, we performed a side-by-side structural and functional comparison of collagen gels with thin films of collagen. Our results indicate that vSMC on thin films of collagen are nearly identical to vSMC on thick gels as determined by morphology, proliferation rate, integrin ligation, tenascin-C expression and intracellular signaling events. These results suggest that the features of collagen gels that direct the observed vSMC responses are adequately reconstituted in the thin films of collagen. These thin films will be useful for elucidating the features of the collagen matrix that regulate vSMC response and may be applicable to high content screening.

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### 1. Introduction

As a component of the vascular extracellular matrix (ECM), type I collagen plays an essential structural role as well as a role in regulating proliferation and migration of vascular smooth muscle cells (vSMC) (Raines et al., 2000; McCullagh et al., 1980; Heeneman et al., 2003). Type I collagen molecules are composed of three subunits arranged in an extended triple helical structure approximately  $1.5 \times 300$  nm long. They assemble into large supramolecular

fibrillar structures that are 10–500 nm wide and up to several hundred microns long (Kadler et al., 1996; Hulmes, 2002; Parry and Craig, 1984). Several integrins including  $\alpha_2\beta_1$ ,  $\alpha_1\beta_1$ ,  $\alpha_{10}\beta_1$ ,  $\alpha_{11}\beta_1$  have been shown to mediate cellular adhesion to native type I collagen (White et al., 2004). For vSMC, the integrins  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  appear to be the main receptors responsible for adhesion to the surrounding fibrillar collagen matrix (Wayner and Carter, 1987; Gullberg and Lundgren-Akerlund, 2002; Xu et al., 2000; Kramer and Marks, 1989). Peptide domains of type I collagen that contribute to the specificity for  $\beta_1$ -integrin receptors have been identified (Tulla et al., 2001; Knight et al., 2000; Xu et al., 2000; Tuckwell et al., 1994). Collagen

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recognition by these integrins requires the presence of the native triple helical structure. This has been demonstrated by studies that show that integrins that recognize native collagen do not bind to heat-denatured collagen (Tuckwell et al., 1994; Davis, 1992; Jones et al., 1997), and by structural studies of complexes of triple helical collagen peptide and  $\beta_1$  integrin (Emsley et al., 2000).

In healthy vascular tissue, vSMC are mainly in a growth-arrested and non-motile state. Vascular injury, such as that which occurs in hypertension and atherosclerosis, can lead to vSMC proliferation and ultimately vascular occlusion apparently as a result of changes in the ECM due the action of matrix metalloproteinases (MMP) (Galis et al., 1994; Nikkari et al., 1995; Cowan et al., 2000). MMPs are thought to reveal cryptic RGD sites in the native collagen protein (Montgomery et al., 1994; Davis, 1992) that allow cell ligation to the matrix through RGD-dependent  $\alpha_v\beta_3$  integrins (Davis, 1992; Jones et al., 1997). Engagement of  $\alpha_v\beta_3$  integrins initiates signaling pathways that result in vSMC growth and migration (Jones et al., 1997), and secretion of the extracellular matrix protein, tenascin-C (TN-C) (Jones and Rabinovitch, 1996). For in vitro experiments, heat-denatured collagen has been used in place of MMP-treated collagen, because it is also recognized by vSMC via RGD-dependent  $\alpha_v\beta_3$  integrins, and causes the cells to exhibit a phenotype characteristic of the diseased state (Davis, 1992). On denatured collagen gels, the cells assume a highly spread morphology, have well-organized cytoskeletal stress fibers, secrete TN-C, and proliferate (Jones et al., 1997). Alternatively, on native fibrillar collagen gels, the cells assume a minimally spread morphology, do not exhibit well-organized cytoskeletal stress fibers or high levels of TN-C secretion and have low proliferation rates. Direct comparisons of cellular response on these model matrices clearly indicate that differences between native collagen and denatured collagen matrices trigger the cells to switch between a non-proliferative and a proliferative phenotype. Studies to understand the signaling mechanism behind the matrix induced proliferation in vSMC suggest that TN-C expression is regulated through a  $\beta_3$ -integrin-stimulated increase in ERK1/2 MAPK phosphorylation (Jones et al., 1999), and may involve the prx1 and prx2 gene products as homeo-domain transcription factors working at the level of the TN-C promoter (Jones et al., 2001). The presence of TN-C in the surround matrix may stimulate clustering of epidermal growth factor (EGF) receptors in cell membranes, leading to an increased cell sensitivity to EGF and an increased proliferation rate (Jones et al., 1997).

The display of different integrin binding sites on denatured and native collagen may be only part of the collagen matrix features that result in the prototypical response of vSMC to collagen. For example, vSMC response to native collagen that is in a fibrillar structure is distinct from native collagen that is monomeric or non-fibrillar (Koyama et al., 1996; Ichii et al., 2001). Surpris-

ingly, native fibrillar collagen, but not native monomeric collagen, has been shown to arrest cell cycle in G1 (Koyama et al., 1996), and to suppress the expression of many of the genes that are upregulated by balloon angioplasty induced vascular injury, including TN-C (Ichii et al., 2001). In these experiments, the integrin binding sites on monomeric and fibrillar native collagen appear to be similar suggesting that other factors such as collagen supramolecular structure and mechanical properties of the collagen fibrils may play mitigating roles in cell signaling.

Dissecting how the complex nature of the extracellular matrix influences cell response requires detailed information about the matrix properties. Such analytical information is not easily determined with conventional tissue culture substrates such as collagen gels. An alternative to conventional tissue culture substrates is the use of ECM proteins organized into ultra thin films on highly controlled surfaces such as alkanethiol monolayers (Chen et al., 1997; Jung et al., 2001; Chen et al., 2000; Ito, 1999; Mrksich, 1998). The advantages of this approach are that thin films of protein can be highly homogeneous, highly reproducible, and independently characterized by a wide range of analytical techniques. Thin films provide the possibility to quantify matrix features such as topology, supramolecular structure, protein conformation, chemical bonds and spatial location. Thin films of collagen may allow investigation of the role of mechanical and other features of collagen fibrils that are responsible for directing cellular response.

Recently, we reported the fabrication of thin films of collagen and their effect on the morphology of vSMC (Elliott et al., 2003a). The thin films are spontaneously assembled by collagen adsorption onto hydrophobic alkanethiol monolayers. They have distinct advantages over collagen gels, which are often unstable and inhomogeneous, difficult to analyze, and which can reduce optical resolution of phase contrast and fluorescence images due to light scattering. The thin films, which range from 3 to 30 nm in average thickness, are highly robust, and can be analyzed by ellipsometry and atomic force microscopy (AFM) to quantify their homogeneity and reproducibility (Elliott et al., 2003a). The denatured collagen thin films appear to be physically smooth on a submicron scale as determined by AFM, suggesting the adsorption of a monolayer of denatured collagen to the hydrophobic surface. The native collagen thin films exhibit supramolecular fibrils that are similar in size to fibrils present in native collagen hydrogels. AFM images from our laboratory provide spatial resolution similar to transmission electron microscopy images of collagen fibrils (Jokinen et al., 2004; Parry and Craig, 1984; Elsdale and Bard, 1972), and indicate that the fibrils of thin films of native collagen are similar in appearance and dimension to those of collagen from tissue (Provenzano et al., 2005; Holmes and Kadler, 2005; Parry and Craig, 1984). The high-resolution spatial information of these films obtained by AFM and optical microscopy provides insight into the nature of fibril formation, adhesion, and interaction

with cells (Elliott et al., 2003a). Furthermore, the excellent optical properties of the thin films facilitates quantitative automated microscopy analysis of many cells in a population. Because the films are thin and homogeneous in thickness, cells in different locations on these samples are in similar focal planes, making it easier to use an automated stage to sample many different fields. We have demonstrated that vSMC on thin films of collagen accurately recapitulate the morphologies and the distribution of sizes of vSMC that are observed for cells on collagen gels (Elliott et al., 2003a). The minimally spread morphology observed when vSMC are cultured on native collagen hydrogels is also observed when the cells are cultured on thin films of native fibrillar collagen. The well spread morphology of vSMC on denatured collagen gels is observed on denatured collagen thin films. These results provide evidence that the matrix signals present in the thin films are sufficient for inducing the morphological changes that occur when the cells are cultured on collagen gels.

In this report, to further confirm that collagen thin films and thick gels induced similar phenotypes in vSMC, we examined integrin specificity, cytoskeletal organization, ERK1/2 phosphorylation, proliferation, and expression of TN-C in vSMC cultured on these substrates. Our results show that intracellular signaling pathways induced in vSMC by thin films of collagen are nearly indistinguishable from those induced by collagen gels. These results are consistent with the idea that the ECM signals from thin films of collagen mimic those present in collagen gels. Because thin films offer analytical and optical advantages, these substrates can improve the quality and quantity of image data, and may be useful for achieving a better understanding of the role that collagen matrices play in directing cellular response.

## 2. Results

### 2.1. Comparison of the physical characteristics of collagen gels and thin films

Fig. 1 presents phase microscopy images of vSMC on collagen gels and on thin films of collagen. Fig. 1b and 1d show a comparison of cells cultured on a denatured collagen gel and a thin film of denatured collagen, respectively. As can be seen, the vSMC on these substrates are well spread and are similar in appearance. Fig. 1a and 1c show images of cells on gels of native fibrillar collagen and thin films of native fibrillar collagen, respectively. Although the cells can be observed on both matrices, light scattering from the fibrillar collagen gel often prohibits a clear optical image of the cells. The thin films allow not only a sharp image of the cells, but also a clear visualization of the intimate interaction between the cells and the fibrils. When fluorescent probes such as tagged antibodies are used, the collagen gels tend to exhibit high backgrounds due to retention of the fluorescent

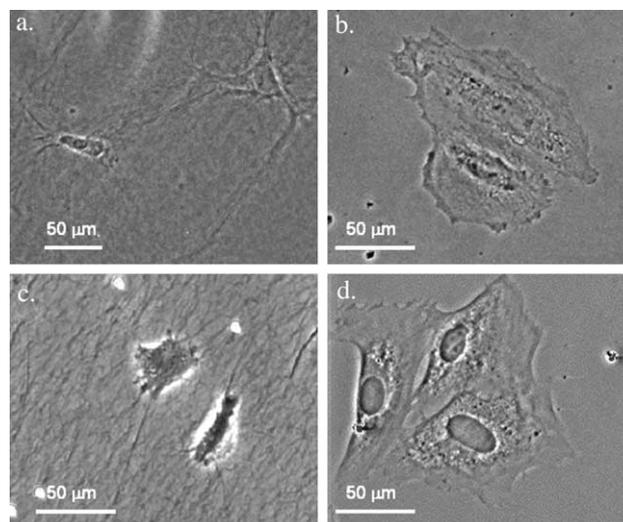


Fig. 1. Phase contrast optical images of vSMC on collagen substrates taken at 20× magnification: (a) native collagen gel, (b) denatured collagen gel, (c) thin film of native collagen and (d) thin film of denatured collagen. White bars are 50  $\mu\text{m}$ .

probes within the matrix, further reducing discrimination of cellular features. In contrast, thin films of fibrillar collagen show little background fluorescence when used with these reagents (data not shown).

Detailed topographical and structural comparison of collagen gels and thin films of collagen at three different length scales are shown in Figs. 2 and 3. Figs. 2a and b, and 3a and b, show collagen substrates prepared with fluorescently labeled collagen and imaged by epifluorescence microscopy. Atomic force microscopy was used to image the substrates at shorter length scales as shown in Fig. 2c through f and 3c through f. Fig. 2 shows fluorescent and AFM images of gels and thin films of denatured collagen. By fluorescence microscopy, the denatured collagen gel (Fig. 2a) appears to be inhomogeneous, with areas of greater and less fluorescence, indicating varying surface concentrations of the denatured collagen. In contrast, thin films of fluorescent denatured collagen (Fig. 2b) appear highly homogeneous.

Fig. 2c through f show AFM images of the gels and thin films of denatured collagen. As can be seen in Fig. 2c, the denatured collagen gel has inhomogeneities on the submicron and micron scale that extend up to 0.3  $\mu\text{m}$  above the surface. The thin film of denatured collagen has a significantly different appearance than the gel. By AFM, the thin films of denatured collagen appear homogeneous (Fig. 2d and e). The features observed are nearly identical to images of the underlying gold substrate (data not shown) suggesting the adsorbed collagen homogeneously covers the surface and has no topographical features. Ellipsometric thickness measurements from previous work (Elliott et al., 2003a) and the work described here indicates the adsorbed protein film is approximately 5 nm thick, which is approximately equivalent to a monolayer of protein.

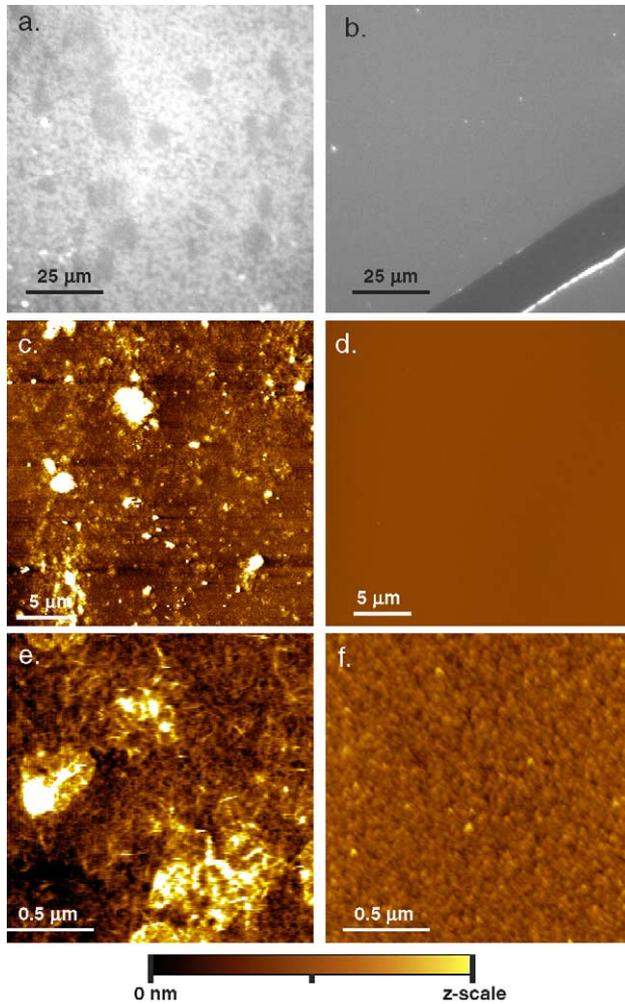


Fig. 2. Fluorescent and AFM images of denatured collagen substrates. Images of Tx-Red-labeled denatured collagen in (a) gel and (b) thin film. The black scratch in the lower right corner of panel b shows the background fluorescence intensity. AFM images ( $25 \times 25 \mu\text{m}$ ) of (c) denatured collagen gel under buffer,  $z=20 \text{ nm}$  and (d) thin film of denatured collagen in air,  $z=20 \text{ nm}$ . AFM images ( $2 \times 2 \mu\text{m}$ ) of (e) denatured collagen gel under buffer,  $z=10 \text{ nm}$  and (f) denatured collagen film in air,  $z=5 \text{ nm}$ .

Fig. 3a and b show fluorescence microscopy of gels and thin films of native collagen fibrils, respectively. As observed with transmission microscopy (Fig. 1a), resolution of individual fibrils is not possible in the collagen gel due to light scattering throughout the gel. In contrast, fluorescent fibrils can be clearly observed in the collagen thin films.

Fig. 3c and e show AFM images of gels of native collagen, and Fig. 3d and f show thin films of native collagen. A mesh of collagen fibrils and significant submicron scale topographical variations can be seen. In Fig. 3e and f, we can observe what appears to be two sizes of collagen fibrils in both the gel and in the thin film. The thin fibrils (filled arrows), are approximately 20–25 nm in diameter, and thicker fibril structures (open arrows) are approximately 150–250 nm in diameter. The inset in Fig. 3e shows a thicker collagen fibril in the gel. The characteristic 67 nm banding in these thicker fibrils can be seen.

In both the collagen gel and in the thin film (Fig. 3e and f, respectively) the larger fibrils appear to be approximately 150–250 nm in diameter and several microns long. The thinner 25 nm diameter fibrils appear to be several microns long in the gel (Fig. 3e), but appear to be only about  $0.3 \mu\text{m}$  in length in the thin film. As has been described previously (Elliott et al., 2003a), other AFM analysis leads us to hypothesize that these small fibrils may act as seeding sites for the larger fibrils in the thin films.

## 2.2. Comparison of biological activity induced by collagen gels and thin films

Some of the advantages of the thin films are that they can be analytically verified with independent measurements, and that they facilitate higher resolution analysis that may be

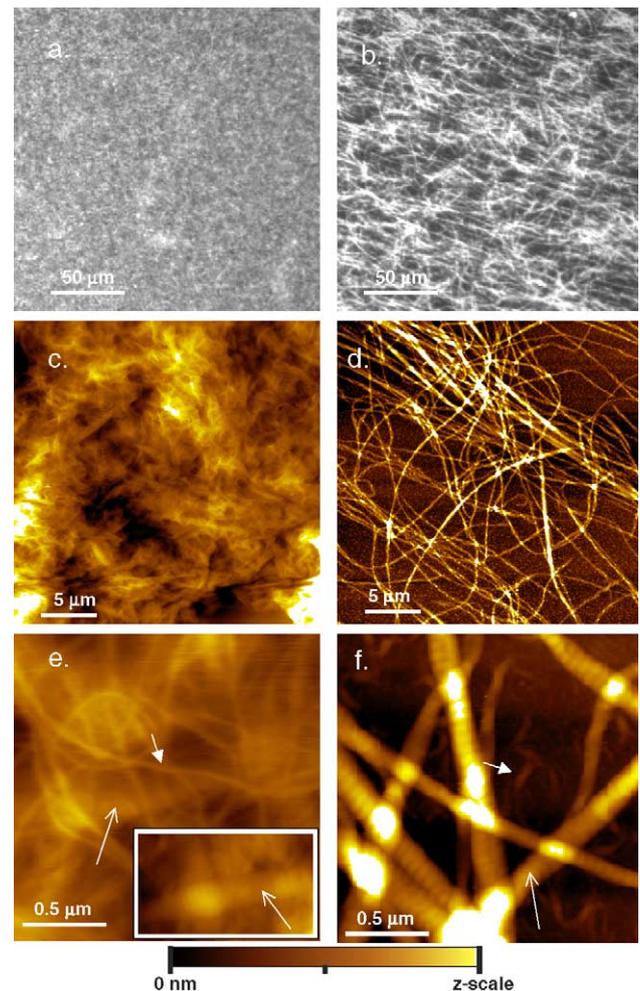


Fig. 3. Fluorescent and AFM images of native fibrillar collagen substrates. Images of Tx-Red-labeled native fibrillar collagen in (a) gel and (b) thin film. AFM images ( $25 \times 25 \mu\text{m}$ ) of (c) native collagen gel under buffer,  $z=1000 \text{ nm}$ , and (d) thin film of native fibrillar collagen in air,  $z=300 \text{ nm}$ . AFM images ( $2 \times 2 \mu\text{m}$ ) of (e) native collagen gel under buffer,  $z=300 \text{ nm}$  and (f) thin film of native collagen in air,  $z=250 \text{ nm}$ . Filled arrows indicate thin 25 nm diameter fibrils. Open arrows indicate 150–200 nm diameter fibrils. Inset in panel (e) is a 200 nm fibril imaged on a native collagen gel in air.

difficult with collagen gels. To be useful, however, cells must respond to these thin films in a manner that is similar to their response in gels. We have compared vSMC response to collagen gels with vSMC response to thin films of collagen using several criteria: adherence and spreading in the presence of a function-blocking antibody or peptide; cytoskeletal organization; ERK phosphorylation; TN-C expression; and proliferation. We have previously shown that vSMC assume distinct morphologies when they are cultured on denatured collagen gels vs. native collagen gels, and that similar morphological differences are observed when the cells are cultured on thin films of denatured collagen vs. thin films of native fibrillar collagen. The cells assume a smaller and minimally spread morphology on gels of native fibrillar collagen and on thin films of native fibrillar collagen, and a well-spread morphology on gels of denatured collagen and on thin films of denatured collagen (see Fig. 1) (Elliott et al., 2003a). Automated microscopy and image analysis of data from several hundred cells on each substrate reveals that the distribution of sizes of cells grown on gels or thin films of native collagen are similar to one another, and statistically distinct from that of cells on gels or thin films of denatured collagen (Elliott et al., 2003a). This suggests that thin films of collagen functionally mimic the corresponding gels. To further compare the response of vSMC to collagen gels with their response to thin films of collagen, we examined the integrin dependence of cell adhesion and spreading, and the activation of signaling pathways in cells cultured on gels or thin films of collagen.

We assessed the molecular recognition of vSMC for thin films of collagen and for collagen gels with the use of agents that block  $\beta_1$ -integrin or RGD-dependent integrin ligation. Adhesion of vSMC to native collagen is mediated by mainly  $\beta_1$ -containing integrins (Gullberg and Lundgren-Akerlund, 2002; Xu et al., 2000; Tuckwell et al., 1994; Wayner and Carter, 1987). To examine whether  $\beta_1$  integrin plays a similar role in adhesion to native collagen gels and to thin films of native collagen, cells were preincubated with a function-blocking anti- $\beta_1$  antibody prior to seeding. Fig. 4a shows that compared to cells in the absence of antibody, the addition of function-blocking anti- $\beta_1$  antibody prevented vSMC spreading on both the gels and the thin films of native fibrillar collagen, and had only a small effect on spreading of cells on the denatured collagen substrates. Cells spread to only 40% of their normal area on native collagen gels, and to only 30% of their normal area on thin films of native collagen. To further examine the effect of the  $\beta_1$ -blocking antibody on vSMC spreading on gels and thin films of native collagen, we quantified the ‘roundness’ of the cells. Roundness for each cell was calculated using the following equation:

$$\text{Roundness} = 4\pi \times \text{Area}/\text{perimeter}^2.$$

In the absence of the function-blocking anti- $\beta_1$  antibody, cells make many connections with the fibrillar matrix via filopodia (see Fig. 4b and c, images on the left), leading to a

value of roundness  $< 1$ . In the presence of the antibody, cells failed to make those extensions, and as a result appear rounder (see Fig. 4b and c, images on the right), and have a calculated value for roundness  $\sim 1$ . Similar effects were observed whether the vSMC were cultured on the gels or the thin films of native collagen. Roundness factors were determined for several hundred cells on the fibrillar collagen gels and on the thin films of fibrillar collagen. These data are presented as histograms in Fig. 4b and c, respectively. It is apparent that in the presence of the anti- $\beta_1$  antibody, cells on both native substrates assume a highly rounded morphology with a roundness factor of approximately 1. Cells are distinctly less round in the absence of antibody, exhibiting a distribution of morphologies, with roundness factors on both gels and thin films of native collagen that center around a value of 0.5. These results indicate that the anti- $\beta_1$  antibody prevents vSMC spreading on both the fibrillar gels and the fibrillar thin films of native collagen. We observed a very small change in the spreading of cells on denatured collagen substrates in the presence of the anti- $\beta_1$  antibody (Fig. 4a). This effect has been previously observed (Jones et al., 1997; Tuckwell et al., 1994) and suggests that vSMC may use  $\beta_1$ -integrins in addition to  $\alpha_v\beta_3$  to recognize denatured collagen.

vSMC adhesion and spreading on denatured collagen gels has been shown to be mediated by  $\alpha_v\beta_3$  integrin recognition of an RGD sequence in the collagen molecule (Jones et al., 1997; Jones et al., 1999). To determine if adhesion and spreading of vSMC to thin films of denatured collagen also occur in an RGD-dependent fashion, we examined cells cultured in the presence of the peptide, GRGDSP. This RGD-containing peptide is a competitive inhibitor of the  $\alpha_v\beta_3$  integrin (Kunicki et al., 1997) and other integrins (Ruoslahti, 1996) that recognize RGD-containing extracellular matrix proteins. As shown in Fig. 5a, preincubation of the vSMC with the RGD-containing peptide reduced cell spreading on both gels and thin films of denatured collagen. Cells on gels and thin films of denatured collagen spread to only 20–30% of the area of control cells in the presence of the RGD-containing peptide (Fig. 5b).<sup>1</sup> Preincubation with RGD-containing peptide had no effect on the area of cells on gels or thin films of native collagen. Preincubation with the GRGESP control peptide had no effect on cell spreading on the denatured collagen substrates and the thin films of native fibrillar collagen. These data indicate that the vSMC respond to both gels and thin films of denatured collagen in a similar RGD-dependent fashion, which is distinctive from the mechanism of interaction with native collagen substrates. Taken together, these blocking studies suggest that vSMC employ the same

<sup>1</sup> It is worth noting that one batch of RGD-containing peptide used in these experiments strongly inhibited cell adhesion ( $< 5\%$  of cell adhered) on both gels and thin films of denatured collagen. In all other experiments cell spreading, and not adhesion, was influenced by the presence of RGD peptide.

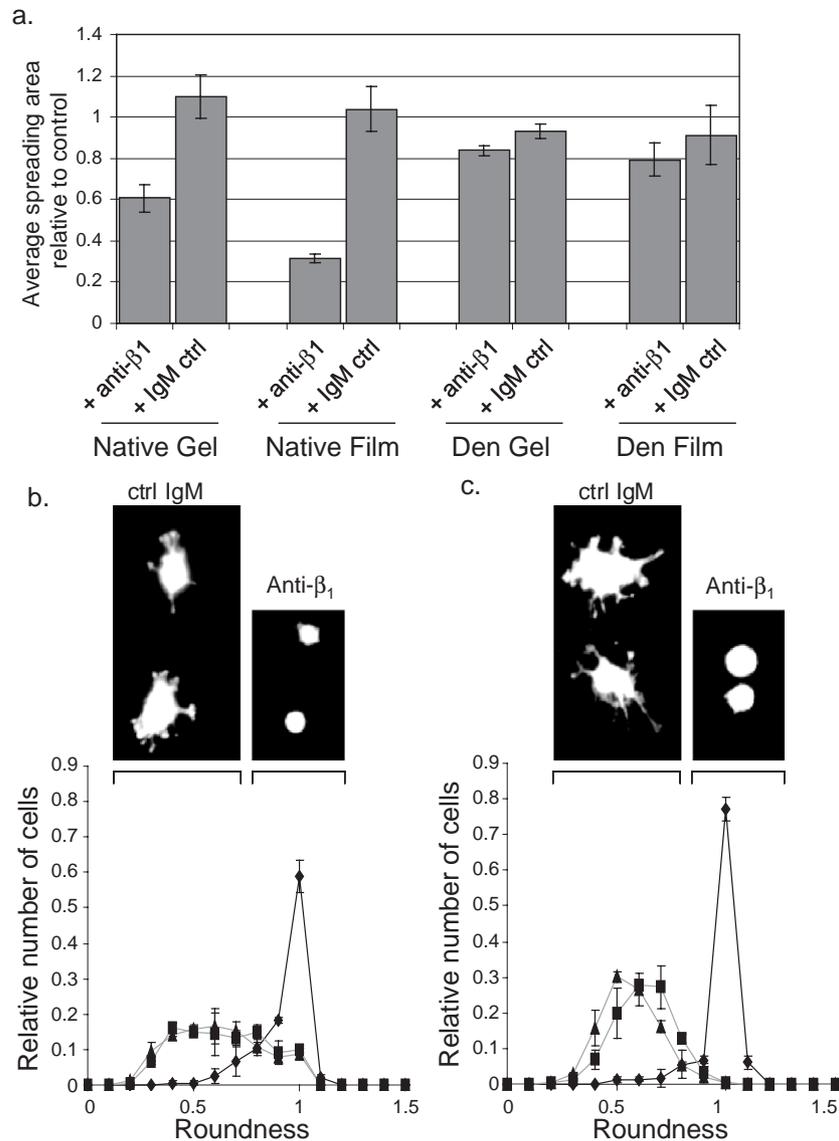


Fig. 4. a) Control antibodies or function-blocking antibodies to  $\beta_1$ -integrin were added to cells in suspension for 15 min prior to plating the cells onto the collagen substrates. The height of the bars indicates the extent of spreading of adhered cells relative to untreated cells after 2 h at 37 °C. Fluorescent micrographs of vSMC and histogram analysis of cell roundness for cells on native collagen gels (b) and thin films of native collagen (c) in the absence of antibody ( $\blacktriangle$ ), in the presence of nonspecific IgM ( $\blacksquare$ ) and in the presence of anti- $\beta_1$  antibody ( $\blacklozenge$ ). Error bars reflect the standard deviation between two replicate experiments.

RGD-dependent integrins when interacting with either the thin films of collagen or thick collagen gels.

Cytoskeletal organization within vSMCs can be influenced by the mechanical and chemical properties of the surrounding ECM (Abedi and Zachary, 1995; Shattil, 1995). The cytoskeletal organization of vSMC on native fibrillar collagen has been shown to be distinct from that of vSMC on denatured collagen (Chapados et al., in press). To show that cellular cytoskeletal features are similar regardless of whether vSMC are cultured on gels or thin films of collagen, we examined vSMC cytoskeletal organization by labeling F-actin with phalloidin conjugated to FITC. Images of vSMC grown on either gels of native collagen fibrils (Fig. 6a) or thin films of native fibrillar collagen (Fig. 6c) show

that on fibrillar collagen, phalloidin staining is primarily at the edges of cells, with little indication of a well-organized intracellular cytoskeletal network. In contrast, cells grown on denatured collagen, either as thick gels (Fig. 6b) or thin films (Fig. 6d), have a well-organized cytoskeleton with long actin stress fibers across the cell body. The distinct differences in cytoskeletal organization on native or denatured collagen, and the similarity of the staining pattern in vSMC regardless of whether they are on thick collagen gels or thin collagen films, suggest that extracellular cues that influence cytoskeletal organization are present in both the gel and thin film substrates.

Additional experiments were performed to test if differences in ERK phosphorylation that are observed in vSMC

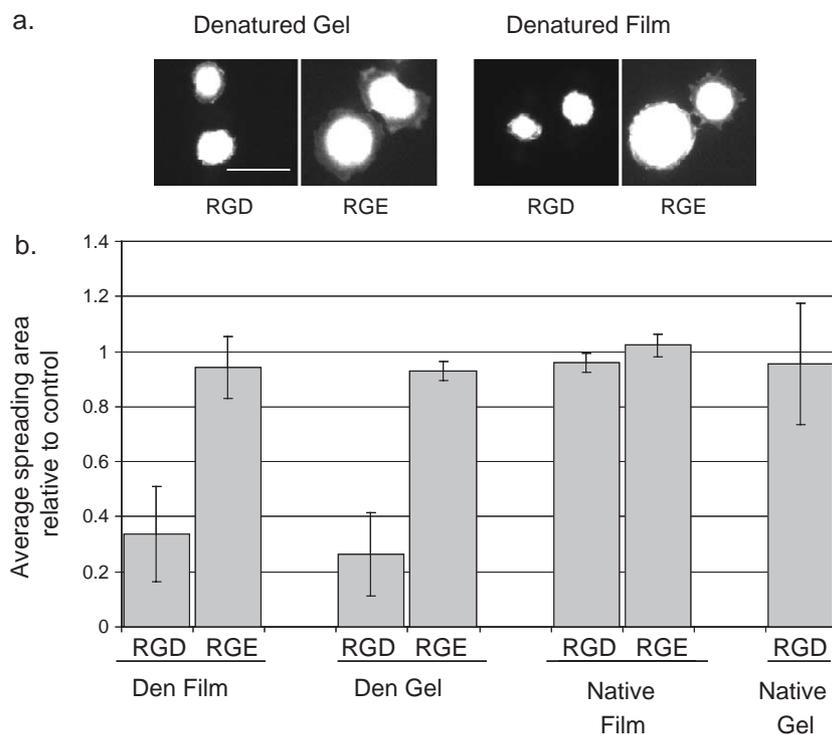


Fig. 5. The peptides GRGDSP or GRGESP were preincubated with cells prior to plating onto collagen substrates. After 2 h at 37 °C, samples were washed, and cells were fixed, stained, and analyzed with automated microscopy. (a) Fluorescent images of vSMC on denatured collagen gels and films show the reduction of spreading in the presence of the RGD-peptide compared to the presence of the RGE-containing peptide. (b) Average cell area reported is relative to average cell area in the absence of peptide. Error bars reflect the standard deviation between 2 replicate experiments.

on native vs. denatured collagen gels are reproduced on thin films of native and denatured collagen. ERK phosphorylation is associated with signal transduction as a result of  $\beta_3$ -integrin engagement in vSMCs on denatured collagen, and is not observed with  $\beta_1$ -integrin binding to native fibrillar collagen gels (Jones et al., 1997). Western blot analysis (Fig. 7) using a probe for phosphorylated ERK1/2, illustrates at least a 5-fold increase in the presence of phosphorylated

ERK1/2 in vSMC cultured on denatured collagen both in the form of thin films or thick gels (5.2 and 5.8-fold increase, respectively). Almost no phosphorylated ERK is detected in cells on native collagen, either as thin films or thick gels.

Comparison of thin films and thick gels of collagen on downstream signaling in vSMC was also assessed by examining the expression of the extracellular matrix protein, TN-C. TN-C expression is correlated with the increased proliferation in several cell types including vSMC (Chiquet-Ehrismann and Chiquet, 2003; Jones and Rabinovitch, 1996). On native fibrillar collagen gels, and in the healthy vascular tissue, vSMC produce low levels of TN-C; but in hypertensive or injured arteries (Jones et al., 1997; Chen et al., 1996), and on denatured collagen gels (Jones and Rabinovitch, 1996; Jones et al., 1997), vSMC produce large quantities of TN-C. Since vSMC binding to thin films or thick gels of collagen appears to be mediated by the same integrins, we expect that vascular smooth muscle cells on thin films of native collagen will express relatively little TN-C, while cells on thin films of denatured collagen will express relatively large amounts of TN-C.

The expression of TN-C is shown in Fig. 8. TN-C, detected by fluorescence after labeling with anti-TN-C antibody, is readily visible associated with cells on both the gels and on the thin films of denatured collagen (Fig. 8b and d, respectively). In contrast, almost no TN-C staining is visible when the vSMCs are cultured on native collagen,

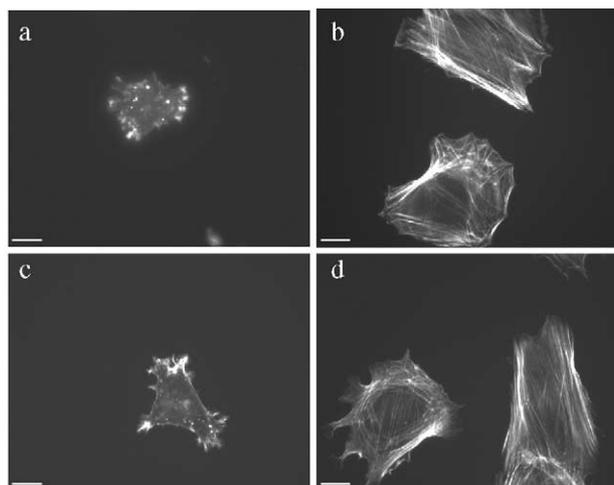


Fig. 6. Fluorescence micrographs of vSMC stained with FITC-phalloidin. Cells are on (a) native collagen gel; (b) denatured collagen gel; (c) thin film of native collagen and (d) thin film of denatured collagen. White bars are approximately 20  $\mu$ m.

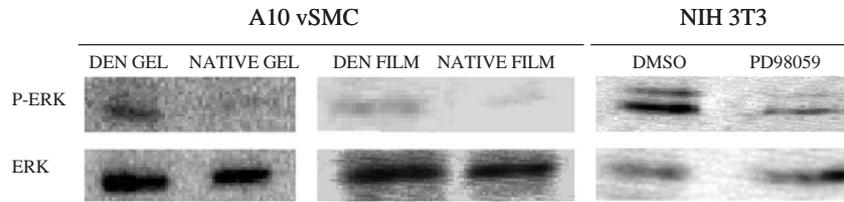


Fig. 7. vSMC were incubated on denatured (Den) or native collagen substrates for 24 h and prepared simultaneously for Western blot analysis. The membranes were probed with antibody against phosphorylated ERK1/2 and visualized with enhanced chemiluminescence. Membranes were also probed with an ERK antibody to verify that protein concentration in each lane were similar. ERK phosphorylation levels are approximately 5-fold higher after culture on denatured films and gels as compared to native collagen films and gels. To verify antibody specificity NIH3T3 cultures were treated with 50  $\mu$ M PD98059 (MEK inhibitor) or vehicle (DMSO) for 3 h and prepared for Western blot analysis. PD98059 treatment increased ERK phosphorylation levels approximately 8-fold compared to vehicle control (DMSO).

either as gels or as thin films (Fig. 8a and c, respectively). Taken together, the ERK1/2 phosphorylation data and the TN-C expression data suggest that the thin film collagen matrices induce intracellular responses in vSMC that are similar to those induced by collagen gels.

Another critical phenotypic difference between vSMC cultured on native and denatured collagen gels is proliferation rate. Native type 1 fibrillar collagen inhibits proliferation of various cell types including vSMC (Yoshizato et al., 1985; Schor, 1980; Koyama et al., 1996), while denatured collagen is known to support cell proliferation (Henriet et al., 2000; Jones et al., 1999). To test if thin films of native and denatured collagen are also capable of controlling the proliferation rate of vSMC, we used automated microscopy to determine the density of adhered cells at 24 h intervals. Automated stage movements allowed unbiased data collection from cells on 50 to 100 fields on each coverslip. Only features that exhibited a Texas Red-labeled periphery and a DAPI stained nucleus were counted as valid cells (see

Materials and Methods). As seen in Fig. 9, the change in number of cells on native collagen after 96 h is essentially zero, whether the substrate is a gel or a thin film. Cell densities are higher, and the rate of change is nearly identical, for cells on denatured collagen whether as thin films or gels. These data indicate that the thin films of collagen are capable of controlling the proliferation state of vSMC to an extent that is similar to that observed on collagen gels.

### 3. Discussion

Cell behavior is highly dependent on physical and chemical signals received from the insoluble extracellular matrix. In addition to supplying specific receptor binding sites, the matrix can provide topographical and mechanical information to cells. To efficiently and accurately determine how the properties of the matrix influence cell behavior, it is critical to be able to prepare experimental systems that allow reproducible control over the matrix properties. ECM protein hydrogels are commonly used as cellular substrates, but it is difficult to control and understand the physiochemical properties of the gel system. There is little consensus on how to prepare them and it is difficult to independently

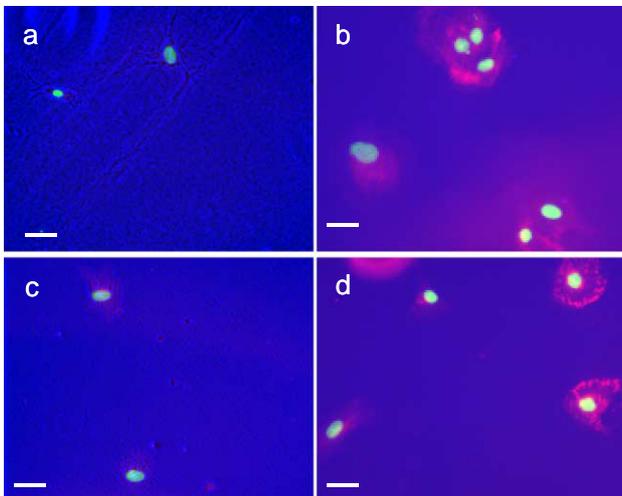


Fig. 8. vSMC were cultured on collagen substrates for 24 h and then fixed and stained with DAPI and an anti-TN-C antibody. For comparison, fluorescence antibody was visualized with identical exposure times. Contrast enhancement settings are identical for all images to facilitate comparative viewing. (a) native collagen gel; (b) denatured collagen gel; (c) thin film of native collagen; (d) thin film of denatured collagen. The phase contrast image is shown in dark blue, DAPI nuclear stain is light blue and TN-C is pink. White bar is approximately 50  $\mu$ m.

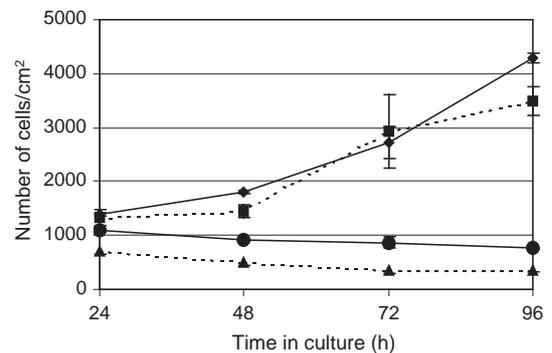


Fig. 9. vSMC were plated onto the different substrates at the same density, and at the indicated times after plating, they were fixed, stained with DAPI and Texas Red-C<sub>2</sub>-maleimide. Cells on 100 random fields were counted on each sample using automated microscopy. Thin films of denatured collagen, ◆; denatured collagen gels, ■; thin films of native collagen, ●; native collagen gels, ▲.

verify that the physical and chemical properties are the same from experiment to experiment and from lab to lab. Developing a substrate that mimics the biological properties of protein hydrogels, but offers a high level of control over the chemical, topographical, and mechanical features of the matrix, and allows independent characterization by analytical methods, has the potential to be a valuable tool for investigating cell-matrix interactions.

Native fibrillar collagen gels and denatured collagen gels have been used extensively as cell culture substrates that are intended to mimic the ECM environment found in tissues. We have developed collagen thin films as alternatives to collagen gels in order to improve analysis of the matrix and to improve the quality of the data from of experiments using collagen matrices. Thin films allow the use of analytical tools to independently and quantitatively verify the characteristics of the films. Optical microscopy, AFM and ellipsometry measurements indicate that the films are highly reproducible in film thickness, topology and homogeneity (Elliott et al., 2003a). The homogeneity of thickness and topology, and the low level of light scattering from thin films are advantageous to data collection by automated optical microscopy, facilitating the collection of large amounts of data. The purpose of this work was to query if vSMC behavior on thin films of collagen is similar to the behavior of vSMC on collagen gels, thus permitting the use of the more analytically addressable collagen thin films for studies of cell-matrix interactions.

Previously, we demonstrated that the thin films of native and denatured collagen induced the distinct morphologies in vSMC that are observed on native and denatured collagen gels. In this study, we directly compare some of the details of cell-matrix recognition and downstream signal processing in vSMC on thin films to vSMC on collagen gels. Integrin blocking studies were used to evaluate whether the chemical moieties that mediate the initial integrin binding interactions are intact on the thin films of native fibrillar collagen and denatured collagen. Function-blocking antibodies to the  $\beta_1$ -integrin, which mediates binding to native triple helical collagen (Emsley et al., 2000; Knight et al., 2000), prevented spreading to the same extent on both the native collagen gels and native collagen thin films. This suggests that the fabrication procedure used for the native collagen thin films does not alter the chemical structures recognized by the  $\beta_1$ -integrins. RGD-containing peptides reduced cell spreading equally on the denatured collagen gels and denatured collagen thin films. This result suggests that the presentation of the RGD-sequence in thin films of denatured collagen is suitable for engaging the  $\beta_3$ -integrins.

In addition to integrin recognition of thin film collagen matrices, similar cytoskeletal organization and actin stress fibers are as visible in the cells cultured on thin films of denatured collagen as they are in cells on denatured collagen gels. In contrast, on gels and thin films of native fibrillar collagen, dense F-actin regions are mainly present in the tips

of the filopodia protruding from the vSMCs. Although the specific properties of the different collagen substrates that dictate vSMC actin organization is not completely understood, our results suggest that these properties are similar whether the collagens are presented as gels or thin films.

Downstream events such as ERK1/2 phosphorylation, and TN-C expression are also similar for cells on native collagen matrices, whether in the forms of gels or as thin films, and these responses are distinct from cells on denatured collagen substrates. The results obtained from the collagen thin films are consistent with previous observations that  $\beta_3$ -integrin engagement with denatured collagen results in an increase in ERK1/2 phosphorylation and TN-C secretion in vSMC (Chapados et al., in press; Boudreau and Jones, 1999), as has been observed for vSMC on collagen gels.

Perhaps the most significant downstream event for vSMC in the diseased state is an increased proliferation rate. We observed nearly identical proliferation rates when vSMC were cultured on gels or thin films of denatured collagen. The proliferation rate was significantly higher than that measured for the vSMC on gels and thin films of native fibrillar collagen. The mechanism that induces vSMC growth arrest in native collagen gels appears to be intact on the native collagen thin films. Overall, the above results suggest that the response of vSMC on thin films of collagen is nearly identical to that on collagen gels, and that the thin films can be used to study the effect of the collagen matrix on vSMC response.

There are several protocols for preparing collagen matrices and coatings for cell studies. The procedures vary in neutralization conditions, addition of cross-linkers, concentration and types of collagen, incubation temperatures and times, and use of plastic and glass substrates. It is not completely known to what extent these preparations are equivalent and how differences in preparation may induce differences in cell responses. We have observed (Elliott et al., 2003a) that merely changing the concentration of native collagen in solution during the fabrication of the native collagen thin films has a significant effect on the vSMC response. High-resolution analysis of the films revealed that collagen concentration controlled the fibrillar density of the film and a native collagen gel-like response only occurred at high fibril densities. The methodology for preparing thin films of collagen that we present here will allow studies that more critically identify the essential parameters of the cell-matrix interactions that govern cell response.

The use of thin collagen films may also enable other studies including elucidating cellular remodeling of the matrix, and the effect of topographical and other physical influences of the matrix on cell response. Furthermore, the physical robustness of thin films and their ease of use with automated microscopy, suggest that this approach may be applicable to high content screening on ECM substrates.

#### 4. Conclusions

The characterization of the cellular response to an alternative method for preparing collagen matrixes to study cell-matrix interactions is described here. The data presented indicate that the thin films of native or denatured collagen appear to invoke the same signaling pathways in vSMC that are initiated when the cells are cultured on collagen gels. Cells recognize the thin films with the same integrins with which they recognize the thick gels of collagen, and they proliferate, express TN-C and organize cytoskeleton in a similar manner. The thin film matrices are highly reproducible and homogeneous, and are superior for optical microscopy compared to thick collagen gels. In addition to the potential advantages of these characteristics to high content screening, the analytical advantages inherent in the thin films may enable more quantitative studies on the mechanisms by which native and denatured collagen matrixes control vSMC behavior. The thin films also provide a means to systematically alter chemistry, supra-molecular structure, and surface topology of collagen substrates. These features may facilitate understanding of the combined effect of integrin engagement, cytoskeletal organization, and mechanical features on gene expression and cell response.

#### 5. Experimental procedures<sup>2</sup>

##### 5.1. Preparation of collagen gels

Purified type I collagen was purchased as a solution of acid-stabilized monomer (Vitrogen; Cohesion Technologies, Inc., Palo Alto, CA). Heat-denatured collagen was prepared by mixing ~3 mg/mL Vitrogen, 0.4 M<sup>3</sup> acetic acid and 10 × Ca<sup>2+</sup> and Mg<sup>2+</sup>-free Dulbecco's phosphate buffered saline (DPBS) in a 16:1:2 volume ratio, boiling the solution for 30 min and neutralizing it with 0.5 M NaOH (Jones et al., 1999). Neutralized denatured collagen solution (350 μL) was placed into the center of a 20 mm diameter ethanol-sterilized silicone gasket (Electron Microscopy Sciences, Fort Washington, PA) that was affixed to a cleaned, ethanol-sterilized glass coverslip (#1, 22 × 22 mm). Samples were air dried overnight in a tissue culture hood, rinsed several times with DPBS, and stored in DPBS at 4 °C until they were used. The silicone gaskets were removed before the cell culture experiments were initiated.

To prepare thick gels of native fibrillar collagen, a variation of a published method (Jones et al., 1999) was used. Gaskets (~16 mm inside dia., 22 mm outside dia.)

were cut from paper labels (Fasson label material, Avery Dennison, Brea, CA) and adhered onto cleaned coverslips before sterilization with 70% ethanol. The paper gaskets aided in preventing the collagen gel from sliding off the glass coverslip. Vitrogen collagen solution (0.8 mL, ~3 mg/mL, 4 °C) was neutralized with 0.1 mL of 10 × DPBS (4 °C) and 0.1 mL of NaOH (0.1 M, 4 °C), and was kept at 4 °C to minimize polymerization. Neutralized native collagen solution (100 μL) was applied to the dried coverslips, making sure that solution fully contacted the paper gaskets. The samples were placed at 37 °C overnight to initiate fibrillogenesis. The native collagen gels were carefully rinsed and stored in DPBS at 4 °C until they were used.

##### 5.2. Preparation of collagen thin film<sup>4</sup>

The preparation of thin films of collagen has been described previously (Elliott et al., 2003a). Coverslips were coated with a 5 nm layer of chromium and a 15–20 nm layer of gold by magnetron sputtering. The semi-transparent gold-coated coverslips were immersed in 0.5 mM 1-hexadecanethiol (Aldrich, Milwaukee, WI) in ethanol for at least 8 h before being rinsed with ethanol and dried with filtered N<sub>2</sub>. The alkanethiol-coated samples could be stored under ethanol for at least 7 days without any loss in performance. To prepare thin films of native collagen, alkanethiol-treated gold-coated coverslips were placed into neutralized solutions of native collagen in DPBS (0.4 mg/mL) and incubated overnight at 37 °C. After incubation, the samples were lifted out of the gelled collagen solutions, and rinsed with a stream of DPBS and then deionized water from sterile Teflon squirt bottles (Nalgene Nunc, Rochester, NY). Once all loosely adhered gel was removed, the samples were dried under a stream of filtered N<sub>2</sub> and immediately placed back into a DPBS solution. Thin films of denatured collagen were prepared by immersing alkanethiol-treated gold-coated glass coverslips into a solution of ~0.3 mg/mL denatured collagen in DPBS. The samples were incubated at 4 °C for at least 12 h, rinsed in DPBS and water, dried under a stream of filtered N<sub>2</sub> and stored in DPBS at 4 °C for less than 8 h before they were used with cells. Prior to seeding cells on the collagen substrates, the thin films and thick gels were conditioned for a minimum of 3 h with 3 changes of cell culture medium containing 2% (v/v) fetal bovine serum (see below). This step was necessary to exchange solution trapped in gels with culture medium, and to normalize the gel and film substrates with respect to possible adsorption of serum proteins.

<sup>2</sup> Indication of specific manufacturers and products is for clarity only and does not constitute endorsement by NIST.

<sup>3</sup> The accepted SI unit of concentration, mol/L, has been represented by the symbol M in order to conform to the conventions of this journal.

<sup>4</sup> Further details describing the preparation of the collagen gels and thin film on gold coverslips and bacterial graded polystyrene will be available at the [www.nist.gov](http://www.nist.gov) website. Additional questions about preparing the substrates in a laboratory or potential collaborations using substrates prepared in our laboratory should be addressed to the corresponding author at [jelliott@nist.gov](mailto:jelliott@nist.gov).

### 5.3. Atomic force microscopy imaging of collagen thin films and collagen gels

The collagen samples were imaged under dry and aqueous conditions using an AFM with a magnetically driven Si tip in intermittent contact mode (PicoScan; Molecular Imaging, Phoenix, AZ). The dried denatured collagen gel and the hydrated native collagen gel, prepared as described above, were rinsed extensively with deionized H<sub>2</sub>O to remove salts and dried under a laminar flow sterile hood for 48 h. The samples were imaged in air before they were placed into a fluid cell and imaged under DPBS. The thin films prepared as described above were imaged in air before they were placed into the wet cell and imaged under DPBS.

### 5.4. Cell culture

The rat aortic vascular smooth muscle cell line, A10 (vSMC; ATCC, Manassas, VA), was maintained in Dulbecco's Modified Eagles Medium (DMEM; Mediatech, Herndon, VA) supplemented with nonessential amino acids, glutamine, penicillin (100 U/ml), streptomycin (100 µg/mL), and 10% (v/v) fetal bovine serum (FBS; Gibco Invitrogen, Carlsbad, CA), and maintained in a humidified 5% (v/v) CO<sub>2</sub> balanced-air atmosphere at 37 °C. Subconfluent cultures were switched to supplemented DMEM containing 2% (v/v) FBS 24 h prior to an experiment. The reduced serum concentration maximizes the extent of cell signaling that is due to the ECM and mimics conditions that have been typically used in characterizing the response of these cells to native and denatured collagen gels (Jones et al., 1997). Cells were removed from tissue culture polystyrene flasks by trypsinization, washed with DMEM/2% FBS and plated in DMEM/2% FBS onto the collagen substrates at a density of 2000 cells/cm<sup>2</sup> except where indicated. Care was taken to ensure the seeding density was homogeneous over the surface of the substrates. Cells were typically incubated at 37 °C for 24 h except in the proliferation and integrin blocking experiments. All experiments were performed with the collagen substrates on the bottom of 8-well polystyrene plates (Nalgene Nunc, Rochester, NY). The mouse embryonic fibroblast cells (NIH3T3; ATCC) used as a control for the Western blotting experiments were cultured in the 10% (v/v) FBS-containing DMEM media described above.

### 5.5. Cell fixation and staining

After incubation, cells on the substrates were washed with warm Hanks Balanced Salt Solution (HBSS; ICN Biomedicals, Costa Mesa, CA), fixed in 4% (v/v) formaldehyde in DPBS (30 min) at room temperature, quenched in 0.25% (m/v) NH<sub>4</sub>Cl in DPBS (15 min) and rinsed with DPBS. Cells were permeabilized and stained (1 h) with

Texas Red-C<sub>2</sub>-Maleimide (10 mg/mL in DMF stock, (Elliott et al., 2003b) dissolved in DPBS (1 µg/mL) containing 0.1% (v/v) Triton X-100. Cells were rinsed once with DPBS, DPBS containing 3% (m/v) bovine serum albumin (BSA) and DPBS. DPBS-glycerol (1:1 v/v) containing 0.25% (m/v) 1,4-diazabicyclo(2,2,2)octane (DABCO, Sigma, St. Louis, MO) to reduce photobleaching, and 1.5 µg/mL 4',6-diamidino-2-phenylindole (DAPI, Sigma) as a nuclear counterstain, was added to each well. Substrates were placed upside down onto a drop of Tris-buffered saline (10 mM Tris, 140 mM NaCl, pH 8.5) containing 90% (v/v) glycerol, 0.25% DABCO and 1.5 µg/mL DAPI on #1 coverglass slides (Nunc, Naperville, IL). The coverslips were clamped to the slides with small alligator clips, rinsed extensively with distilled water, dried under a stream of air and sealed at the edges with nail polish. Throughout the fixation and staining procedure, cell samples were always kept immersed in solution. This avoided potential disruption of the cell structure by dewetting of the substrate, which the denatured collagen samples were particularly susceptible to.

### 5.6. Automated fluorescence microscopy

The fixed and stained cells were examined by phase contrast and fluorescence microscopy using a using a 10× objective on an inverted microscope (Zeiss Axiovert S100TV, Thornwood, NJ) outfitted with a computer controlled stage (LEP, Hawthorne, NY), an excitation filter wheel (LEP, Hawthorne, NY), and a CCD camera (Cool-Snap *fx*, Roper Scientific Photometrics, Tucson, AZ). Hardware operation, and image digitization and analysis were under software control (ISee Imaging, Cary, NC). A modular software routine controlled automated movement of the stage, autofocusing, and collection of data from 50 to 100 independent fields (870 × 690 µm) of cells per coverslip. At each field cellular fluorescence from Texas Red, and then DAPI, were collected by automated switching of the appropriate excitation filters and passing the emitted light through a multipass beam splitter (set# 84000; Chroma Technology Inc., Brattleboro, VT). For quantitative analysis, appropriate thresholding criteria allowed cell areas, as determined by cellular Texas Red fluorescence, to be accurately distinguished from the non-fluorescent non-cell areas. The number of nuclei, and therefore the number of cells, was determined from the corresponding images collected with the DAPI filter. The requirement for spatial correspondence of DAPI and Texas Red fluorescence ensures that only cell areas with nuclei are used during data analysis. The cell area and roundness of individual cells and the cell density were determined with image processing software (ISee Imaging) as previously described (Elliott et al., 2003a,b). Data used to determine cell density, average cell area and cell roundness histograms were collected from at least 200 cells on each substrate.

### 5.7. Proliferation assay

Thick gel and thin film collagen substrates (8 of each) were prepared and divided among four 8-well plates. Each plate contained duplicates for each type of collagen substrate. vSMC were seeded on the collagen substrates at 1100 cells/cm<sup>2</sup> and incubated at 37 °C. After 24 h, all substrates were rinsed twice with warm DMEM/2% FBS to remove non-adhered cells, and fresh DMEM/2% FBS was added to each well before returning plates to the incubator. After an additional 1 h incubation, the first plate was fixed and stained. The cell densities measured on these samples were considered to be the initial adhesion densities on each substrate. The remaining plates were fixed after 48, 72, and 96 h from seeding, respectively, with a medium change at 68 h. All plates were stained immediately after fixation. Cell density on each substrate was determined by automated microscopy and image analysis of 50 fields/substrate. The requirement for the presence of DAPI stained nuclei within a Texas Red stained cell object ensured that only valid cells were counted during the automated analysis. An increase in cell density over time was considered to be evidence of cell proliferation.

### 5.8. Integrin blocking studies

Cells were trypsinized, rinsed and resuspended in 6.5 ml of DMEM/2% FBS at  $1.25 \times 10^4$  cells/mL in 15 mL Falcon tubes. Anti  $\beta_1$ -integrin (anti-rat CD29, IgM; Research Diagnostics, Flanders, NJ), non-specific mouse IgM (Sigma), or DPBS vehicle were respectively added to the suspensions (10  $\mu$ g/mL final antibody concentration), and the tubes were incubated at 37 °C for 15 min with gentle mixing every 5 min. Cells were seeded onto the collagen substrates at 2000 cells/cm<sup>2</sup>, and allowed to adhere for 2 h at 37 °C. Cells were then fixed, stained and analyzed by automated microscopy to determine the extent of cell adhesion and spreading. A similar protocol was used for the RGD peptide blocking studies. Trypsinized cells were resuspended as above, and GRGDSP, GRGESP (BaChem, King of Prussia, PA) or DPBS vehicle, respectively, were added to the suspensions (0.3 mg/mL final peptide concentration). Cells were incubated at 37 °C for 15 min, plated on the collagen substrates, and then fixed, stained and analyzed by automated microscopy.

### 5.9. Immunofluorescence and phalloidin staining

For TN-C immunostaining, fixed cells were permeabilized with 0.1% (v/v) Triton X-100 in DPBS (5 min), blocked with DPBS containing 3% (m/v) BSA and 5% (v/v) donkey serum at room temperature (1 h), stained overnight at 4 °C with 2  $\mu$ g/ml rabbit anti-chicken TN-C (Chemicon, Temecula CA) in blocking solution. The samples were rinsed multiple times in DPBS and incubated with 10  $\mu$ g/mL rhodamine-labeled donkey anti-rabbit antibody (Chemicon),

in blocking solution at room temperature (1 h). For filamentous actin (F-actin) staining, cells were permeabilized with 0.1% (v/v) Triton X-100 in DPBS (5 min), rinsed with DPBS, blocked with DPBS containing 3% (m/v) BSA (30 min), stained with fluorescein-isothiocyanate (FITC)-phalloidin (Sigma) in blocking solution (200 nM, 1 h), and rinsed with blocking solution. The immunostained and phalloidin-stained samples were rinsed extensively with DPBS and samples were mounted on slides in Tris-buffered saline containing 90% (v/v) glycerol, 0.25% (m/v) DABCO and 0.05  $\mu$ g/mL DAPI. Cells were imaged with phase and fluorescence microscopy using the appropriate filter sets.

### 5.10. Western blot analysis

vSMC cultured on collagen substrates and control NIH3T3 cells cultured on tissue culture polystyrene were scraped into RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% (m/v) deoxycholate and 1% (v/v) Nonidet P-40) containing aprotinin (20  $\mu$ g/ml), leupeptin (20  $\mu$ g/ml), phenylmethylsulfonyl fluoride (10  $\mu$ g/ml), sodium orthovanadate (1 mM), sodium pyrophosphate (10 mM) and sodium fluoride (10 mM) followed by centrifugation (9300  $\times g$  for 8 min at 4 °C). The protein concentration of the supernatant was assessed using Bio-Rad Reagent (Bio-Rad Laboratories, Hercules, CA) and equal amounts of total protein were separated by SDS-PAGE and analyzed by Western blotting. Membranes were probed with a phosphorylation state-specific antibody against ERK1/2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or an antibody against ERK1/2 (Santa Cruz Biotechnology) followed by incubation with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and visualization by enhanced chemiluminescence. Antibody specificity was verified with NIH3T3 cultures treated with 50  $\mu$ M PD98059 (MEK inhibitor) or vehicle (DMSO) for 3 h. PD98059 treatment is known to decrease ERK phosphorylation levels in fibroblasts (Dudley et al., 1995). Quantitative comparison of the phospho-ERK levels was performed by collecting digital images of the membranes (Q Imaging, BC, Canada) and quantifying the band densities with NIH Image J software. Images of the membranes used for Fig. 7 were contrast adjusted to optimize visual presentation.

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