Mechanical Strain of Rat Vascular Smooth Muscle Cells Is Sensed by Specific Extracellular Matrix/Integrin Interactions

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Abstract

Cyclic mechanical strain (1 Hz) causes a mitogenic response in neonatal rat vascular smooth muscle cells due to production and secretion of PDGF. In this study, the mechanism for sensing mechanical strain was investigated. Silicone elastomer strain plates were coated at varying densities with elastin, laminin, type I collagen, fibronectin, or vitronectin. Strain was applied by cyclic application of a vacuum under the dishes. Cells adhered, spread, and proliferated on each matrix protein, but the mitogenic response to strain was matrix dependent. Strain increased DNA synthesis in cells on collagen, fibronectin, or vitronectin, but not in cells on elastin or laminin. When strain was applied on matrices containing both laminin and vitronectin, the mitogenic response to strain depended upon the vitronectin content of the matrix. Fibronectin, in soluble form (0–50 μg/ml), and the integrin binding peptide GRGDTP (100 μg/ml) both blocked the mitogenic response to mechanical strain in cells grown on immobilized collagen. Neither soluble laminin nor the inactive peptide GRGESP blocked the response to strain. GRGDTP did not alter the mitogenic response to exogenous PDGF or α-thrombin but did prevent the secretion of PDGF in response to strain. Furthermore, GRGDTP, but not GRGESP, prevented strain-induced expression of a PDGF-A chain promoter 890 bp–chloramphenicol acetyltransferase construct that was transiently transfected into vascular smooth muscle cells. Finally, the response to strain was abrogated by antibodies to both β1 and αβ1 integrins but not by an antibody to β1 integrins. Thus interaction between integrins and specific matrix proteins is responsible for sensing mechanical strain in vascular smooth muscle cells. (J. Clin. Investig. 1995. 96:2364–2372.) Key words: mechanical strain • RGD peptides • extracellular matrix • platelet-derived growth factor • gene regulation

Introduction

Vascular smooth muscle (VSM)1 cells in situ are constantly exposed to cyclic mechanical strain. Alterations in blood flow and pressure are thought to play an important role in the development of the vasculature and adaptation of adult blood vessels (1). Mechanical forces may also contribute to the pathologic alterations of the vessel wall in hypertension (2, 3). The effect of mechanical forces on the growth and differentiated properties of cells in vitro is not well understood. Little is known about how individual cells respond to mechanical strain or the underlying molecular mechanisms by which mechanical events are transduced into physiological responses.

Methods have recently been developed to subject cells to mechanical strain in vitro (4, 5). Using a device which imparts strain by application of a vacuum to silicone elastomer plates, Sumpio et al. (6) reported that VSM cells alter their orientation in response to mechanical strain. Using a similar device, our laboratory showed that cyclic mechanical strain induces DNA synthesis in VSM cells via the autocrine production of PDGF (7) and that the mitogenic potential of angiotensin II and α-thrombin are enhanced by mechanical strain (8). Additionally, our group showed that exposure of VSM cells to mechanical strain alters the expression patterns of smooth muscle and nonmuscle myosin (9). These studies taken collectively suggest that VSM cells respond to mechanical strain with complex changes in both DNA synthesis and expression of specific gene products.

Physical forces can lead to changes in gene expression that are important both in normal development and in pathological conditions. Werb and co-workers have shown that shape changes can induce or suppress the expression of metalloproteinases (10). Gimbrone and co-workers have found in endothelial cells that the PDGF-B gene is induced by fluid shear stress via a specific DNA response element (11).

How cells sense mechanical perturbations is not known. It has been shown that mechanical forces can activate adenyl cyclase (12–14), phospholipase C (15, 16), and activate mechanosensitive ion channels (17). Ingber proposed that interaction of extracellular matrix (ECM) proteins with their integrin receptors plays a central role in transmitting mechanical signals to and from the cytoskeleton (18). Wang et al. showed that magnetic beads coated with RGD peptides are capable of transferring mechanical stress to the cytoskeleton (19). Stretching of a flexible culture substratum leads to altered cytoskeletal organization in some systems (6), and shear stress causes focal adhesions to reorganize in endothelial cells (20). Whether or not the cytoskeleton (through its interaction with integrins and the ECM) participates in signal generation after mechanical perturbation remains unknown.

In this study we examined the role of the ECM–integrin interaction in the ability of VSM cells to increase PDGF secretion and DNA synthesis in response to mechanical strain. We found that particular matrix proteins, when immobilized on deformable surfaces, are necessary to confer this response to strain. Soluble matrix proteins, RGD peptides, and certain anti-integrin antibodies block this response. Thus, the ability of VSM
cells to sense mechanical strain is dependent on the interaction between specific integrins and the extracellular matrix subjected to strain.

Methods

Materials. All materials were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. BSA was fraction V, fatty acid poor (Miles Scientific Div., Milwaukkee, WI, Naperville, IL). [3H]Thymidine and [14C]acetly-CoA were from New England Nuclear/ Du Pont (Wilmington, DE). Flex plates were from Flexcell Corp. (McKeesport, PA). Peptides GRGDTP and GRGESP were purchased from GibCo-BRL Gaithersburg, MD. Highly purified a-thrombin was generously supplied by John W. Fenton II (Albany Medical College of Union University, Albany, NY) and PDGF-BB was purchased from GIBCO (Madison, WI). Bolton-Hunter reagent was obtained from ICN Radiochemicals Div., ICN Biomedical Inc. (Irvine, CA). A function-blocking antibody to rat β1 integrin was obtained from PharMingen (San Diego, CA). Anti-human α5, β1 (P1F6) (21) and α1 (HSP10) were obtained from Dr. Dean Sheppard (University of California, San Francisco). Anti-β1- was obtained from Dr. Donna Mendrick (Harvard University, Cambridge, MA).

Cell culture. Primary cultures of VSM cells from newborn rats were established by Peter Jones (University of Southern California, Los Angeles, CA). From these primary cultures the R22 D cell line was established (22) and generously supplied to us by Dr. Jones at passage 15. The cells were maintained in MEM with 10% FBS, 2% tryptophan phosphate broth, penicillin (50 U/ml), and streptomycin (50 μg/ml) in a humidified atmosphere of 5% CO2 at 37°C. Culture medium was changed every other day until confluent. Cells were subcultured with trypsin-verseae. Cells were used from passages 16–29 for these studies.

Application of cyclic strain to cultured cells. For most experiments, cells were grown to confluence in six-well collagen-coated silicone elastomer-bottomed culture plates (Flex I; Flexcell Corp.). Cells were subjected to mechanical deformation with the Flexcell Stress Unit (Flexcell Corp.). The stress unit is a modification of the unit initially described by Banes and co-workers (4, 5) and consists of a computer-controlled vacuum unit and a base plate to hold the culture dishes. The vacuum (15–20 kPa) is repetitively applied to the rubber-bottomed dishes via the base plate which is placed in a humidified incubator with 5% CO2 at 37°C. The computer system controls the frequency of deformation and the negative pressure applied to the culture plates.

Coating of plates with extracellular matrix proteins. Extracellular matrix proteins were applied to the silicone elastomer by adsorption. Solutions containing collagen (6 μg/cm2), poly-L-lysine (10 μg/cm2), fibronectin (5 μg/cm2), elastin (10 μg/cm2), laminin (2 μg/cm2), or vitronectin (0.005–5.0 μg/cm2) were placed in the dishes and allowed to air dry overnight. For plates with mixed matrices containing laminin and vitronectin, commercial plates coated with laminin (1012 sites/cm2), Flexcell Corp.) were coated with various quantities of vitronectin as described above. All plates were washed with PBS, followed by BSA (1 mg/ml to block uncoated sites on the silicone) for 1 h at 37°C. The plates were then rinsed with culture medium to remove unadsorbed protein. Efficiency of protein adsorption was determined by 125I-labeled matrix proteins. Matrix proteins were iodinated using Bolton-Hunter reagent obtained from ICN. Proteins were labeled using modifications of the protocol originally described by Bolton and Hunter (23). The proteins were suspended in phosphate buffer (pH 7.5) at a concentration of 1 mg/ml and reacted with the labeling reagent for 2 h at 0°C. Unreacted reagent was separated from the labeled protein by gel filtration chromatography with disposable column (10 DG; Bio Rad Laboratories, Richmond, CA). The radio specific activity of the protein solution was calculated and used to estimate the amount of adsorbed protein.

[3H]Thymidine incorporation. Cells were grown in six-well Flex plates until confluent and were growth arrested by placing them in "quiescence" medium containing 5 mg/ml transferrin and 0.5 mg/ml BSA for 72 h. For measurement of [3H]thymidine incorporation into DNA, 1 μCi/ml of [3H]thymidine was added to the growth medium of each well and incubated at 37°C for 6 h. Cells were then washed three times with assay medium (140 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM Na2HPO4, 25 mM glucose, 25 mM Hepes/NaOH, pH 7.20, and 0.5 mg/ml BSA) and extracted with 15% TCA at 4°C for at least 30 min. The rubber bottom of the Flex plates containing the TCA-precipitable material was washed with water, removed from the plate, and placed directly into a scintillation vial for counting (7).

Cell number and morphology. Determinations of cell number were made using a hemocytometer on trypsinized cell preparations. After subjecting the cells to the described protocol, medium was removed, and the cells were washed extensively with PBS. The cells were then fixed for 5 min with 100% methanol. The methanol was removed and the silicone membranes were allowed to air dry. The cells were stained with Giemsa (Sigma Chemical Co.) for 5 min and then washed with distilled water. The cells were then examined and photographed using bright-field microscopy with an inverted microscope (Nikon Inc., Melville, NY).

Analysis of PDGF-A chain promoter activity. A PDGF-A chain promoter 890 bp–chrylamphenicol acetyltransferase (CAT) construct was obtained from Tucker Collins (Harvard Univ. Initiative Grant). The CAT gene is a reporter gene that is transcribed into a constitutively expressed protein, Rous sarcoma virus–β-galactosidase. Transfection was accomplished using DEAE-dextran with chloroquine (25). The cells were allowed to recover for 24 h in complete medium and were then transferred to quiescence medium and subjected to 24 h of mechanical strain. CAT activity was measured by a modification of the method of Siesler and colleagues (26). Cells were lysed with lysis buffer (Reporter; Promega Corp.). The resulting lysates were added to assay buffer containing [14C]acety-CoA (0.02 mM, 0.5 μM) and unlabeled chlamrophendicol (1.6 mM). The mixtures were incubated at 37°C for 1–4 h.

The lysates were extracted with ethyl acetate. The organic phase was counted in a scintillation counter. Cell lysates were normalized to adjust for transfection efficiencies by adding equal amounts of β-galactosidase activity (assay kit; Promega Corp.). Mock transfections were performed to determine background.

Flow cytometry. Cells were harvested in saline/trypsin/verseae (0.05% trypsin) and were incubated in normal goat serum for 10 min at 4°C to inactivate trypsin and to block nonspecific binding. Cells were pelleted, resuspended in PBS, and incubated with the integrin antibodies (mouse) for 20 min on ice. Medium was aspirated, cells were washed with PBS and incubated with phycoerythrin-conjugated anti–mouse IgG (H+L, F(ab')2, Boehringer Mannheim Biochemicals, Indianapolis, IN) secondary antibody. Cells were washed and resuspended in PBS and subjected to flow cytometric analysis using a FACScan* (Becton Dickinson and Co., San Jose, CA [21]).

Results

Extracellular matrix composition determines the mitogenic response of vascular smooth muscle cells to mechanical strain. The role of specific ECM molecules in conferring the mitogenic response of VSM cells to mechanical strain was examined. Silicone elastomer culture plates were coated with various ECM molecules by adsorption. The number of matrix molecules adherent to the silicone plates was determined using 125I-labeled proteins and was ~1012 sites/cm2 for collagen, fibronectin, and laminin. For vitronectin, the number of sites was varied between 5 × 107 and 2 × 1012 by adjusting the quantity of protein adhered to the plates (Table 1). Control cells not subjected to mechanical strain were grown on similar silicone plates coated with the same matrix components. All cells were plated in serum-free medium and allowed to adhere for 12 h before initiation of mechanical strain. Thymidine incorporation was determined after 48-h exposure to cyclic mechanical strain. Cells

Mechanical Strain Sensed by Matrix Proteins
Table I. Site Density of Matrix Proteins Adsorbed to Silicone Elastomer Dishes

<table>
<thead>
<tr>
<th>ECM component</th>
<th>Coating density</th>
<th>Sites/cm²</th>
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<tbody>
<tr>
<td>Collagen (type I)</td>
<td>6.0 µg/cm²</td>
<td>1 × 10¹²</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>5.0 µg/cm²</td>
<td>2 × 10¹⁰</td>
</tr>
<tr>
<td>Laminin</td>
<td>2.0 µg/cm²</td>
<td>1 × 10¹⁰</td>
</tr>
<tr>
<td>Vitronectin</td>
<td>0.005 µg/cm²</td>
<td>5 × 10⁸</td>
</tr>
<tr>
<td></td>
<td>0.05 µg/cm²</td>
<td>6 × 10⁸</td>
</tr>
<tr>
<td></td>
<td>0.5 µg/cm²</td>
<td>4 × 10⁸</td>
</tr>
<tr>
<td></td>
<td>5.0 µg/cm²</td>
<td>2 × 10⁸</td>
</tr>
</tbody>
</table>

Purified matrix proteins were labeled with ¹²⁵I and the radiospecific activity was calculated. Except where indicated, 5 µg/cm² of the matrix proteins was adsorbed to the silicone elastomer plates (see Methods). After adsorption, blocking with BSA, and washing (see Methods), plates were counted to determine the number of adsorbed sites of the indicated proteins.

plated on dishes with no coating, poly-L-lysine, elastin, or laminin showed no significant response to mechanical strain (Fig. 1 A). In contrast, cells plated on collagen or fibronectin responded with dramatic increases in thymidine incorporation of 3.7- and 8.2-fold, respectively. Cells plated on vitronectin also exhibited a significant mitogenic response to mechanical strain (Fig. 1 B). For vitronectin, we examined the effect of coating density on mitogenic response to strain. Response increased with vitronectin density and appeared to saturate at densities higher than 0.5 µg/cm² (4 × 10¹¹ sites/cm²).

The failure of strain to generate a mitogenic response on laminin was not due to a general loss of mitogenic responses on this matrix protein. Cells cultured on laminin exhibited a 19-fold increase in thymidine incorporation (from 2,875±345 to 48,650±1,270 cpm/dish) in response to a mixture of PDGF (10 ng/ml) and thrombin (1.5 nM). In cells plated on mixed matrices containing an excess of laminin with varying densities of vitronectin (Table II), cells exhibited an increasing mitogenic response to strain as a function of the vitronectin content of the matrix (Fig. 2). Thus, cells fail to respond to strain when plated on pure laminin-coated dishes but do respond to growth factors on this matrix. More importantly, cells also respond to strain on laminin if small quantities of vitronectin are added to the laminin matrix.

To determine whether the different matrix proteins altered the ability of the cells to adhere to the plates or to spread normally, cell number and morphology were examined for cells on each substrate. Cells added to plates with no coating or poly-L-lysine adhered to the plates but failed to spread (data not shown). Cells plated on laminin, collagen, and fibronectin attached to the dishes with approximately equal efficiency (Table III). As expected from the thymidine data, cell number

Table II. Site Density of Vitronectin Adhered to Laminin-coated silicone Elastomer Dishes

<table>
<thead>
<tr>
<th>Vitronectin density</th>
<th>Sites/cm²</th>
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<tbody>
<tr>
<td>0.005</td>
<td>8 × 10⁹</td>
</tr>
<tr>
<td>0.05</td>
<td>1 × 10¹¹</td>
</tr>
<tr>
<td>0.5</td>
<td>9 × 10¹¹</td>
</tr>
<tr>
<td>5</td>
<td>5 × 10¹²</td>
</tr>
</tbody>
</table>

Purified vitronectin was labeled with ¹²⁵I and the radiospecific activity was calculated. Vitronectin, at the indicated concentrations, was adsorbed to silicone elastomer plates containing 10¹⁰ sites/cm² laminin (see Methods). After adsorption, blocking, and washing, plates were counted to determine the number of adsorbed vitronectin sites.

Figure 1. Effect of extracellular matrix proteins on mitogenic response to strain. (A) VSM cells were plated on Flex I (carboxyl surface) strain plates that were coated with nothing (None), poly-L-lysine (P-Lys), elastin (Elas), laminin (Lam), type I collagen (Coll), or fibronectin (Fib) at the densities indicated in Methods. (B) Plates were coated with vitronectin at the indicated densities. Cells were plated in serum-free medium and allowed to adhere to the dishes overnight. Medium was changed and cells were exposed to cyclic strain or no strain for 48 h. [³H]Thymidine was added to each well 6 h before harvest. [³H]Thymidine incorporation into DNA was determined as described in Methods. Basal thymidine incorporation levels were (A) 1,505±158 (no coating), 765±82 (poly-L-lysine), 4,205±310 (elastin), 8,253±425 (laminin), 3,525±210 (collagen), 1,575±107 (fibronectin). Basal (nonstrain) thymidine incorporation in (B) was 1,235±20 (vitronectin, 0.005 µg/cm²), 2,740±210 (0.05 µg/cm²), 5,272±38 (0.5 µg/cm²), and 6,140±475 (5.0 µg/cm²). Data are presented as the fold increase in thymidine incorporation of cells subjected to strain as compared to unstrained cells on the same surface coating. Data are the mean±SE for triplicate determinations in one of two similar experiments.

Figure 2. Effect of mixed laminin/vitronectin matrices on mitogenic response to strain. VSM cells were plated on silicone elastomer dishes with mixed matrices containing laminin and vitronectin (Table II). After exposure to 48 h strain, fold thymidine incorporation was determined as in Fig. 1. Basal (nonstrain) thymidine incorporations were 450±35 (control), 417±27 (0.005 µg/cm², vitronectin), 437±42 (0.05 µg/cm²), 538±57 (0.5 µg/cm²), and 612±52 (5.0 µg/cm²) cpm/dish.
increased to a greater extent in response to strain on fibronectin than on the other substrates (Table III). Although morphological appearance varied to some degree on these substrates, the cells rapidly attached and spread on all of them (Fig. 3). Furthermore, 48 h of mechanical strain did not grossly alter their morphology. Thus, the dramatic differences in response to mechanical strain shown in Fig. 1 and Table III cannot be explained simply by the failure of cells to adhere to particular matrix proteins. Rather, specific matrix-cell interactions must be involved in transmitting mechanical signals to the cell. We therefore attempted to interrupt the response to mechanical strain by perturbing the matrix-integrin interaction with soluble proteins and peptides.

Soluble matrix proteins or peptides perturb the ability of VSM cells to sense mechanical strain. Since cells adhered to fibronectin-coated plates showed the largest increases in [³H]-thymidine incorporation in response to strain, we asked whether fibronectin would interfere with the response to strain if present in the soluble form rather than adherent to the dishes. Soluble fibronectin (1–100 µg/ml) was added to both control and strained VSM cells that were plated on collagen-coated silicone plates (Fig. 4 A). In the absence of soluble fibronectin, strain increased thymidine incorporation by 3.1-fold. Soluble fibronectin (25 µg/ml) blocked the response to strain on collagen-coated plates by > 90% (closed circles, Fig. 4 A). This inhibitory effect of soluble fibronectin was dose dependent, with 50% inhibition at 10 µg/ml. Unstrained cells were not affected by soluble fibronectin (open circles, Fig. 4 A). This inhibition of the mitogenic response to strain by soluble fibronectin contrasts

**Figure 3.** Effect of mechanical strain and extracellular matrix on cell morphology. VSM cells were plated in serum-free medium on silicone elastomer dishes that were coated with fibronectin and subjected to no strain (A) or strain (B) for 48 h. VSM cells plated on laminin (C), elastin (D), or collagen (E) were subjected to mechanical strain (48 h). Cells were fixed and stained as described in Methods. ×120.
markedly with the enhancement of the response observed when fibronectin was fixed to the stretch plates (Fig. 1).

Soluble laminin over the same concentration range used for fibronectin (Fig. 4 B) did not significantly affect the mitogenic response to strain on collagen-coated plates. Therefore, unlike fibronectin, laminin did not affect the mitogenic response whether soluble or fixed to the dishes.

To further test the involvement of matrix-integrin interactions in transducing the growth response to mechanical strain, we examined the effect of an integrin-binding peptide GRGDTP (100 μg/ml) on strain-induced DNA synthesis (27). Addition of GRGDTP to the medium before the start of mechanical strain completely blocked the strain-induced increase in DNA synthesis (Fig. 5 A). The strain-induced increase in cell number was also blocked by GRGDTP (cell number on control dishes increased from 2.6 x 10^4 to 4.0 x 10^4 after 48 h strain but was unchanged in the presence of GRGDTP (100 μg/ml)). At concentrations of 10–50 μg/ml, GRGDTP caused lesser, dose-dependent reductions in strain-induced thymidine incorporation (data not shown). Thymidine incorporation was unaffected by the inactive control peptide GRGESP (100 μg/ml). At 100 μg/ml, GRGDTP caused only minor changes in cell shape (Fig. 6), making it unlikely that the failure to respond to strain was due to loss of contact with the substratum. Moreover, the lack of response to strain in the presence of GRGDTP was not artifically due to detachment of cells during the strain period. Only a tiny fraction of the cells were found in the medium after the strain period in either the absence or presence of RGD (55 cells/dish, control strain; 69 cells/dish, strain with GRGDTP).

Since strain-induced DNA synthesis in this system is due to autocrine production of PDGF (7), the failure to respond to strain in the presence of GRGDTP could be due to a failure to respond to PDGF or a failure to synthesize or secrete PDGF in response to strain. The mitogenic response to either PDGF-BB (10 ng/ml) or α-thrombin (1 U/ml) was unaffected by GRGDTP (100 μg/ml) or GRGESP (100 μg/ml) (Fig. 5 B and C). This finding suggested that GRGDTP interferes with the production or secretion of PDGF (or other growth factors) in response to strain.

The ability of the cells to secrete PDGF or other growth factors in response to mechanical strain in the presence of RGD peptide was tested by transferring conditioned medium from strained and unstrained cells to unstrained PDGF-responsive cells (Table IV). Conditioned medium from the strained cells (48 h, 1 Hz) produced a 1.7-fold increase in thymidine incorporation in test cells when compared with medium from unstrained cells. This mitogenic response to the conditioned medium was absent when the medium was taken from strained cells exposed to GRGDTP (Table IV). Since the cells remain responsive to PDGF in the presence of GRGDTP (Fig. 5 B), these findings suggest that GRGDTP prevents the strain-induced production or secretion of PDGF.

**Figure 5. RGD peptides specifically block strain-induced [3H]thymidine incorporation.** In three separate experiments, the effects of the peptides GRGDTP (100 μg/ml) (RGD), GRGESP (100 μg/ml) (RGE), or no peptide (Con) on mitogenic response to 48 h of mechanical strain (A), PDGF-BB (10 ng/ml, 24 h) (B), or α-thrombin (8 nM/ml, 24 h) (C) were analyzed. [3H]-Thymidine was added for the final 6 h of each incubation. Data presented as fold increase in [3H]-thymidine incorporation over cells not receiving the indicated mitogenic stimulus. Data are the mean±SE for triplicate determinations in one of two similar experiments.
 Antibodies to fibronectin/vitronectin receptors inhibit strain-induced DNA synthesis. To begin identification of the integrin receptor(s) involved in transducing the mechanical signal, we screened existing function-blocking antibodies to integrins of the fibronectin/vitronectin receptor families for cross-reactivity with the rat VSM cells used in these studies. Using FACS we identified an mAb, clone P1F6, to the human vitronectin receptor, $\alpha_v\beta_3$, that cross-reacts with rat cells (Fig. 8). Additionally, the cells were found to cross-react with a non-function-blocking antibody to the human $\alpha_\text{v}$, integrin subunit (H5P10).

Finally, we asked whether the $\alpha_v\beta_3$ antibody would interfere with the response to mechanical strain. Control cells exposed to 48 h of mechanical strain exhibited a 3.2-fold increase in $[^{3}H]$thymidine incorporation; cells treated with random IgG responded similarly. However, thymidine incorporation after strain in cells treated with anti-$\alpha_v\beta_3$ was reduced by 90% (Fig. 9 A). A rat anti-$\beta_3$ antibody (28) also reduced the mitogenic response to strain by ~90%. A non-function-blocking antibody which recognizes rat $\alpha_\text{v}$ (H5P10) did not block the response to strain (Fig. 9 B). Moreover an antibody to rat $\beta_1$ (29) did not significantly blunt the response to strain. None of the antibodies we tested significantly altered the mitogenic response to PDGF (data not shown). Thus the response to mechanical strain appears to be mediated through specific fibronectin/vitronectin receptors, including $\alpha_v\beta_3$- and $\beta_3$-containing integrins.

Table IV. Effect of RGD Peptide on Secretion of Growth Factors by Cells Exposed to Strain

<table>
<thead>
<tr>
<th>Condition</th>
<th>Thymidine incorporation cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>27,942±2,238</td>
</tr>
<tr>
<td>GRGDTP</td>
<td>20,648±4,821</td>
</tr>
</tbody>
</table>

Cells were incubated with GRGDTP (100 μg/ml) as indicated and were subjected to mechanical strain or no strain for 48 h. Medium was collected and transferred to experimental cells that had not been subjected to strain. 24 h later, thymidine incorporation was determined. Data are mean±SE of triplicate determinations.

Figure 6. Effect of GRGDTP on morphology of strained cells. Quiescent cultures of VSM cells on collagen-coated silicone elastomer plates were treated with vehicle (A), GRGDTP (100 μg/ml) (B), or GRGESP (100 μg/ml) (C) and subjected to mechanical strain (48 h). Cells were fixed and stained as described in Methods. x120.

Discussion

Mechanical deformation is a prominent feature of the environment of VSM cells in vivo and may play an important role both in developmental and pathological changes in the structure of

Figure 7. RGD peptides block strain-induced PDGF-A chain promoter–driven CAT activity. VSM cells were cotransfected (see Methods) with a PDGF-A chain promoter (890 bp) CAT construct and Rous Sarcoma Virus–β-gal. After 24 h of recovery, cells were exposed to either GRGDTP (100 mg/ml) or GRGESP (100 mg/ml) and to cyclic strain for 24 h. The lysates were harvested, assayed for β-galactosidase and CAT activities, and CAT normalized to β-galactosidase. Data presented as mean±SE for quadruplicate determinations in one of two similar experiments.
blood vessels. We have previously reported that in neonatal VSM cells, cyclic mechanical strain causes an increase in DNA synthesis via the autocrine production of PDGF (7). The present study was designed to learn more about the detection and signaling of mechanical forces.

The experiments reported in this study show that in VSM cells, the mitogenic response to mechanical strain is conferred through specific interactions with the extracellular matrix. Cells plated on silicone elastomer coated with collagen, fibronectin, or vitronectin increased DNA synthesis in response to mechanical strain, while cells plated on laminin, elastin, poly-L-lysine, or no coating responded minimally or not at all (Fig. 1). Although basal rates of DNA synthesis varied greatly on these various matrix proteins, there was no obvious correlation between basal thymidine incorporation on any given matrix protein and the response to strain on that protein. In addition, the failure of laminin to elicit a mitogenic response to strain cannot be explained by a nonspecific interference with mitogenic responses. Cells cultured on laminin failed to respond to strain but responded normally to PDGF and thrombin. More importantly, cells cultured on laminin (Fig. 2) were capable of responding to strain when the coating also contained small quantities of vitronectin.

The role of specific ECM proteins in conferring a response to strain was further assessed by exposing cells to these proteins in soluble form. When soluble, these proteins cannot confer information about surface deformation. When cells adhered to collagen were exposed to soluble fibronectin, the strain response was obliterated as fibronectin concentration increased. On the other hand, soluble laminin had no effect on the mitogenic response to strain. These data suggest that fibronectin, but not laminin, actually interferes with the detection of mechanical strain when presented to the cell in the medium instead of being fixed to the deforming substrate.

The state of extracellular matrix proteins is important in determining biological responses in other systems. For example, Werb and co-workers showed that immobilized RGD peptides or fibronectin fragments induce metalloproteinases by synovial fibroblasts, while intact fibronectin results in low expression of metalloproteinase (10, 30). Schwartz showed that insoluble, but not soluble, fibronectin activates Na/H exchange by clustering and immobilizing integrins (31).

These data taken together suggest that specific extracellular matrix proteins, including fibronectin and vitronectin, are involved in the cellular detection of mechanical strain. Preliminary data examining the induction of smooth muscle myosin in response to strain suggests that the matrix protein(s) responsible for conferring a given response to strain may not be invariant. Laminin, which does not confer a mitogenic response to strain, may participate in the strain response by adapting to strain and transmitting the signal (32). These data suggest that the cell–matrix interactions involved in the detection of mechanical strain are more limited than the interactions involved in adherence and spreading. Along these lines, Clyman and co-workers found that adhesion of VSM cells to many substrates requires a β1 integrin subunit, while migration specifically requires occupancy of αvβ3 integrins (35). Using endothelial cells, Schwartz and Denninghoff found that α-integrins are involved in calcium signaling but play only a minor role in adhesion (36). These data from several systems indicate that the cell–matrix interactions involved in signaling biological responses may differ from those involved in adhesion.
In our studies, fibronectin and vitronectin conferred the largest response to mechanical strain. This finding suggests that the primary method of sensing mechanical strain may be through interaction of fibronectin/vitronectin with specific integrin receptors. There is a high degree of redundancy of the integrin receptors for extracellular matrix ligands. For example, fibronectin can bind to integrins αβ1, αβ1, αβ1, αβ1, αβ1, αβ1, and αβ5 (37). Vitronectin has been shown to bind to αβ1, αβ1, αβ1, and αβ1 (38). Because both fibronectin and vitronectin confer a response to mechanical strain, our initial screening of integrins has centered on the subset of molecules thought to bind these proteins.

Most of the currently available function-blocking integrin antibodies were raised against human proteins and do not cross-react with the rodent forms. There are however, some anti-human integrin antibodies that do cross-react with rat integrins. We found mAbs to αβ5 (PIF6) and α, (HSP10) cross-reacted with rat VSM cells (Fig. 7). Liaw and co-workers found that rat VSM cells also express αβ5 (39). Function-blocking integrin antibodies were used to interfere with the mitogenic response to mechanical strain. Both αβ5 and β5 antibodies almost completely blocked this response, while the non–function-blocking α, antibody HSP10 did not significantly affect the response to strain. The virtually complete blockade of the strain response by both anti-αβ5 and anti-β5 antibodies may be explained in several ways. First, the anti-β5 antibody may cross-react with β5. Alternatively, the strain response could require participation by both integrins. In this regard, it is interesting that an antibody to the most prevalent β chain (β1) caused only partial inhibition of the strain response, suggesting that this β chain may not be of major importance in conferring the mitogenic response to strain. In view of the limited availability of reagents for rat integrins, these studies may give only a partial view of the role of integrins in mechanical signaling. However, taken in conjunction with the data obtained with various ECM proteins (Figs. 1–4), they clearly implicate the α family of fibronectin/vitronectin receptors in conferring mitogenic responses to mechanical forces.

To further demonstrate that occupancy of specific matrix receptors is involved in the detection of strain, RGD peptides were added to cells while they were subjected to mechanical strain (Figs. 5–7). GRGDTP (50-100 μg/ml) completely blocked strain-induced DNA synthesis while the inactive GRGESP had no effect. The failure of cells to undergo a mitogenic response to strain in the presence of RGD peptides could be due to altered secretion of PDGF or to an altered response to the secreted PDGF. Lin and Grinnell showed decreased PDGF-induced receptor autophosphorylation in cells on relaxed collagen matrices compared with stressed collagen gels (40). In our system, cells exposed to GRGDTP still respond to PDGF (Fig. 5 B), but conditioned medium from strained cells treated with GRGDTP was not mitogenic. Furthermore, GRGDTP inhibited the induction by strain of a PDGF-A chain promoter CAT construct. Thus, the extracellular matrix is responsible for conferring the signal(s) needed to enhance growth factor expression after mechanical strain.

The data presented in this study suggest that cells sense mechanical strain through interaction of integrin receptors in the plasma membrane with specific matrix RGD sites fixed to the moving substrate. Soluble RGD, fibronectin, and certain antiintegrin antibodies disrupt this specific interaction and prevent detection of mechanical strain without disrupting adhesion.

It is possible that detection of motion and subsequent signal generation involves movement of cytoskeletal elements bound to integrins (19). Work is currently under way to further characterize specific integrins and cytoskeletal elements involved in the detection and signaling of mechanical strain.

Acknowledgments

We thank Drs. Dean Sheppard and Tucker Collins for supplying essential reagents and Drs. Caroline Damsky and Dean Sheppard for critical reading of the manuscript. This work was supported by National Institutes of Health grant HL-41210, grants from the Research Evaluation and Allocation Committee and Academic Senate research committees of the University of California, San Francisco, and, during the tenure by H. E. Ives, an Established Investigator award from the American Heart Association. K. Sudhir was supported by a C. J. Martin fellowship of the National Health and Medical Research Council of Australia and funds from the Cardiac Research foundation (University of California, San Francisco).

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