

Mechanical Strain Induces Growth of Vascular Smooth Muscle Cells via Autocrine Action of PDGF

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Abstract. The effect of cyclic mechanical strain on growth of neonatal rat vascular smooth muscle (VSM) cells were examined. Cells were grown on silicone elastomer plates subjected to cyclic strain (60 cycle/min) by application of a vacuum under the plates. A 48 h exposure to mechanical strain increased the basal rate of thymidine incorporation by threefold and increased cell number by 40% compared with cells grown on stationary rubber plates. Strain also increased the rate of thymidine incorporation in response to α -thrombin (from 15- to 33-fold), but not to PDGF. As determined by thymidine autoradiography, strain alone induced a fourfold increase in labeled nuclei at the periphery of dishes, where strain is maximal, and a 2–3-fold increase at the center of dishes. Strain appeared to induce the production of an auto-

crine growth factor(s), since conditioned medium from cells subjected to strain induced a fourfold increase in DNA synthesis in control cells. Western blots of medium conditioned on the cells subjected to strain indicate that the cells secrete both AA and BB forms of PDGF in response to strain. Northern blots of total cell RNA from cells exposed to strain for 24 h show increased steady-state level of mRNA for PDGF-A. Lastly, polyclonal antibodies to the AA form of PDGF reduced by 75% the mitogenic effect of strain and polyclonal antibodies to AB-PDGF reduced mitogenicity by 50%. Antibodies to bFGF did not significantly reduce the strain-induced thymidine incorporation. Thus, the mechanism of strain-induced growth appears to involve the intermediary action of secreted PDGF.

REPETITIVE physical deformation is a prominent feature of the environment of vascular smooth muscles in situ. In hypertension, where mechanical strain on the arterial wall has been reported to increase by as much as 15% (13), there are numerous, marked alterations in the growth, responsiveness, and biochemical phenotype of the vascular smooth muscle cell (7). The mechanism of this important response remains unknown. The vast majority of studies examining the growth of vascular smooth muscle cells have been done under standard static in vitro culture conditions. Thus, little is known about the role which cyclic mechanical strain may play in the responses of vascular smooth muscle cells to growth factors and other stimuli.

Cyclic strain has been found to exert important effects on phenotypic expression and growth of a number of cell types. Sumpio et al. (18) examined the effects of strain on aortic endothelial cells and found altered synthesis of cytoskeletal proteins and reorganization of the cytoskeleton. Sumpio et al. (15) and Upchurch et al. (20) found that mechanical deformation suppressed prostacyclin secretion by arterial and venous endothelial cells. Banes et al. (1, 2) found that fibroblasts from intact chicken tendons showed reduced synthesis of tubulin in response to cyclic tension. Strain may also effect cell proliferation. Cyclic strain has been found to increase cell number and DNA synthesis in cultured bone (6), epithelial cells (3), and endothelial cells (17).

The effects of cyclic strain on vascular smooth muscle (VSM)¹ cells have not been studied extensively in the past. Leung et al. found a 2–4-fold increase in rates of collagen, hyaluronate, and chondroitin 6-sulfate synthesis by arterial smooth muscle cells grown on elastin membranes subjected to cyclic strain compared with controls subjected to agitation or to no motion (10). Kollros et al. used a similar model system and found that rabbit arterial smooth muscle cells responded to cyclic strain with increased collagen production (9). This effect was blocked by application of exogenous cAMP. Sumpio et al. reported that cyclic strain (3 cycles/min) enhanced collagen production and altered the orientation of cultured vascular smooth muscle cells (19), but actually reduced the rate of DNA synthesis (16).

The prominence of strain as a feature in the environment of vascular smooth muscle cells in situ led us to examine further the effects of repetitive strain on the growth of these cells. We studied the effects of cyclic strain on the basal growth of vascular smooth muscle cells and on the responses of these cells to growth factors. We also studied the effects of strain on early signaling events after growth factor activation. We find that strain does not appear to affect a variety of ion transport events associated with cell growth in vascular smooth muscle cells, but does induce secretion of both

1. Abbreviation used in this paper: VSM, vascular smooth muscle.

A and B chains of PDGF. Antibodies to the AA and AB forms of PDGF both blunt the mitogenic response to mechanical strain, implicating secreted PDGF as an important intermediary in the response to mechanical strain.

Materials and Methods

Materials

All materials were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. BSA was fraction V, fatty-acid poor (Miles Inc., Naperville, IL). [³H]thymidine was purchased from New England Nuclear (Boston, MA). 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) and fura-2 were purchased from Molecular Probes, Inc. (Eugene, OR). Photographic emulsion NTB2, D19 developer, photographic fixer, and hypo clearing agent were purchased from Eastman Kodak Co. (Rochester, NY). Ionomycin was purchased from Behring Diagnostics (Marsburg, Germany). Highly purified human α -thrombin was generously supplied by John W. Fenton II (Albany Medical College of Union University, Albany, NY). PDGF was homodimeric BB form obtained from Amgen Biologicals (Thousand Oaks, CA). PDGF antibodies for the Western blots were polyclonal rabbit anti-human PDGF-AA or -BB purchased from Genzyme Corp. (Cambridge, MA). Neutralizing antibodies for PDGF were polyclonal rabbit anti-human PDGF-AA and polyclonal goat anti-human PDGF-AB obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Neutralizing antibody to bFGF was monoclonal mouse anti-bovine bFGF obtained from Upstate Biotechnology, Inc.

Cell Culture

Primary cultures of VSM cells from newborn rat were established by Peter Jones (University of Southern California, Los Angeles, CA). From these primary cultures, the R22 D cell line was established (8) and generously supplied to us by Dr. Jones at passage 15. The cells were maintained in minimum essential medium with 10% FBS, 2% tryptose phosphate broth, penicillin (50 U/ml), and streptomycin (50 U/ml) in a humidified atmosphere of 5% CO₂, 95% air at 37°C. Culture medium was changed every other day until cells were confluent. Cells were subcultured with trypsin-versene and 0.2% pancreatin and cells from passages 16–29 were used for the current studies.

Application of Cyclic Strain to Cultured Cells

Cells were grown to confluence in six-well collagen-coated silicone elastomer-bottomed culture plates (Flex culture plates; Flexcell Corp., McKeesport, PA). Cells were subjected to mechanical deformation with the Flexercel Stress Unit (Flexcell Corp.). The stress unit is a modification of the unit initially described by Banes et al. (1, 2) and consists of a computer-controlled vacuum unit and a baseplate to hold the culture dishes. Vacuum (~15–20 kPa) is repetitively applied to the rubber-bottomed dishes via the baseplate, 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.

[³H]Thymidine Incorporation

Cells were grown in the six-well Flex plates until confluent and were growth-arrested by placing them in "quiescence" medium containing 5 μ g/ml transferrin and 0.5 mg/ml BSA for 72 h. Cells were then treated with the indicated agonists and/or subjected to cyclic strain for the specified periods. For measurement of [³H]thymidine incorporation into DNA, 1 μ gCi/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 (containing 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 30 min. The rubber bottom of the Flex plates containing the TCA-precipitable material was removed and placed directly into scintillation vials for counting.

Cell Number Determination

Cells were harvested with 0.25% trypsin-versene and 0.2% pancreatin, and cell number was measured by a Coulter Cell Counter (Coulter Electronics Ltd., Hialeah, FL).

[³H]Thymidine Autoradiography

Monolayers of cultured VSM cells grown on Flex culture plates were incubated with serum-free medium for 72 h to achieve quiescence as described above. Cells were then subjected to cyclic strain or no strain for 24 or 48 h. During this period cells were incubated with 0.5 μ Ci/ml [³H]thymidine (84.2 Ci/mmol; New England Nuclear) at 37°C. Cells were then washed three times with HBSS and fixed in ice-cold fresh acetic acid/methanol (1:3) for 10 min. To remove unincorporated thymidine, cells were washed three times for 10 min with 10% TCA at 4°C. They were then washed twice with distilled water and twice with 100% methanol. Plates were air dried and the rubber bottoms containing the cells were removed from the Flex culture plates. Under a safelight, the rubber bottoms were dipped into Kodak NTB2 emulsion diluted 1:1 with distilled water in water bath at 40°C. They were air dried for 20 min and transferred to light-tight boxes for exposure at 4°C for 24 h. Rubber bottoms were developed with Kodak D19 for 10 min with gentle agitation. After a brief wash in distilled water, rubber bottoms were placed in Kodak fixer for 3 min and then in hypo clearing agent for 1 min. After washing in distilled water and drying, rubber bottoms were stained with Giemsa stain for 1 min. They were then washed with 8.3 mM phosphate buffer, pH 7.2, for 10 min and rinsed thoroughly under running tap water. Rubber bottoms were then examined by bright field microscopy in a Nikon inverted microscope.

Northern Blots

Total cellular RNA was isolated from VSM cells by guanidine thiocyanate extraction. 15 μ g of total RNA was electrophoresed on 1% agarose gels containing formaldehyde and transferred to nylon hybridization transfer membrane (Amersham Corp., Arlington Heights, IL). Blots were hybridized to a full length cDNA probe for the PDGF-A message which was labeled with [α -³²P]dCTP (3,000 Ci/mmol; Amersham Corp.) using the random primers method. Blots were washed and autoradiographed with x-ray film overnight at -70°C. Blots were then stripped and, for normalization, rehybridized with β_2 -microglobulin cDNA labeled with the random primers method.

Western Blots

15 ml of conditioned culture media was concentrated to 1 ml in a Centriprep-10 filter (10-kD cut-off, Amicon Corp., Danvers, MA). This material was incubated for 4 h at 4°C with 2 μ g each of polyclonal rabbit anti-human PDGF-AA and -BB (Genzyme Corp., Cambridge, MA). Proteins were precipitated with 20 μ l of protein A agarose bead preparation (Sigma Chemical Co.) and centrifuged in a microfuge. Pellet was resuspended in 50 μ l of a nonreducing buffer (Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.02% bromophenol blue) and boiled for 5 min. 15 μ l of this material was applied to each of two 12.5% SDS-polyacrylamide gels. Proteins were electrophoresed under nonreducing conditions, then transferred by electrophoresis to Hybond ECL filters (Amersham Corp.) in transfer buffer (50 mM Tris, 380 mM Glycine, 20% methanol). Filters were blocked with 10% nonfat dry milk in TBS (20 mM Tris, pH 7.5, 50 mM NaCl, and 0.1% Tween-20), then washed and incubated with the primary antibody of interest for 1 h. After washing, blots were incubated with the HRP-conjugated secondary antibodies (anti-rabbit), washed and incubated for 1 min with enhanced chemiluminescence reagents (Amersham Corp.). Blots were exposed to x-ray film for from 30 s to 5 min to obtain ideal exposure.

Data Presentation and Statistics

All data were presented as mean \pm SE for the indicated number of observations (*n*). Comparisons were made using *t* test. Values for *P*<0.05 were considered significant.

Results

To study the effect of cyclic strain on growth of VSM cells, cells were grown in rubber-bottomed flex culture plates that were subjected to strain by cyclic application of a vacuum (see Materials and Methods). Controls were grown in the same dishes not exposed to cyclic strain. In quiescent cells without added mitogens, cyclic strain (60 cycles/min) caused a 2.7 \pm 0.3-fold increase in the rate of [³H]thymidine incorporation at 48 h (Table I) and a 40% increase in total cell

Table I. Effect of Cyclic Strain on Thymidine Incorporation in VSM cells

Condition	Thymidine incorporation
Control	1924 ± 211
Strain	5203 ± 562*

Quiescent cultures of VSM cells in rubber-bottomed flex culture plates were subjected to cyclic mechanical strain for 48 h. Control cells were grown under identical conditions without application of the strain-producing vacuum. During the final 6 h of the incubation, [³H]thymidine was added to each well. Cells were harvested and incorporation of radioactivity was determined. Data is mean ± SE for triplicate determinations in a single experiment typical of six similar experiments.

* *P* < 0.001, strain versus control.

number after 48 h (Table II). In cells grown on static rubber plates, α-thrombin (1 U/ml) induced (at 48 h) a 15-fold increase in the rate of DNA synthesis over control. This response was more than doubled in thrombin-treated cells that were also exposed to strain (Fig. 1). PDGF (5 ng/ml) caused a 27-fold increase in the rate of DNA synthesis at 48 h and a 2.1-fold increase in cell number over control. This response to PDGF was not significantly enhanced by strain (Table II and Fig. 1). At smaller doses of PDGF (0.5 ng/ml), strain also did not enhance the response to PDGF (data not shown). This may be due to the fact that strain appears to act through autocrine production of PDGF (see below).

We next determined the time of exposure to strain which would elicit a maximal increase in DNA synthesis and compared this to the time needed for growth factors to induce maximal DNA synthesis. Cells were exposed to strain for various times from 0 to 54 h. To determine the rate of DNA synthesis, [³H]thymidine was added for the final 6 h of the strain period. DNA synthesis, as measured by thymidine uptake, was maximal after a 36–48-h exposure to strain (Fig. 2). This time is markedly different from the time required for maximal increase in DNA synthesis after α-thrombin. After thrombin (1 U/ml), thymidine incorporation peaks in 18 h. A smaller dose of thrombin (0.01 U/ml) produced a smaller response, but the time of peak thymidine incorporation was still at 18 h. Likewise, DNA synthesis after PDGF peaked at 12–18 h (data not shown). Thus, the mechanism by which strain enhances DNA synthesis requires much longer to be expressed than what is observed with polypeptide growth factors.

Table II. Effect of Cyclic Strain on Proliferation of VSM Cells

Condition	Cell number (×10 ⁵)
Control	1.72 ± 0.56 (6)
Strain	2.42 ± 0.49* (6)
Thrombin	2.95 ± 0.12 (3)
Strain + thrombin	3.70 ± 0.10‡ (3)
PDGF	3.63 ± 0.09 (3)
Strain + PDGF	3.81 ± 0.03§ (3)

Quiescent cultures of VSM cells plated at a density of 1.5 × 10⁵ cells per dish were treated with α-thrombin (1 U/ml) or PDGF (5 ng/ml) in rubber-bottomed flex culture plates that were subjected to cyclic strain versus control cells not subjected to strain. After a 48-h incubation, cells were harvested by trypsinization and counted in a Coulter Counter. Data is mean ± SE for the number of wells in parentheses.

* *P* < 0.05, strain versus Control.

‡ *P* < 0.01, strain + thrombin versus thrombin.

§ NS, strain + PDGF versus PDGF.

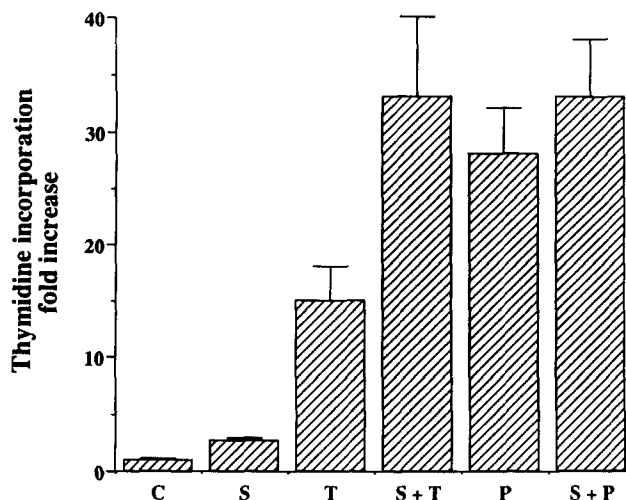


Figure 1. Effects of cyclic strain on growth factor-induced DNA synthesis in vascular smooth muscle cells. Quiescent cultures of VSM cells were treated with α-thrombin (10⁻³ U/ml) or PDGF (0.5 ng/ml) in rubber-bottomed flex culture plates that were subjected to cyclic strain versus control cells not subjected to strain. After a 24-h incubation, [³H]thymidine was added to each well, followed by an additional 6 h of exposure to strain. Cells were then harvested and incorporation of radioactivity was determined. Data is presented as mean ± SE of at least three observations in six similar experiments.

If DNA synthesis is induced by a direct action of strain, the rate of DNA synthesis should be proportional to the degree of strain applied to the cell. In the system we are using, it has been established by mathematical calculations and empirical observations that maximal strain (~25%) occurs at the outer rim of the dish, while cells at the center are strained

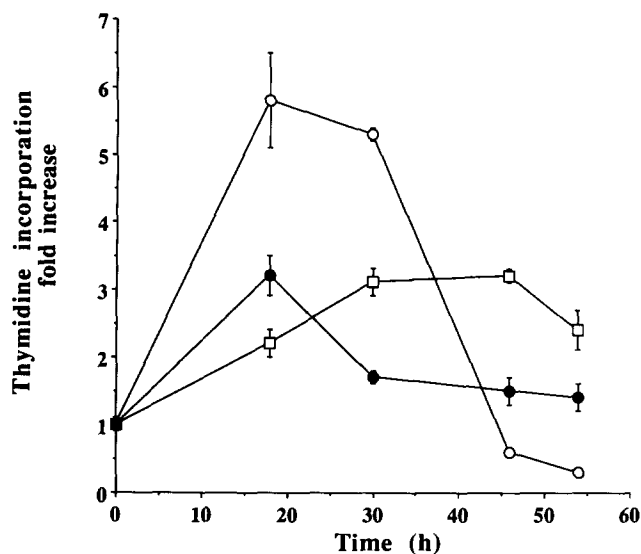


Figure 2. Effect of time of exposure to strain or α-thrombin on rate of [³H]thymidine incorporation. Quiescent cultures of VSM cells grown in rubber-bottomed flex culture plates were exposed to α-thrombin (1.0 U/ml, ○; 0.2 U/ml, ●) or strain (□) for the indicated times. [³H]thymidine was added to each well 6 h before harvest. [³H]thymidine incorporation into DNA was determined as described in Materials and Methods. Data is mean ± SE for triplicate determinations in one of two similar experiments.

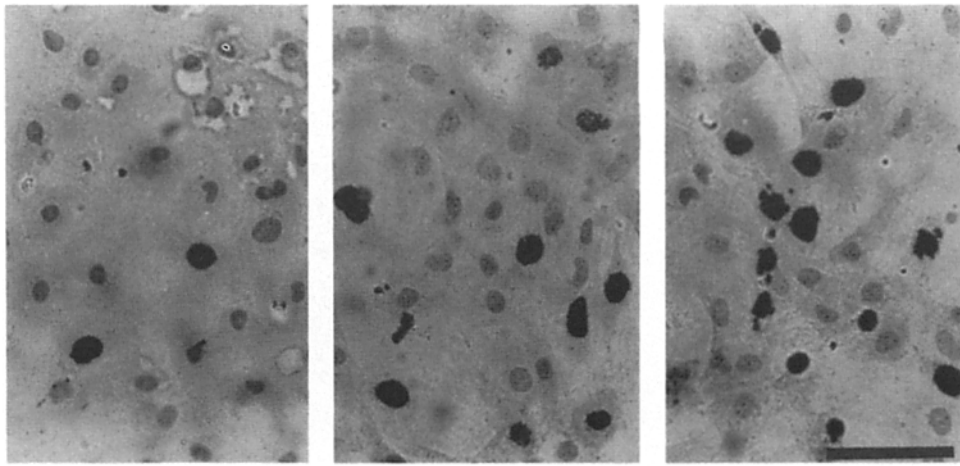


Figure 3. Effect of strain on nuclear labeling with thymidine. Quiescent cultures of VSM cells were incubated with [³H]thymidine for 48 h and autoradiography was performed as described in Materials and Methods. (*Left*) Flex plate that was not strained plates; (*Middle*) center of strained plate; (*right*) periphery of strained plate. Bar, 140 μ m.

by at most 3% (5). To measure DNA synthesis in these regions of the dish, thymidine autoradiography was performed after a 48-h strain (Fig. 3). In dishes not subjected to strain, $10 \pm 1\%$ ($n = 4$) of nuclei were labeled, while in strained dishes, $38 \pm 4\%$ ($n = 3$) of nuclei at the periphery were labeled and $27 \pm 3\%$ ($n = 3$, $P < .001$ compared to control or periphery) of nuclei at the center were labeled. Thus, at the periphery where strain is greatest, the stimulation of DNA synthesis was greatest. However, thymidine incorporation at the center of the dishes was considerably more than would be expected if there were a linear relationship between strain and DNA synthesis. One possible explanation for this nonlinear relationship between growth and strain is that cells may be sufficiently sensitive to strain that only a minimal level of strain is needed to elicit the maximal growth response. Alternatively, strain may induce the cells to produce a paracrine growth factor which would then influence all cells in the dish.

To determine whether VSM cells subjected to strain produce a growth factor, we examined the induction of growth in test VSM cells which were incubated with media that had been conditioned on plates of cells that were static or subjected to strain for 48 h. The test cells were placed in rubber-bottomed dishes, but were not subjected to strain, and were incubated with the conditioned media for an additional 48 h, after which thymidine incorporation was assessed (Table III). The results show that medium from cells subjected to strain markedly increases DNA synthesis in the test cells, while medium from unstrained cells did not. Thus, strain induces the cells to secrete one or more paracrine growth factors.

One growth factor which is known to be secreted by vascular smooth muscle cells is the A chain of PDGF (14). To determine whether the cells subjected to strain for 48 h secrete either A- or B-chains of PDGF, 15 ml of medium was removed from such cells, concentrated, and immunoprecipitated with a combination of polyclonal antibodies to PDGF-AA and PDGF-BB. Immunoprecipitates were electrophoresed under non-reducing conditions and blots were probed separately with the two PDGF antibodies. The Western blots (Fig. 4) revealed significant increases in both AA and BB homodimeric forms of PDGF in the medium after 48 h of mechanical strain, indicating that both chains are secreted into the medium in response to strain.

To determine whether mechanical strain induces mRNA of PDGF-A, Northern blots of total cell RNA obtained from cells exposed to strain or α -thrombin were probed with a labeled cDNA probe for the PDGF A chain message (Fig. 5). Autoradiogram of the blot (quantitated by densitometry) reveals a threefold increase in the steady state level of mRNA for PDGF-A. This is comparable to the induction of this message by α -thrombin (21).

To determine whether the secreted PDGF actually contributes to the mitogenic response to strain, cells were subjected to strain and incubated for 48 h with neutralizing polyclonal antibodies to the AA or AB forms of PDGF (Fig. 6). After subtraction of DNA synthesis in control dishes, the antibody to AA PDGF reduced DNA synthesis in the cells exposed to strain by $>75\%$ (Fig. 6 A). This was not due to a toxic effect of the antibody, as a similar concentration of the same antibody had no effect on the mitogenic response to α -thrombin (data not shown). Furthermore, unrelated mixed rabbit IgG at the same concentration had no effect on growth of control cells or cells subjected to strain (data not shown). Another polyclonal antibody, raised to the AB form of PDGF reduced DNA synthesis by approximately 50% (Fig. 6 B). Lastly, a polyclonal antibody to bFGF did not significantly reduce the mitogenic response to strain (Fig. 6 C). Thus PDGF, but not bFGF, appears to play an important role in the mitogenic response to strain.

Table III. Effect of Conditioned Media on Growth of VSM Cells

Condition	Thymidine incorporation
Control medium	8687 \pm 470
Medium from unstrained cells	8119 \pm 264
Medium from strained cells	33626 \pm 5093*

To determine whether strained VSM cells secrete growth factors, quiescent VSM cells not subjected to strain were incubated with control or conditioned media for 48 h. Control medium was MEM, transferrin (5 μ g/ml) and BSA (0.5 mg/ml). Conditioned media were from cells grown in rubber dishes that has been strained or not strained for 48 h.

* $P < .0001$ compared with medium from unstrained cells.
 $n = 6$ in all cases.

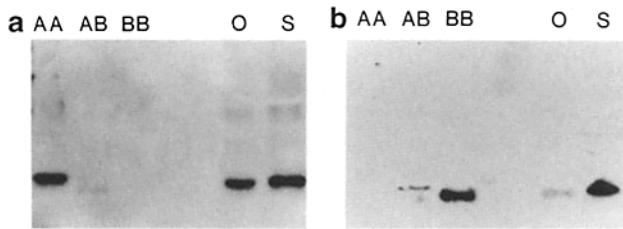


Figure 4. Secretion of PDGF-AA and -BB by cells subjected to strain. Quiescent cultures of VSM cells were plated on flex plates in fresh serum-free medium. After a 48-h incubation, the medium from control cells (O) or cells exposed to strain (S) was removed, concentrated, immunoprecipitated, and electrophoresed as described in Materials and Methods. Gels were blotted and probed with anti-PDGF-AA (a) or anti PDGF-BB (b). Standard lanes contained, as indicated, 10 ng PDGF-AA and 100 ng PDGF-AB or -BB (5a) or, 10 ng PDGF-BB and 100 ng PDGF-AB or -AA (5b). Data is representative of three similar experiments.

Discussion

Cell shape and cell deformation play an important role in the biology of many cell types. Like all muscle cells, VSM cells are physically deformed in the course of performing their biological function. It is therefore logical to ask what effect deformation may have in the responses to important biological agents, such as growth factors.

The experiments reported here show that cyclic strain may play a critical role in vascular smooth muscle cell growth. In quiescent cells without mitogens, cyclic strain significantly increased DNA synthesis and cell number in cultured vascular smooth muscle cells. When cyclic strain was applied with a potent mitogen, α -thrombin, there was a significant increase in the rate of DNA synthesis at 48 h compared to either stimulus when applied separately. While interpretation of the instantaneous rate of DNA synthesis at 48 h may be clouded by the different time courses of the response to mitogens and to strain (Fig. 2), the thrombin-induced increase in total cell number after 48 h is also enhanced by mechanical strain (Table II). This suggests that strain and thrombin may cooperatively increase proliferation of VSM cells. On the other hand, with PDGF there was no enhancement of the mitogenic response by simultaneous application of mechanical strain. This difference between α -thrombin and PDGF may be explained by the finding that

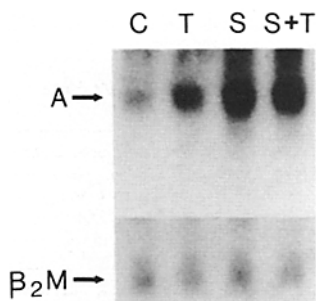


Figure 5. Increase in mRNA for PDGF-A in response to mechanical strain. Quiescent cultures of VSM cells in flex plates were exposed to nothing (C), α -thrombin (1 U/ml) for 8 h (T), mechanical strain for 24 h (S) or both (S+T). Total cell RNA was isolated, electrophoresed, and probed with a cDNA probe for the PDGF-A message as described in Materials and Methods. Band shown (A) is the 1.7 kb PDGF-A transcript. Autoradiograms were quantitated by densitometry, (9.6 arbitrary units [C], 17.5 U (T), 34.9 U (S), and 29.3 U (T+S)). Data is representative of three similar experiments.

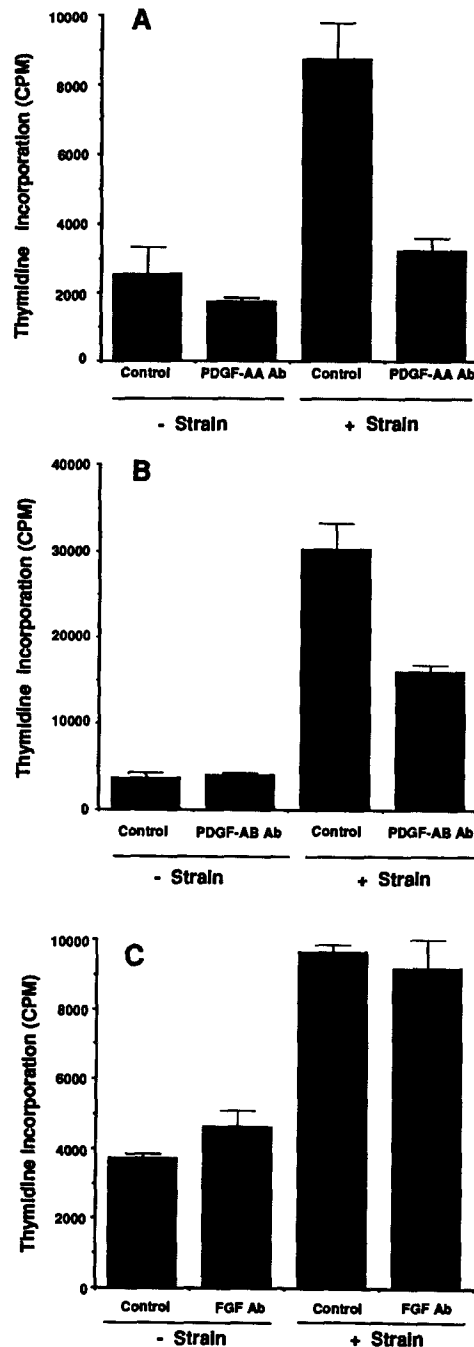


Figure 6. PDGF antibodies block mitogenic response to mechanical strain. Quiescent cultures of VSM cells in flex plates were exposed in separate experiments to the indicated antibodies (3 μ g/ml) and to cyclic strain for 48 h. During the final 6 h of the incubation, [3 H]thymidine was added to each well. Cells were then harvested and incorporation of radioactivity was determined. (a) Experiment 1—PDGF-AA antibody; (B) experiment 2—PDGF-AB antibody; (C) experiment 3—bFGF antibody. Data is mean \pm SE for triplicate determinations. Each figure is typical of two similar experiments in each case.

strain appears to act by inducing the production and secretion of PDGF, obviating any additional effect of exogenously added PDGF (see below).

Our finding that mechanical strain induces growth of cultured VSM cells differs from the findings of Sumpio and

Banes (16), who showed that application of strain at 3 cycle/min for 7 d reduced the proliferation of porcine vascular smooth muscle cells for the first 24–48 h. This initial loss of cells was actually followed by an increased proliferative rate, suggesting that a portion of the cells may have been destroyed by mechanical strain, a phenomenon not observed in our study. Other potential reasons for the discrepancy between the two studies are the different rate of cycling used and the use of neonatal rat cells in our study vs. adult porcine cells by Sumpio. This raises the possibility, requiring further study, that different subtypes of vascular smooth muscle cells (neonatal vs. adult) may respond differently to strain.

One concern raised by these experiments is the potential role of stirring on the access of nutrients and/or growth factors to cells grown on a moving surface. Previous work on this issue indicates that growth of cells in culture is generally not limited by the diffusion of nutrients but rather that it can be altered by changes in cell shape (4, 22). Our results also argue against a significant role for stirring, since improved stirring would be expected at the center of the dish, where vertical motion is the greatest, while the growth response was greatest at the periphery of the dish, where vertical motion is minimal but strain is maximal (Fig. 3). More importantly, the mitogenicity of medium taken from the cells subjected to strain (Table III) indicates that stirring is clearly not the sole mechanism by which growth is induced on the rubber plates. Rather, it appears most likely that strain is inducing the production of an autocrine growth factor which secondarily causes the cells to divide.

In studies on the time course of induction of DNA synthesis, we found a marked difference in the time needed to achieve the maximal rate of DNA synthesis between thrombin and simple application of strain. For thrombin and PDGF, maximal DNA synthesis was 12–18 h after addition of the thrombin, independent of the dose used. For strain, maximal DNA synthesis occurred 36–48 h after initial application of the stimulus. This difference makes it unlikely that strain of the cells leads to activation of conventional growth factor receptors through some form of mechanical stimulation. Rather, this data supports the idea, proposed above, that strain induces slow production and release of growth factors which may then diffuse across the dish and induce cell growth through a paracrine mechanism.

As a first step in determining the mitogen(s) produced by strained VSM cells, we probed Western blots of the concentrated medium with antibodies to intact PDGF AA and BB. Both forms were apparently secreted in response to mechanical strain. Presumably PDGF-AB is also present in the medium but we have not yet shown this directly. Based on a comparison of the staining intensity of the bands from the immunoprecipitated medium with the intensity of the PDGF standards, there is between 2 and 3 ng/ml of both forms of PDGF secreted into the medium during the period of mechanical strain. This quantity is sufficient to elicit a mitogenic response. Moreover, neutralizing antibodies to the AA and AB forms of PDGF strikingly reduced the otogenic response in cells exposed to mechanical strain. While neither antibody totally eliminated the mitogenic response, and there was some experimental variation in the effects of the antibodies, these data clearly implicate PDGF as an important intermediary in the response to strain. It is, of course, possible that other mitogens are produced by the cells during

mechanical strain. bFGF appears not to be involved in the response. Further analysis of the conditioned medium from cells subjected to strain is currently underway. Lastly, induction of PDGF A-chain expression has previously been implicated as a mediator in the autocrine or paracrine stimulation of growth (11, 12, 14). We find that the steady state level of mRNA for this gene is increased in strain. In separate work (Wilson, E., T. Collins, and H. E. Ives, manuscript in preparation), we have shown that this increase in message is due to increased transcription of the PDGF A-chain gene, further implicating this factor in the response to strain.

In summary, this study shows that cyclic strain is an important regulatory factor in the growth of vascular smooth muscle cells. Strain appears to act via production of paracrine growth factors like PDGF. The mechanism(s) by which physical deformation leads to increased gene expression and protein secretion remains an important unsolved problem.

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References

1. Banes, A. J., J. Gilbert, D. Taylor, and O. Monbureau. 1985. A new vacuum-operated stress-providing instrument that applies static or variable duration cycle tension or compression to cells in vitro. *J. Cell Sci.* 75:35–42.
2. Banes, A. J., G. W. Link, Jr., J. W. Gilbert, R. T. S. Tay, and O. Monbureau. 1989. Culturing cells in a mechanically active environment. *Am. Biotechnol. Lab.* 8:12–22.
3. Brunette, D. M. 1984. Mechanical stretching increases the number of epithelial cells synthesizing DNA in culture. *J. Cell Sci.* 69:35–45.
4. Curtis, A. S., and G. M. Seehar. 1978. The control of cell division by tension or diffusion. *Nature (Lond.)* 274:52–53.
5. Gilbert, J. A., A. J. Banes, G. W. Link, and G. J. Jones. 1989. Surface strain of living cells in a mechanically-active, in vitro environment. In ANSYS Conference Proceedings. D. E. Dietrich, editor. Swanson Analysis Systems, Houston, PA. 13.2–13.7.
6. Hasegawa, S., S. Sato, S. Saito, Y. Suzuki, and D. M. Brunette. 1985. Mechanical stretching increases the number of cultured bone cells synthesizing DNA and alters their pattern of protein synthesis. *Calcif. Tissue Int.* 37:431–436.
7. Ives, H. E., and T. O. Daniel. 1990. Vascular diseases of the kidney. In *The Kidney*. B. M. Brenner and F. C. Rector, Jr., editors. W. B. Saunders Co., Philadelphia, PA. 1497–1550.
8. Jones, P. A., T. Scott-Burden, and W. Gevers. 1979. Glycoprotein, elastin, and collagen secretion by rat smooth muscle cells. *Proc. Natl. Acad. Sci. USA.* 76:353–357.
9. Kollros, P. R., S. R. Bates, M. B. Mathews, A. L. Horwitz, and S. Glagov. 1987. Cyclic AMP inhibits increased collagen production by cyclically stretched smooth muscle cells. *Lab. Invest.* 56(4):410–417.
10. Leung, D. Y., S. Glagov, and M. B. Mathews. 1976. Cyclic stretching stimulates synthesis of matrix components by arterial smooth muscle cells in vitro. *Science (Wash. DC)* 191:475–477.
11. Raines, E. W., S. K. Dower, and R. Ross. 1989. Interleukin-1 mitogenic activity for fibroblasts and smooth muscle cells is due to PDGF-AA. *Science (Wash. DC)* 243:393–396.
12. Rao, G. N., M. A. Corson, and B. C. Berk. 1991. Uric acid stimulates vascular smooth muscle cell proliferation by increasing platelet-derived growth factor A-chain expression. *J. Biol. Chem.* 266:8604–8608.
13. Safar, M. E., P. A. Peronneau, J. A. Levenson, J. A. Toto-Moukoko, and A. C. Simon. 1981. Pulsed doppler: Diameter, blood flow velocity, and volumic flow of the brachial artery in sustained essential hypertension. *Circulation.* 63:393–400.
14. Sjolund, M., U. Hedin, T. Sejersen, C.-H. Heldin, and J. Thyberg. 1988. Arterial smooth muscle cells express platelet-derived growth factor

- (PDGF) A chain mRNA, secrete a PDGF-like mitogen, and bind exogenous PDGF in a phenotype- and growth state-dependent manner. *J. Cell Biol.* 106:403-413.
15. Sumpio, B. E., and A. J. Banes. 1988. Prostacyclin synthetic activity in cultured aortic endothelial cells undergoing cyclic mechanical deformation. *Surgery (St. Louis)*. 104:383-389.
 16. Sumpio, B. E., and A. J. Banes. 1988. Response of porcine aortic smooth muscle cells to cyclic tensional deformation in culture. *J. Surg. Res.* 44:696-701.
 17. Sumpio, B. E., A. J. Banes, L. G. Levin, and G. Johnson, Jr. 1987. Mechanical stress stimulates aortic endothelial cells to proliferate. *J. Vasc. Surg.* 6:252-256.
 18. Sumpio, B. E., A. J. Banes, M. Buckley, and G. Johnson, Jr. 1988. Alterations in aortic endothelial cell morphology and cytoskeletal protein synthesis during cyclic tensional deformation. *J. Vasc. Surg.* 7:130-138.
 19. Sumpio, B. E., A. J. Banes, W. G. Link, and G. Johnson, Jr. 1988. Enhanced collagen production by smooth muscle cells during repetitive mechanical stretching. *Arch. Surg.* 123:1233-1236.
 20. Upchurch, G. R., Jr., A. J. Banes, W. H. Wagner, F. Ramadan, G. W. Link, R. H. Henderson, and G. Johnson, Jr. 1989. Differences in secretion of prostacyclin by venous and arterial endothelial cells grown *in vitro* in a static versus a mechanically active environment. *J. Vasc. Surg.* 10:292-298.
 21. Weiss, R. H., and H. E. Ives. 1991. Dissociation between activation of growth-related genes and mitogenic responses of neonatal vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* 181:617-622.
 22. Whittenberger, B., and L. Glaser. 1978. Cell saturation density is not determined by a diffusion-limited process. *Nature (Lond.)*. 272:821-823.