The elastin-laminin receptor functions as a mechanotransducer in vascular smooth muscle

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Spofford, Christina M., and William M. Chilian. The elastin-laminin receptor functions as a mechanotransducer in vascular smooth muscle. Am J Physiol Heart Circ Physiol 280: H1354–H1360, 2001.—Laminin and elastin, two major constituents of the extracellular matrix, bind to cells via the elastin-laminin receptor (ELR), a receptor distinct from integrins. Despite the ubiquitous nature of elastin and laminin in the matrix, the consequences of activation of the ELR are unknown. Because integrins are capable of mechanosensitive transduction, we hypothesized that the ELR would exert a similar function. Accordingly, we examined the effects of cyclical stretch on canine coronary smooth muscle gene expression and proliferation that are mediated by the ELR. Northern blot analyses showed a 31% decrease in serum-induced expression of c-fos when cells were stretched for 30 min on elastin, but no change in expression was observed on collagen. Serum-induced proliferation of stretched cells was markedly attenuated on elastin when compared with collagen. Both the molecular (decreased c-fos expression) and biological (decreased proliferation) responses on elastin were restored after blockade of the ELR with the elastin fragment hexapeptide (valine-glycine-valine-alanine-proline-glycine, VGVAPG). The inhibition was specific for this peptide, as another hydrophobic hexapeptide (valine-serine-leucine-serine-proline-glycine, VSLSPG) did not inhibit the responses. These results demonstrate that cyclic stretch inhibits c-fos expression and proliferation of coronary vascular smooth muscle cells grown on elastin matrixes, a mechanosensitive response that is transduced by the ELR.

VASCULAR CELLS in vivo are primarily subjected to two hemodynamic forces: shear stress from flowing blood and cyclical strain from pulsations in pressure with each cardiac cycle. During the cardiac cycle, large arteries can stretch by 9–10%, which can be increased to 15% with hypertension (15, 20, 45, 46). Many vascular adaptations to hypertension, such as matrix remodeling and vascular smooth muscle (VSM) hypertrophy, can be prevented if the arterial bed is protected from a rise in pressure (5). This suggests that VSM cells are capable of sensing and responding to increased stretch. However, the cellular mechanisms underlying the mechanotransduction of stretch are incompletely understood.

Extracellular matrix (ECM) receptors, when coupling cells to the matrix, are plausible transducers of stretch. These receptors are transmembrane proteins, capable of linking the ECM to the actin cytoskeleton; thus deformation of the cytoskeleton is transmitted intracellularly via these linkages. The integrin family of receptors has been studied extensively as mechanotransducers of stretch in VSM (23, 44, 51). Many integrins, e.g., \( \alpha_5\beta_1 \), \( \alpha_4\beta_5 \), \( \alpha_v\beta_3 \), \( \alpha_5\beta_1 \), and \( \alpha_{10}\beta_3 \), are capable of binding ECM proteins through a conserved arginine-glycine-aspartic acid (RGD) motif (40). Other matrix proteins, such as laminin, can bind to integrins in VSM (\( \alpha_5\beta_1 \)) through non-RGD motifs (9, 40). The binding of integrins to the matrix proteins initiates coupling to focal adhesion contacts, which trigger cellular signaling pathways (23, 24, 31). Although the integrins are clearly involved in myriad cellular functions, including acculturation to stretch, other transmembrane receptors may play pivotal roles as well. We make this point because two major constituents of the ECM are elastin and laminin. Although laminin can bind to VSM cells via the \( \alpha_{5}\beta_1 \)-integrin (9), elastin lacks known amino acid sequences necessary for binding to integrins. However, elastin and laminin can adhere to cells by a distinct transmembrane protein, the elastin-laminin receptor (ELR), which recognizes hydrophobic motifs in the proteins (16). This receptor is distinct from the integrin family and shows sequence homology to the alternatively spliced variant of human \( \beta\)-galactosidase (19).

Because of the promiscuity of elastin in the vessel wall, we postulate that the ELR may mechanotransduce stretch in VSM. The ELR is a heterotrimeric transmembrane receptor that binds specific hydrophobic sequences in elastin and laminin and induces various signal transduction pathways (16, 22, 35, 43). Within the vessel wall, the ELR is located on both endothelial and VSM cells where it has been shown to participate in endothelial control of vascular tone (11, 12). It seems probable that the ELR, in addition to the previously described roles, can function as a mechanotransducer.
transducer in VSM cells. Indeed, previous work in our laboratory (27) and others (51) has shown that gene expression and proliferation in stretched VSM is different in cells grown on collagen vs. elastin. Whereas several laboratories have focused on the role of integrins in mediating these adaptations, to date none have examined a role for the ELR.

Under basal conditions, the expression of the early response gene, c-fos, is very low in many cell types but is quickly and transiently induced by multiple extracellular signals (6, 30, 39, 48). The c-Fos protein acts as a transcription factor at the activator protein-1 site where it controls expression of many target genes. These characteristics provided the foundation for our hypothesis that the expression of this gene would be sensitive to mechanical stretch and, based on our previous studies (27), likely to be modulated in a matrix-dependent fashion. Likewise, cellular proliferation, as a biological output of altered gene expression and signaling cascades, would also be affected by stretch in a substrate-dependent manner. Accordingly, we further hypothesized that the responses to stretch on elastin matrixes would signal through the ELR and could therefore be abrogated by blockade of this receptor.

MATERIALS AND METHODS

Cell culture. Adult VSM was isolated from canine coronary arteries cleaned of fat, adventitia, and endothelium. With the use of an explant technique, freshly isolated left anterior descending arteries were plated luminal side down in 35-mm cell culture dishes containing DMEM supplemented with 10% FBS, 2 mM l-glutamine, 1 mM sodium pyruvate, 200 IU/ml penicillin, and 200 μg/ml streptomycin ( Gibco-BRL, Rockville, MD). To decrease variability seen with phenotypic drift characteristic of smooth muscle cells cultured for extended periods of time, we used cells from early passages (passage 2 to passage 9). Cells were maintained in a humidified atmosphere at 37°C in the presence of 5% CO2 until reaching confluence. Immunocytochemistry for smooth muscle α-actin and calponin (Sigma, St. Louis, MO) was used to verify smooth muscle cells, and the purity of the cultures was assessed by trypan blue exclusion. Comparisons were made to the number of cells seeded at the beginning of the experiment and reported as a percent increase in cell number.

RNA isolation/Northern blot analysis. RNA was isolated from cells using TRIzol reagent (GIBCO-BRL) according to the manufacturer’s directions. Total RNA was quantified using a spectrophotometer, and 20 μg RNA was loaded on a denaturing 1.2% agarose gel with formaldehyde. The RNA was electrophoresed at 4 V/cm and transferred to a positively charged nylon membrane (Boehringer Mannheim, Indianapolis, IN) by capillary blotting. The membrane was cross-linked with ultraviolet light and prehybridized using PerfectHybe reagent (Sigma) according to the manufacturer’s directions. cDNA probes from murine c-fos and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 2.2 and 1.4 kb, respectively) were radiolabeled with [32P]dCTP (Amersham Pharmacia, Piscataway, NJ) and hybridized overnight (5 × 106 counts·min⁻¹·ml⁻¹) at 68°C. The blot was washed and placed on Kodak BioMax MS film with a BioMax intensifying screen at ~80°C for 4–16 h. The film was developed and scanned, and the relative band intensity of each signal was quantified using image-processing software (Image 1.28; National Institutes of Health Research Services Branch, Bethesda, MD). Densitometric units were normalized to the ethidium bromide-stained 28S ribosomal subunit or GAPDH signal. There is continued debate in the literature as to the best housekeeping gene for normalization of molecular data (49, 54). In this study, the early time points (15 and 30 min) were studied using GAPDH, whereas the latter time points (60 and 120 min) were studied using GAPDH. To help discern whether there was a difference in the data sets based on which gene was used as the housekeeping gene, we repeated some of the early time points with GAPDH. We found no significant difference in the percent change from static control between the data sets normalized to either gene. This suggests that stretching in the presence of serum does not uniformly alter the expression of c-fos and GAPDH, genes both transcribed by RNA polymerase II.

Protocols. Cells were stretched for 48 h to assess changes in proliferation or for 15, 30, 60, or 120 min to assess changes in c-fos expression. The ELR antagonist hexapeptide valine-glycine-valine-alanine-proline-glycine (VGVAPG; Sigma) and the biologically inactive control peptide valine-serine-leucine-serine-proline-glycine (VSLSPG; custom synthesis, Medical College of Wisconsin Protein & Nucleic Acid Facility) were solubilized in DMSO (final concentration 0.02% vol/vol). Both peptides (10⁻⁵ M) were added to cells in low serum media for a 2-h preincubation period before the initiation of stretch. Once the preincubation was completed, serum was added, and the cells were stretched. Previous studies have revealed that the optimal concentration of VGVAPG to block elastin-ELR binding was 10⁻⁵ M, which elicited biological blockade of the receptor without causing cells to lift from the culture dish. The control peptide, VSLSPG, was found to be applied to the culture dishes. Cycling parameters were chosen for comparability with previous work (25, 27, 36, 37) and result in cyclical stretch of ~10% at the periphery.

Cellular proliferation. Proliferation was assessed by direct determination of the cell numbers. Trypsinized cells were plated (2.5 × 10⁴ cells/well) on type I collagen- or elastin-coated Flexercell dishes with rigid or flexible bottoms. Cells were grown arrested in 0.1% FBS for 3 days, with media replacement each day. After growth arrest, 10% serum was added, and cells were stretched for 48 h. At the end of the stretching period, cells were trypsinized and counted using a hemacytometer. To confirm complete trypsinization, cells were visualized before counting. Cellular viability was assessed by trypan blue exclusion. Comparisons were made to the number of cells seeded at the beginning of the experiment and reported as a percent increase in cell number.

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ineffective at eluting the ELR from elastin affinity columns, suggesting that a sequence-specific interaction beyond similar charge, hydrophobicity, and secondary structure is necessary for elastin-ELR interactions (29). In pilot experiments, the basal expression of c-fos was undetectable under growth arrest conditions, and we could not discern any effects of stretch. However, we found that, when stretch and 10% serum were applied simultaneously after 3 days of growth arrest, we could adequately detect and modulate the c-fos message.

**Statistics.** Dimensionless quantities (relative densitometric units) of the c-fos and GAPDH signals from multiple similar experiments were calculated as a percent change from unstretched control under each condition. Combined data were expressed as means ± SE. For comparisons of two groups, we used the Student’s unpaired t-test (two tail). We used ANOVA followed by Scheffe’s multiple comparison test for comparisons of three or more groups. Significance was set at $P \leq 0.05$.

**RESULTS**

**Stretch-mediated changes in c-fos expression are matrix dependent.** When cells were grown on type I collagen, the serum-induced expression of c-fos (normalized for differences in loading by GAPDH or 28S rRNA) did not differ between stretched cells and the unstretched control at any time point studied (Fig. 1). Under both conditions, c-fos expression increased with time to a peak value (30 min) and then decreased to undetectable values (120 min), which is characteristic of c-fos expression kinetics. However, when cells were grown on elastin matrix, the expression of c-fos was significantly decreased at 15 and 30 min of stretch ($-17.1 \pm 5.0\%$ and $-31.1 \pm 6.9\%$, respectively). At 60 min, expression was not significantly different between stretched and static groups, and by 120 min c-fos was undetectable (Fig. 2).

**Stretch-mediated changes in c-fos expression on elastin are dependent on the ELR.** To examine the role of the ELR in mediating changes in gene expression, cells were treated with $10^{-2}$ M VGVAPG for 2 h before stretch and during the stretching period. Our results show that, in the presence of ELR blockade, c-fos expression was not different from the unstretched control ($10.83 \pm 10.8\%$ at 15 min and $1.01 \pm 4.2\%$ at 30 min), suggesting paramount importance for this receptor in mediating stretch-induced reductions in c-fos expression on elastin matrices (Fig. 3).

**Proliferation of stretched VSM cells is matrix dependent.** The increase in cell number, as measured by percent change from numbers of cells plated, was used to determine biological adaptations to stretch. On elastin under static conditions, serum induces robust proliferation ($1,189 \pm 82\%$ increase in cell number), but this serum effect was reduced by $50\%$ ($621 \pm 96\%$) when cells were stretched on elastin (Fig. 4A). In contrast, there was not a significant difference in serum-induced proliferation between stretched and unstretched cells on collagen ($1,073 \pm 111\%$ vs. $1,051 \pm 88\%$, Fig. 4B).
Decreased proliferation with stretch on elastin matrix is mediated by the ELR. To assess the role of the ELR in mediating stretch-dependent decreases in proliferation on elastin matrix, we antagonized the normal elastin receptor interaction with the competitive elastin hexapeptide VGVAPG. Treatment with the ELR inhibitor restored the proliferative capacity of VSM cells stretched on elastin, suggesting a prominent role for this receptor in signaling stretch to VSM cells grown on elastin matrixes. The control hydrophobic hexapeptide VSLSPG did not attenuate the decreased proliferation with stretch on elastin matrixes (Fig. 4A).

DISCUSSION

These data provide the first direct evidence that the ELR functions as a mechanotransducer of stretch in VSM. The major findings of our study are 1) modulated expression of c-fos with stretch is matrix dependent, 2) decreases in c-fos expression with stretch on elastin matrix are dependent on ELR signaling, 3) stretch-mediated proliferation is matrix dependent, and 4) decreased proliferation with stretch on elastin matrix is mediated by the ELR. To place our findings and conclusions in perspective, our discussion will focus on aspects of the methodology and cogent literature relating to heterogeneity of VSM and mechanotransduction.

Methodological considerations. Mechanical stress is a prominent feature of the VSM cell environment in vivo. Physiological stretch of the vessel wall may play an important role in both embryonic development of the cardiovascular system and maintenance of vascular homeostasis. Increased levels of stretch may act as inductive or progressive stimuli for vascular pathologies. With these processes in mind, understanding the cellular adaptations to cyclical stretch are of paramount importance.

Standard cell culture methodologies have generally ignored the impact of mechanical activity in the regulation of biological behavior. Cultured cells are typically maintained on hard culture dishes in a static environment. Mechanically active environments provide a means for studying cellular behavior under physical stress. The Flexercell system generates a gradient of stretch, with maximal stretch (24%) at the periphery, whereas cells at the center are stretched very slightly (3%; see Ref. 3). It is likely that the forces applied with this system are analogous to the pulsatile stresses exerted on arterial medial cells in situ. However, it is worth noting that, in vivo, increased tension and stretch are borne by the already tensioned actin filaments. The cell will not be stretched beyond its relaxed length as occurs in the cultured cells on flexible membranes. Although we do not have results to address this issue, we can state that the matrix composition results in dramatic changes in stretch-mediated gene expression and cellular proliferation.

In an effort to reduce the potential for cellular damage, which may occur with large strains, we conducted the study at physiological levels of strain (10% change in resting length). Therefore, these data represent an
average response of cells exposed to various degrees of stretch. In contrast, uniaxial strain systems have also been used to study stretch-dependent gene expression (28). A recent study investigated stretch-induced cell-cycle arrest using both equiaxial strain with a Flexer-cell system and uniaxial strain. The results showed induction of $G_1$ cell cycle arrest with both systems, suggesting that this response does not depend on the type of stretch imposed (7).

Various methods have been described for assessing cellular proliferation. Because VSM both in vivo and in vitro can become polyploid (33, 34), we chose to manually count cells rather than use the [3H]thymidine uptake assay. Thymidine uptake may provide ambiguous results about proliferation in the event of polyploidy.

FBS is a crucial component in many cell culture studies. In this report, FBS was used in conjunction with stretch to assess changes in gene expression and proliferation. As mentioned previously, this was essential because, in quiescent coronary VSM without FBS, stretch did not produce changes in c-fos expression. We found that FBS induction was critical in our study. Because FBS contains soluble fibronectin and vitronectin (14), we cannot rule out the possibility that our c-fos modulation is due to the effects of fibronectin or vitronectin signaling (either alone or in conjunction with collagen signaling). Nevertheless, potential signaling pathways initiated by fibronectin and vitronectin engagement could interact with ELR signaling, resulting in matrix-dependent changes to stretch. Further studies are necessary to determine whether this potential interaction is present and, if so, biologically significant.

To circumvent the problems associated with cell culture, studies have been performed using isolated arteries (1, 2, 30, 32). These studies have elucidated cellular responses to pressure without removing normal cell-cell and cell-ECM interactions. In studies by Allen et al. (1, 2), increased pressure led to protooncogene expression in rat mesenteric arteries, which was normalized by myogenic constriction and inhibited by blockade of tyrosine kinases with genistein. These studies suggest that wall stress may be the signal for coupling physical stress to gene expression and that the cellular signaling may involve tyrosine kinases. Although our results differ in important aspects from Allen et al. (decrease vs. increase in c-fos expression, respectively), we emphasize that the different conditions of these experiments (cultured cells vs. intact vessel, elastin vs. native matrix, cyclical stretch vs. static pressure, serum-induced c-fos vs. no serum) make direct comparison of these studies difficult. Importantly, our results extend these previous investigations, in as much as we have documented a role for the ELR in smooth muscle mechanotransduction.

**Heterogeneity of VSM.** We have previously reported that stretch-mediated gene expression is differentially regulated in VSM from different arterial beds (27), which may parallel functional properties of those cells in vivo. Likewise, it has been shown that neonatal smooth muscle cells behave differently than adult cells, even within the same vascular bed (13, 42, 50). There are conflicting reports about whether proliferation of VSM cells is decreased (10, 47) or increased with stretch (8, 26, 50). Some of these disparate experimental findings may be explained by VSM cell origin (splanchnic mesoderm vs. neuroectoderm), species (human vs. rat), vascular site (vein vs. conduit artery vs. resistance artery), or location (tunica intima vs. tunica media). Supporting this theory were previous results that showed proliferative responses of VSM to stretch were dependent on the artery from which the cells were derived (26, 52). Clearly, more studies are needed to determine how physical forces influence cellular behavior in different vascular domains.

**Receptors as mechanotransducers of stretch.** Ingber and Jamieson (21) proposed the tensegrity paradigm to account for force transmission across cellular membranes, which leads to adaptive responses. This paradigm emphasizes integrin receptors linking ECM domains to intracellular cytoskeletal elements but does not consider the possibility of ELR signaling. Several studies have illustrated the importance of matrix composition on cellular responses to stretch. Wilson et al. (50) have shown that late-passage (passage 16 to passage 29) neonatal rat VSM cell grown on vitronectin or fibronectin proliferate with stretch via autocrine platelet-derived growth factor production. The stretch-induced proliferation was not present when cells were grown on laminin matrixes, suggesting specific cell-matrix interactions may differentially signal the transduction of stretch (50). In another study, Reusch et al. (37) showed that expression of smooth muscle myosin heavy chains (SM-1 and SM-2) was induced in neonatal VSM cells stretched on laminin but not pronectin (poly-RGD). These investigators also found that stretched neonatal VSM cells activated different mitogen-activated protein kinase pathways, depending on the matrix. Interestingly, the result was not seen in adult cells, suggesting a functional difference in cells derived from animals of different ages (36). Our study extends these previous observations by showing that stretch-induced, matrix-specific gene expression and cellular proliferation occur on elastin in addition to collagen matrixes. Importantly, we found that a control hexapeptide (VSLSPG) with a similar sequence, charge, hydrophobicity, and secondary structure as VGYAPG did not restore stretch-mediated proliferation and gene expression patterns seen in the unstretched controls. This minimizes the possibility that the ELR binds nonspecifically to any hydrophobic sequence.

Three theories could account for the alterations seen with stretch of VSM cells on elastin. First, the vascular myocyte could use a yet undescribed integrin linkage capable of recognizing specific elastin sequences. Second, the vascular myocyte could be synthesizing matrix proteins, such as collagen, which can bind to integrins. Last, the VSM could utilize alternative receptors, such as the ELR, which underlies the response to stretch. The first two possibilities seem remote because the physiological and molecular responses demonstrated...
in this study were different depending on the substrate; therefore, we favor the last explanation for the elastin-specific responses.

Our data demonstrate that coronary VSM cells are capable of responding to stretch in a matrix-dependent manner, and responses on elastin are mediated by the ELR. Specifically, on elastin, VSM cells decreased expression of the early response gene, c-fos, and showed resultant decreases in proliferation. In contrast, cells stretched on type I collagen matrixes failed to show stretch-dependent changes in gene expression or proliferation. When cells were stretched in the presence of the elastin hexapeptide VGVAPG, matrix-dependent changes seen on elastin were lost, suggesting a role for this receptor in transducing stretch.

**Pathological implications.** The ELR may have a role in the pathogenesis of hypertension and atherosclerosis. Two hallmark features of atherosclerosis are migration of smooth muscle cells through the internal elastic laminae of the vessel wall and neointimal thickening (38). It is interesting to speculate that degradation of elastin and the subsequent loss of elastin/ELR interactions may be a prerequisite for the formation of the lesion. Although vascular lesions are rich in collagen, they contain negligible amounts of elastin; thus VSM may be able to proliferate in lesions because the normal static influence of elastin is absent. Likewise, ductus arteriosus smooth muscle cells, which are similar to cells from atherosclerotic plaques, lack functional ELR (17, 18). Moreover, the elastase inhibitor elafin prevents VSM cell migration and proliferation after viral myocarditis (53). The hypertrophic response of coronary arterioles in hypertension includes intussusception and perivascular fibrosis and increased collagen biosynthesis by vascular myocytes (4, 41). This complex process changes the vascular ECM composition, which strengthens the vessel. As with atherosclerotic processes, this alteration is likely to disrupt normal signaling pathways via increased collagen signaling and decreased elastin signaling. Collectively, these data suggest that disruption of the normal matrix-receptor interaction could disrupt homeostatic signal transduction pathways and may contribute to SMC migration and proliferation. If, on the other hand, normal elastin-elastin receptor interactions occur, VSM cells may be prevented from initiating migratory and proliferative processes common in vascular diseases such as atherosclerosis and hypertension.

It seems plausible that physiological levels of strain engender a quiescent phenotype in smooth muscle attached to elastin. This hypothesis is supported by our present findings in vitro, in addition to in vivo observations, where VSM normally proliferates at very low levels. In conclusion, we have demonstrated that a physiological level of cyclical stretch on elastin confers a quiescent phenotype in VSM that is mediated by the ELR.

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