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Mechanical Stress Regulates Syndecan-4 Expression and Redistribution in Vascular Smooth Muscle Cells

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Abstract—Syndecan-4 is a unique membrane-associated heparan sulfate proteoglycan that colocalizes with integrin heterodimers in focal adhesion complexes. Because focal adhesions serve as a putative mechanotransduction system, we postulated that physical forces that are sensed by focal adhesions may regulate the expression and intracellular distribution of syndecan-4 and thereby modulate cell movement and orientation. In this report, syndecan-4 was identified as a transcriptionally regulated, immediate-early gene in response to the application of oscillatory stress. This fluctuation was associated with coordinate changes in the concentration and compartmentalization of syndecan-4 proteins. Specifically, syndecan-4 was lost from the dorsal aspect of the cell membrane and translocated from its intracellular pool to the ventral cell surface. Dissociation of syndecan-4 and vinculin from focal adhesions may contribute to promoting cell motility, because overexpression of syndecan-4, in part, blocked this dissociation and also retarded mechanical stretch-induced cell migration. These studies suggest that mechanical stress induces cell locomotion, in part, by the dynamic regulation of syndecan-4 expression and relocation. **(*Arterioscler Thromb Vasc Biol*. 2002;22:61-68.)**

Key Words: restenosis ■ hypertension ■ atherosclerosis ■ heparan sulfate ■ syndecan

Vascular smooth muscle cells (SMCs) are subjected to a dynamic, mechanical environment modulated by pulsatile pressure and oscillatory shear forces. Although these forces are important regulators of normal cell function, under certain conditions they may contribute to the development of a pathological state. For example, hypertension, which increases transmural stresses due to mechanical distension of the arterial wall, leads to vascular wall hypertrophy and is an independent risk factor for the development of atherosclerosis.[1] Strong correlations have also been noted between regions of elevated wall stresses and the propensity for rupture of either an atherosclerotic plaque or an aortic aneurysm.[2,3] Likewise, the restenosis response that often follows balloon angioplasty may be due, in part, to the extreme vessel wall strains that are associated with this mechanical intervention.[4] Although these observations support the notion that wall stress is a potent stimulus for pathophysiological adaptations of the vessel wall, the underlying molecular and cellular mechanisms that eventually lead to a pathological end point remain largely undefined.

Syndecan-4 is a cell surface heparan sulfate proteoglycan (HSPG) widely expressed in tissues derived from ectoderm, mesenchyme, and endoderm in a position-, time-, and development-dependent manner.[5] The ectodomain of syndecan-4 bears 3 distinct heparan sulfate (HS) chains that are capable of binding a variety of ligands that modulate events relevant to acute tissue repair and chronic injury responses, including cell migration and proliferation, cell-substrate interactions, and matrix remodeling.[6,7] In this regard, syndecan-4 serves as a coreceptor for a variety of soluble ligands, including fibroblast growth factors (FGFs), transforming growth factors-1 and -2, and vascular endothelial growth factor, as well as CC and CXC chemokines. Moreover, as a consequence of the large number of matrix proteins with associated heparin-binding domains, syndecan-4 serves as an important cell adhesion molecule. It is particularly notable that syndecan-4 is exclusively recruitable into focal adhesions, where it contributes to the structural and signaling functions of this macromolecular complex.[8] Significantly, focal adhesions serve as 1 of a number of cellular mechanotransduction systems, coupling and integrating mechanical stimuli with intracellular chemical cascades that are relevant for modulation of cell shape, phenotypic transformation, polarization, proliferation, and motility. Thus, it is conceivable that physical forces that are sensed by the focal adhesion complex regulate the expression and self-assembly of at least some of its structural constituents. Indeed, although mechanical stretch does not effect integrin expression levels, it does alter their intracellular distribution and induces the phosphorylation of several focal adhesion proteins, including paxillin and focal adhesion kinase.[9,10]

In the present study, we used a device that applies homogeneous and uniform biaxial mechanical strain to cultured vascular SMCs, thus allowing precise control of applied stress, to address the following hypotheses: (1) brief, oscil-

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latory, mechanical strain induces changes in syndecan-4 expression as a consequence of transcriptional, translational, and posttranslational events; (2) the recruitment of syndecan-4 into focal adhesions is altered by mechanical strain; and (3) cyclic strain–mediated changes in the concentration and distribution of cell surface syndecan-4 directly affects cell motility behavior. The results described below indicate that an applied mechanical stimulus in the form of an applied stress induces receptor shedding and tightly regulates syndecan-4 expression and distribution in vascular SMCs. Likely, changes in the amount of syndecan-4 expressed on the cell surface as well as receptor shedding into the pericellular environment will help to define stress-related, proatherogenic events.

Methods

Materials

Immortalized rat pulmonary arterial SMCs (PAC-1) were a gift of Dr A. Rothman (UCSD Medical Center, San Diego, Calif). Both syndecan-4 ectodomain (S4ED) and syndecan-4 cytoplasmic domain (S4CD) antibodies were provided as a gift from Dr N. Shworak at Harvard University, Boston, Mass. They are polyclonal rabbit antisera raised against CEPKELEENEVIPKP peptides derived from S4ED and CLGKKPIYKKAPTNE, from S4CD. The pcDNA3.1/syndecan-4 vector was donated by Dr J.T. Gallagher at the University of Manchester, Manchester, UK. Syndecan-4$^{-/-}$ mouse fibroblast lysates were kindly supplied by Dr K. Ishiguro at Nagoya University, Nagoya, Japan. Mouse monoclonal anti-α5 integrin antibody and goat anti-rabbit (GAR)–IgG-FITC were obtained from Immunotech. The nonimmune IgG of rabbits and mice, GAR-IgG–alkaline phosphatase (AP), horse anti-mouse (HAM)-IgG-AP, and avidin-AP were ordered from Vector. AlexaFluor™ 488 goat anti-mouse–IgG, AlexaFluor™ 568 GAR-IgG, and rhodamine-phalloidin were purchased from Molecular Probes. Fetal bovine serum was purchased from Hyclone. EZ-link™-sulfo-NHS-LC-biotin and bicinchoninic acid assay kits were obtained from Pierce. Growth medium powder (medium 199 and Dulbecco's modified Eagle's medium) and dissociation buffer were purchased from Gibco BRL Life Technologies. Mouse monoclonal anti-vinculin antibodies, heparitinase I, protein A beads, and all other chemical reagents, unless otherwise indicated, were obtained from Sigma.

Cyclic Strain Assay

Cells were plated onto silicone elastomer–bottomed culture dishes, as detailed elsewhere,[11] and a uniform, biaxial strain profile was generated by using a StrainMaster (Z-Development Inc).[12] Before cell plating, the silicone membrane culture dishes were autoclaved, rinsed in 10 mL Hanks' balanced salt solution (HBSS), and coated overnight with 2 μg/mL fibronectin/HBSS. PAC-1 cells were then seeded onto the membrane after a brief rinse of the membrane with HBSS and incubated with medium 199 (Mediatech) containing 10% fetal bovine serum (Hyclone) until 60% to 80% confluence was observed. Cells were arrested in low-serum medium (0.5% fetal bovine serum) for 12 hours before the onset of experiments.

Western Blotting

Cells were detached from the culture dish, lysed, digested by 1 U/mL heparitinase I at 37°C for 4 hours, fractionated by SDS–polyacrylamide gel electrophoresis (PAGE), and transferred to a nitrocellulose membrane. The membrane was blocked and probed overnight by primary antibodies (S4ED or S4CD) at 1:1000 dilution and by the GAR-IgGs conjugated with AP-IgG at a dilution of 1:2000 for 3 hours.

Immunoprecipitation

PAC-1 cells were deglycosylated in the low-serum medium (0.5% fetal bovine serum) containing 2 U/mL heparitinase I at 37°C for 4 hours. After thorough washes in phosphate buffered saline (PBS), cells were lysed and cell proteins precleared with rabbit nonimmune IgG plus protein A–agarose beads. Supernatants were then incubated overnight with either S4CD or S4ED antisera (1:1000 dilution) at 4°C. Syndecan-4–IgG complexes were collected, washed, and separated by SDS-PAGE. Gels were examined by either Coomassie blue staining or immunoblotting. Of note, we observed that biotinylation of soluble proteins in the culture medium before immunoprecipitation with S4ED increased our sensitivity to subsequently detect precipitated, shed syndecan-4 protein.

Subfractionation

The plasma and internal membranes were isolated by a cationic colloidal silica isolation technique.[13,14]

FACS Analysis

Expression of cell surface syndecan-4 was measured by fluorescence-activated cell sorter (FACS) analysis modified from Longley's method.[15] Cells were detached and incubated with S4ED antibodies at a 1:50 dilution in 0.5% bovine serum albumin/PBS on ice for 1 hour. After extensive washes in PBS, incubation was resumed on ice for 30 minutes in the presence of GAR-IgG conjugated with FITC diluted 1:50. Cells were then rinsed in PBS and resuspended in 0.5 mL of 0.1% paraformaldehyde/PBS. Samples were analyzed by the FACScan/Lysys II system (Becton Dickinson) supported by the CellQuest program. Cells incubated with secondary antibody alone were used as negative controls, which consistently revealed a computed mean fluorescence intensity of <10.

Immunofluorescence Cell Staining

Cells grown on fibronectin-coated membranes before and after exposure to cyclic strain were fixed, permeabilized, blocked, and then incubated overnight with either monoclonal mouse vinculin antibody (1:400 dilution) or S4ED antibody (1:800) at 4°C. In select cases, cells were incubated in deionized water for 30 minutes at 37°C before fixation and permeabilization to reveal the dorsal location of syndecan-4 and vinculin.[16] Cells were incubated with AlexaFluor™ 488 goat anti-mouse–IgG and AlexaFluor™ 568 GAR-IgG at a 1:1000 dilution. Samples were examined with a Zeiss LSM410 laser confocal microscope equipped with differential interference contrast (DIC) Apochromat optics and an argon-krypton laser.

Ribonuclease Protection Assay

Radiolabeled riboprobes were generated by using the Riboprobe® System-T7 (Promega) from 50 ng pcDNA3.1(+)/rAsyndecan-4 and pPMG/glyceraldehde-4-phosphate dehydrogenase (GAPDH, PharMingen) templates. Sample RNA was hybridized to riboprobes according to the manufacturer's instructions (RPAII™ kit, Ambion).

Nuclear Runoff Transcription Assay

Nuclei were collected and in vitro transcription was undertaken at 30°C for 2 hours in the presence of 150 μCi [α-^{32}P]UTP. Nonradiolabeled riboprobes were synthesized as outlined above, except that the isotope was excluded. Radiolabeled nuclear RNA extracts were then hybridized for 36 hours at 65°C with a Zetabind® membrane (Cuno Laboratory Products) containing 50 ng nonradiolabeled riboprobes of antisense syndecan-4 and GAPDH genes.

In Vitro Migration Assay

Cells were pretreated and seeded as described above, except that a plastic divider was placed in the midline of a dish for plating transfectants of control and syndecan-4 plasmids into separate wells. Cells were maintained in growth medium until they achieved 80% confluence. The divider was then removed, and the rearrangement and migration of cells were photomicrographed by a phase-contrast microscope after an application of 10% cyclic strain for 24 hours (spreading) and 36 hours (migration). Cell movement was measured from the initial field edge toward the moving front by using IP Laboratory Spectrum software (Scanalytics, Inc). The distance *(D)* from the initial field edge *(a,b)* to the moving front *(x,y)* was determined by using the expression $D=k[(x-a)^2+(y-b)^2]^{0.5}$, where k is a constant. The means±SD of at least 30 measurements in each group, derived from 3 independent experiments, were plotted.

Statistics

ANOVA (single factor) was used to compare differences among average values obtained from within groups. Significance was accepted at $P<0.05$.

Results

Effect of Cyclic Strain on Syndecan-4 mRNA Expression in PAC-1 Cells

Cyclic strain produced a rapid, though transient, upregulation in syndecan-4 mRNA expression (Figure 1). Normalized to the amount of GAPDH, syndecan-4 mRNA was induced within 30 minutes (2.62±0.46-fold, $P=0.025$, n=4) and exhibited a maximal increase (3.89±0.14-fold, $P<0.001$, n=5) at 1 hour, with a decline thereafter to prestrained levels within 24 hours. A decrease in strain magnitude from 10% to 3% did not alter the expression pattern (data not shown). Of note, at a strain amplitude of 10% and a cycle frequency of 1 Hz, significant cell loss was not observed during the 24-hour study interval, as determined by total cell protein levels that decreased by <8% ($P>0.05$, n=3; data not shown). Thus, all subsequent experiments were performed at 1 Hz and a 10% strain amplitude.

To characterize the role of transcriptional regulation in the alteration of syndecan-4 mRNA levels, a nuclear runoff transcription assay was performed (Figure 1B). An increase in the syndecan-4–GAPDH ratio (≈ 2.9-fold) was observed after 1 hour but not after 24 hours of cyclic strain, indicative of a rapid induction of syndecan-4 promoter activity. In principle, suppression of mRNA decay in the cytosol might provide an additional mechanism for transient accumulation of syndecan-4 mRNA. However, addition of actinomycin completely abolished strain-induced upregulation of syndecan-4 mRNA, which suggests that transcription is the dominant mechanism of this effect. Notably, cycloheximide treatment did not inhibit syndecan-4 mRNA induction in response to cyclic strain (Figure 1C). It suggests that syndecan-4 is an immediate-early gene, because the rapid induction of its mRNA is independent of new synthesis of transcription factors.[17]

Effect of Cyclic Strain on Syndecan-4 Protein Expression

Immunoprecipitation with S4ED anti–syndecan-4 polyclonal antibodies identified 2 species in PAC-1 cells with apparent molecular weights of 50 and 70 kDa, likely representative of core protein oligomers. It should be noted that reblotting with anti–syndecan-4 antibodies was performed in independent experiments, during which the 50- and 70-kDa bands were identified as syndecan-4. Deglycosylated syndecan-4 core proteins have a well-documented tendency to self-associate, forming noncovalently linked multimers that migrate on polyacrylamide gels at 35 kDa,[18,19] 44 to 46 kDa,[15,16,20] and 70 kDa.[16] The extent of oligomer formation is often dependent on the source of the examined tissues,[21] the relative expression level of the protein,[6] the complex pattern of unsaturated glucuronates remaining on the core protein after enzymatic deglycanation,[22] and the intercalation of membrane lipids with core proteins,[23,24] as well as the sensitivity and avidity of antibodies to diverse syndecan-4 epitopes. The specificity of the antibody used in our study has been characterized in detail by Shworak et al[7] and confirmed by others.[18] Moreover, with the use of identical reagents and protocols, these 2 species were not observed in syndecan-4$^{-/-}$ mouse fibroblasts, whereas transfection of PAC-1 cells with pcDNA3.1(+)/syndecan-4 significantly enriched the 50-kDa species (Figure 4A).

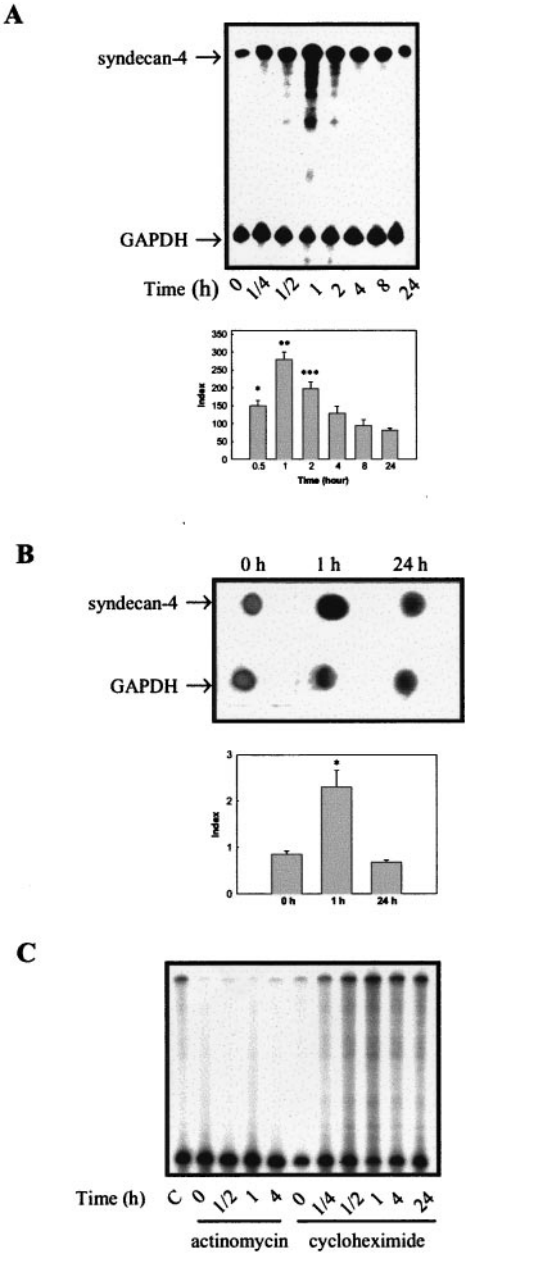

Figure 1. Syndecan-4 mRNA expression in response to cyclic strain. A, Total RNA was harvested from cells exposed to 10% cyclic strain for 0, 0.25, 0.5, 1, 2, 4, 8, and 24 hours and subjected to ribonuclease protection assay. Means±SD are plotted. *$P=0.025$, n=4 (0.5 hours vs control), **$P<0.001$, n=5 (1 hour vs control), ***$P=0.007$, n=4 (2 hours vs control). B, The transcriptional activities of the syndecan-4 gene in the absence (control) and presence of cyclic strain for 1 and 24 hours were examined by nuclear runoff transcription assay. Indexes represent the average ratios of syndecan-4 to GAPDH of 3 independent experiments. *$P=0.034$, 1 hour vs 0 hours. In C, cells were pretreated by actinomycin (5 μg/mL) or cycloheximide (5 μg/mL) for 2 hours in the absence of stretch, after which the indicated agents remained in the medium and 10% cyclic strain was applied to the cells for various times. Ribonuclease protection assay was carried as described in A. C indicates control.

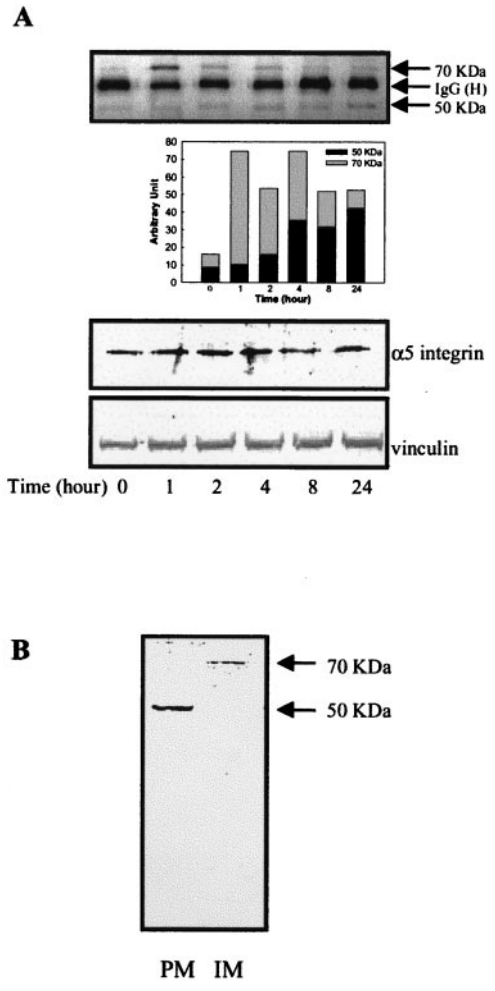

Figure 2. Effects of cyclic strain on expression and compartmentalization of syndecan-4 proteins. Cells before and after exposure to cyclic strain for 1, 2, 4, 8, and 24 hours were detached; 100 μg cell protein from each sample was used for immunoprecipitation with S4ED antibodies. Proteins were fractionated by 15% SDS-PAGE and visualized by Coomassie blue staining (top panel of A). Immunoprecipitated bands were reconfirmed by reblotting with anti–syndecan-4 antibodies. With the use of identical reagents and protocols, protein bands were not observed in syndecan-4 $^{-/-}$ mouse fibroblasts. The expression of α5 integrin and vinculin was determined by Western blotting (middle and bottom panels of A). In B, the plasma membranes (PM) and internal membranes (IM) were subfractionated by differential centrifugation on a Nycodenz™ gradient, deglycosylated by heparitinase I, and immunoblotted by S4CD antibodies.

Cyclic strain differentially modulated the levels of both the 50- and the 70-kDa species (Figure 2A). The 70-kDa band was elevated at 1 hour, followed by a gradual decline that paralleled an increase in the 50-kDa species. In contrast, the expression of α5 integrin and vinculin, 2 proteins associated with fibronectin-induced focal adhesions, was unaffected by cyclic strain. Subfractionation studies revealed that the 50-kDa species was predominantly localized in association with the plasma membrane, whereas the 70-kDa species was concentrated within internal membrane fractions (Figure 2B). These data suggest that mechanical forces alter both the expression of syndecan-4 and its distribution between cell compartments.

Effect of Cyclic Strain on the Redistribution of Cell Surface Syndecan-4

A unique property of syndecan-4 is its recruitment to focal adhesions[25] and the capacity of thrombin, epidermal growth factor, and phorbol myristate acetate[26] to induce cleavage of its ectodomain. To analyze whether cyclic strain causes a redistribution or loss of this molecule from the cell surface, the levels of syndecan-4 on the cell surface and in the culture medium were characterized. Biotinylation of the cell monolayer selectively labeled syndecan-4 on the dorsal aspect of the cell membrane (please see www.ahajournals.org, Figure IA). This technique, which has been referred to as "differential cell surface biotinylation," has been used as a biochemical marker of polarity.[27] Immunoprecipitation revealed a 50-kDa, membrane-associated species, in agreement with subfractionation studies, that became less abundant with the duration of mechanical stretch. A higher-molecular-weight band ($\approx$100 kDa) was also observed as the 50-kDa species decreased, suggesting that higher-order oligomers might form before the displacement of syndecan-4 from the cell membrane. FACS analysis confirmed that syndecan-4 was lost from the cell surface within 1 hour of exposure to cyclic strain (Figure IB). However, while the level of syndecan-4 on the dorsal cell surface remained low throughout the late period of cyclic strain (>1 hour), total cell surface syndecan-4, as determined by FACS, gradually increased from 4 to 24 hours. These data suggest that the reappearance of syndecan-4, after the initial onset of cyclic strain, is largely confined to the underlying cell-substrate interface.

The loss of syndecan-4 from the cell surface suggests that mechanical strain induces protein shedding. Indeed, a 50-kDa species was detected as early as 1 hour after the onset of cyclic strain, with a maximum level observed at 4 hours (Figure IC). At 24 hours, an additional 30-kDa band was also noted, suggesting possible degradation or proteolytic cleavage of the core protein. Constitutive and inducible shedding of a 20-kDa syndecan-4 ectodomain has been previously identified in wound fluids and cell culture medium,[5] with proteolytic cleavage of the core protein at a site near the plasma membrane as the presumed mechanism. Our ability to detect a 50-kDa protein in the conditioned medium is probably indicative of core protein aggregation or, potentially, loss of the noncleaved protein by a process of membrane budding or vesicle formation.

Effect of Cyclic Strain on Syndecan-4 Recruitment Into Focal Adhesions

Redistribution of cell surface syndecan-4, mediated by cyclic strain, may imply changes in the extent or formation of focal adhesion complexes. Utilizing indirect immunofluorescence microscopy, we investigated the association of syndecan-4 with vinculin as a focal adhesion marker. In the absence of cyclic strain, syndecan-4 was observed at the perimeter of leading lamellipodia, some of which were colocalized with vinculin (Figure 3A). Incomplete colocalization of syndecan-4 with vinculin might be due to the nonrigid nature of the silicone membrane contact surface[28,29] or the pretreatment of cells in quiescent medium.[30] Syndecan-4 staining was also identified in a diffuse pattern radiating from the nucleus and was accompanied by apparent sequestration in granular structures. Localization of syndecan-4 to leading lamellipodia

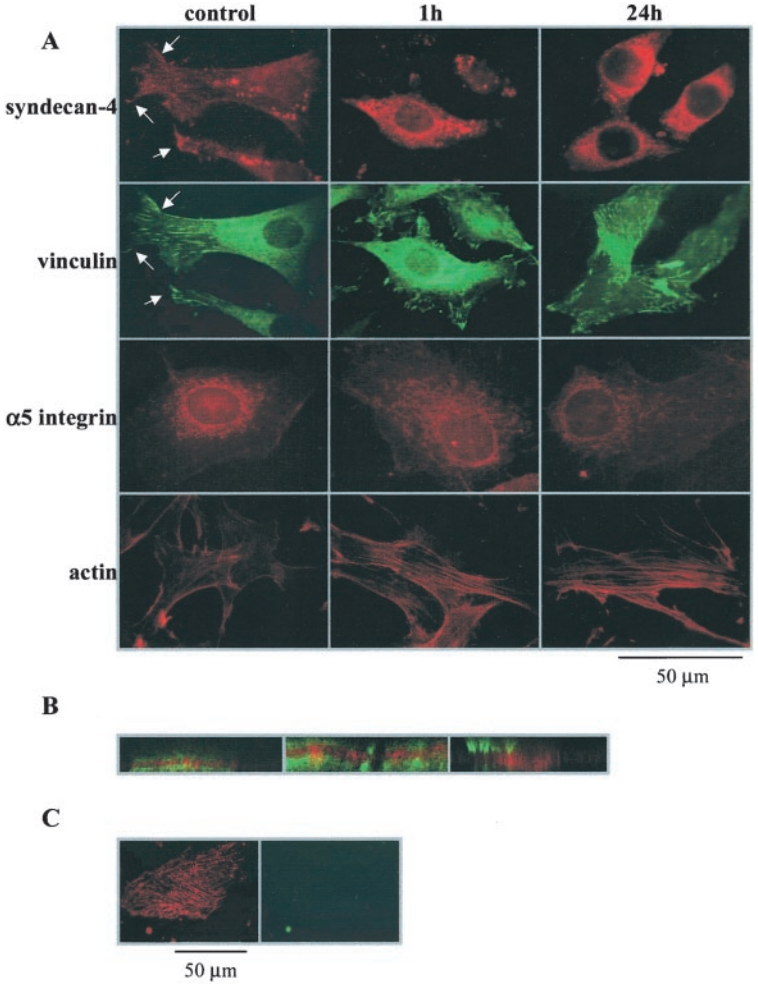

Figure 3. Immunofluorescence and confocal microscopic imaging of syndecan-4 and vinculin in stretched cells. A, Syndecan-4, vinculin, α5 integrin, and actin microfilaments were examined by immunofluorescence microscopy as outlined in Methods. Panels from left to right: control (prestrain); 1 hour of cyclic strain; 24 hours of cyclic strain. B, Optical confocal sectioning of syndecan-4 (red) and vinculin (green) distribution in the *z* direction. Panels from left to right: Control (prestrain); 1 hour of cyclic strain; 24 hours of cyclic strain. C, After exposure to 10% cyclic strain for 24 hours and cell removal from the silicone membrane by hypotonic lysis, immunostaining revealed that syndecan-4, but not vinculin, was bound to the fibronectin-coated silicone membrane.

was lost with the acute onset of cyclic strain (<1 hour). After 24 hours of mechanical deformation, syndecan-4 staining was uniformly concentrated in the vicinity of the nucleus. Likewise, the longitudinal orientation of F-actin microfilaments and the perinuclear localization of vinculin were more pronounced. In contrast, the distribution of α5 integrin was not significantly altered by cyclic strain.

Confocal microscopy was used to optically section cells to further define the distribution of syndecan-4 and vinculin (Figure 3B). After 24 hours of repetitive mechanical stretch, vinculin and syndecan-4 were localized predominately to dorsal and ventral aspects, respectively, of cross-sectional cell images. This polarized distribution was further supported by immunofluorescent staining of the silicone membrane after cell removal by hypotonic lysis (Figure 4C). Syndecan-4 staining resembled a fibril-like network, similar to that associated with cell-mediated reorganization of substrate-adsorbed fibronectin.[31] Furthermore, little vinculin was bound to the membrane, consistent with cross-sectional cell imaging.

Overexpression of Syndecan-4 Limits Stretch-Induced Cell Movement

Mechanical deformation stimulated cell migration, as well as the reorganization of randomly plated cells into cordlike structures. The ability of syndecan-4 to modulate stretch-induced cell migration was investigated by overexpression of the core protein in PAC-1 cells. Ribonuclease protection assay, Western blotting, and FACS analysis confirmed that syndecan-4 levels were increased, including that found on the cell surface (Figure 4A). After 24 hours of stretch, overexpressers remained distributed in a random surface pattern (Figure 4B). Moreover, cell movement was significantly reduced for transfectants that overexpressed syndecan-4 but not for those cells transfected with a control plasmid (118.4±13.6 vs 30.4±12.9, $P<0.001$, n=30; Figure 4C). Interestingly, confocal microscopy demonstrated that overexpression of syndecan-4 was associated with increased retention of vinculin in the ventral plasma membrane (Figure 4D). These studies suggest that the loss of syndecan-4 contributes to stretch-induced cell movement and aggregation.

Discussion

Although the mechanical effects of elevated blood pressure provide an important stimulus for the development of vascular wall hypertrophy and atherosclerosis, the molecular and cellular events underlying this process have only recently become the focus of experimental study. In part, this field of investigation has been motivated by the development of mechanostimulus systems based on the distention of a flexible substrate, thereby providing a convenient route for the in vitro application of a well-defined stress to cultured cells. Because vascular SMCs regulate the synthesis, degradation, and organization of the extracellular matrix, it is probable that

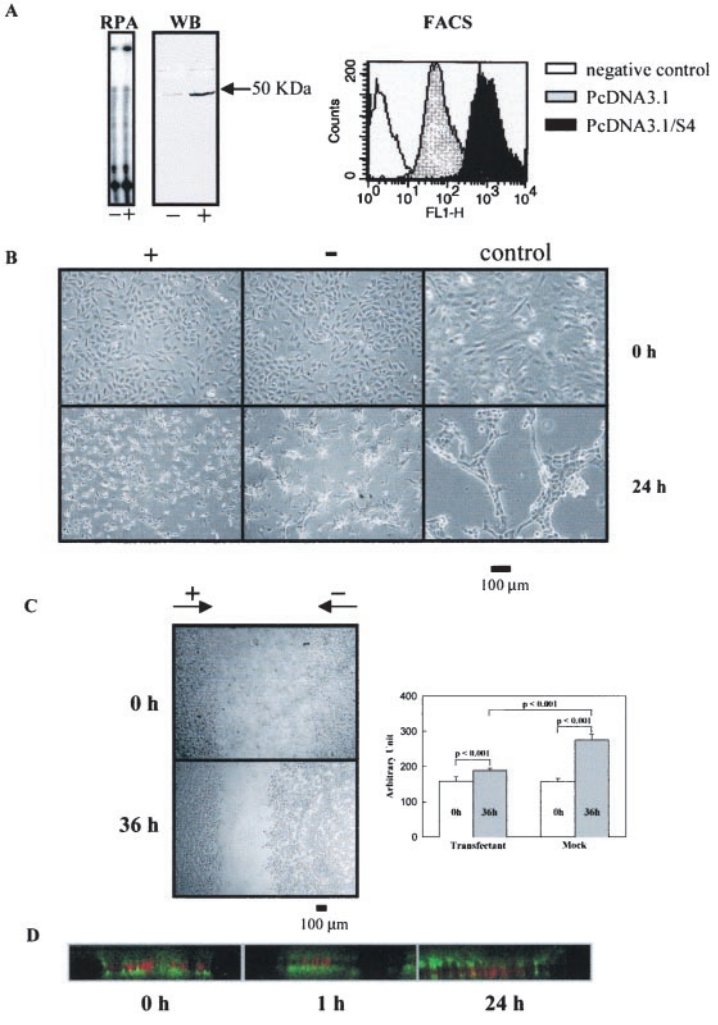

Figure 4. Effect of overexpression of syndecan-4 on stretch-induced cell migration. A, Cells were transfected by pcDNA3.1/syndecan-4 and pcDNA3.1 alone by Lipofectamine™, and the overexpression of syndecan-4 was characterized by ribonuclease protection assay (RPA), Western blotting (WB), and FACS analysis. Cells transfected by either pcDNA3.1/syndecan-4(+) or pcDNA3.1(−), and nontransfected cells (control) were exposed to cyclic strain and photographed as described in Methods. Cell spreading and migration are presented in B and C, respectively. D, Cells were optically sectioned, and syndecan-4 (red) and vinculin (green) distribution was defined in the *z* direction.

these cells play a central role in responding to local changes in arterial wall mechanics. Cheng et al,[12] for instance, have observed that large, mechanical deformations in vitro (≥14% strain) lead to direct SMC injury, with the release of FGF-2 from intracellular stores and, as a consequence, the initiation of cell proliferation. This sequence of events is similar to that observed after experimental balloon injury in vivo. Likewise, others have observed that at similar strain amplitudes, SMCs generate superoxide anions and hydrogen peroxide, as well as express other proinflammatory factors such as monocyte chemotactic protein-1.[32–34] Notably, mechanical stretch is also capable of regulating gene expression at strains well below amplitudes that cause SMC injury (≤10% strain amplitude) and FGF-2 release. However, of 5000 genes that were screened in human aortic SMCs by a transcription profiling strategy, Feng et al[35] identified only 5 genes with altered expression patterns at low strain amplitudes. Three genes were upregulated (>2.5-fold)—cyclooxygenase-1, tenascin-C, and plasminogen activator inhibitor-1, and 2 were downregulated (>2.5 fold)—matrix metalloproteinase-1 and thrombomodulin. It was suggested that by increasing the expression of plasminogen activator inhibitor-1, an inhibitor of plasminogen activators, and reducing the levels of matrix metalloproteinase-1, the effect of strain on SMCs was to promote extracellular matrix accumulation by altering the balance of matrix synthesis and degradation. Significantly, this work emphasized that the application of mechanical stretch in a range similar to that used for our investigation does not provide a stimulus for a generalized transcriptional response.

In this report, we have identified that syndecan-4 is induced as an immediate-early gene in response to cyclic strain in a manner that is transcriptionally regulated. Primer extension analysis of the human, rat, and mouse syndecan-4 genes reveal that the 5′ flanking sequence contains binding sites for Sp1, activator protein-2, nuclear factor-κB, MyoD, histone H4 transcription factor-2, and lipopolysaccharide-binding protein-1.[36] The presence of these binding sequences suggests that growth factors,[37,38] cytokines,[39] and proteases[38] may regulate syndecan-4 expression at a transcriptional level. Indeed, Cizmeci-Smith et al[37] have observed that FGF-2, platelet-derived growth factor, and serum induce syndecan-4 expression in vascular SMCs. No other inducers of syndecan-4 expression have been identified, with the exception of PR-39, a member of the cathelin protein family that is produced by circulating granulocytes and macrophages, and albumin-bound linoleic acid.[40] The mechanism by which PR-39 and linoleic acid upregulate syndecan expression is not well understood. Although "stretch response elements" have been reported in the promoter region of the c-*fos*, atrial natriuretic factor, and β-myosin heavy-chain genes in cardiac myocytes, a consensus sequence has not been clearly identi-

fied.[41] Nonetheless, cyclic strain–mediated signaling pathways have been directly linked to specific *trans*-acting factors, such as nuclear factor-κB and activator protein-2.[42,43] As noted, both factors have corresponding *cis*-acting elements in the syndecan-4 promoter region.

In response to cyclic strain, changes in syndecan-4 expression were associated with coordinate changes in concentration and compartmentalization of syndecan-4 proteins. In particular, accelerated loss of syndecan-4 from the surface of cultured SMCs was noted almost immediately after the initiation of mechanical stretch. Cell surface HSPGs are shed as part of normal turnover by proteolytic cleavage of the core protein. Although the proteolytic enzyme that is responsible for core protein cleavage has not been identified, recent data suggest that shedding can be regulated by various external stimuli. Syndecan-4 shedding has been induced by growth factors and proteases, such as epidermal growth factor and thrombin, respectively; soluble virulence factors of microbial pathogens; and cellular stress (eg, ceramide and hyperosmolarity).[7] Mechanical stretch appears to provide an additional stimulus for syndecan-4 shedding and supports the notion that HSPG shedding is a regulated host response to tissue injury. Soluble agonists that stimulate syndecan shedding act through specific signaling pathways. For example, cellular stress–related agonists induce shedding by way of the c-Jun NH_2-terminal kinase (JNK) mitogen-activated protein (MAP) kinase pathway, whereas epidermal growth factor works through the extracellular signal–regulated kinase (ERK)/MAP kinase pathway.[26,38] Other signaling pathways appear to regulate shedding because of direct stimulation of protein kinase C (PKC). All of these pathways appear to converge on a protein tyrosine kinase(s) in a fashion not yet defined. Notably, ERK, JNK, and PKC are all activated in response to mechanical stress.[44,45] Moreover, whereas use of a tyrosine kinase inhibitor appears to prevent strain-induced cell alignment and reorientation, this effect has not been observed in response to PKC and ERK inhibitors.[46,47] The role of JNK as a mediator of strain-induced cell migration has not been well defined.

Prior reports have emphasized that shed syndecans retain the ligand-binding activities of their cell surface counterparts. For example, purified syndecan-1 ectodomains bind neutrophil elastase and cathepsin G, thereby reducing the affinity of these proteases for their physiological inhibitors and enhancing their activity.[48] In binding a variety of growth factors and cytokines, shed syndecans are also capable of increasing their effective concentration near the site of release, presumably enhancing the probability of interactions with high-affinity cell surface receptors. Thus, acute or chronic changes in the local mechanical environment within the vascular wall likely induce SMCs to shed syndecans as an adaptive response to injury that ultimately may contribute to a cascade of proatherogenic events. However, it also bears emphasis that the shedding of cell surface HSPGs rapidly reduces the amount of HS on the cell surface. As a coreceptor for binding of growth factors and matrix proteins to cell surfaces, it has been suggested that syndecan shedding might provide another mechanism for modulating cell responses to both soluble and insoluble HS-binding proteins.[7] For example, cell proliferation in response to FGF-2 is reduced in cells treated with either HS-digesting enzymes or agents that interfere with HS sulfation, such as sodium chlorate. Our studies demonstrate that strain-induced loss of cell surface syndecan-4 was associated with increased SMC motility on fibronectin-coated substrates and that this effect was abrogated in cells overexpressing syndecan-4. These data confirm that acute changes in cell surface syndecan-4 levels due to receptor shedding can have a significant impact on receptor signaling events related to cell migration.

It is also worthy of comment that with the onset of mechanical strain, relatively little syndecan-4 was localized to the basal cell surface or associated with vinculin. Recent studies have revealed that cyclic strain can induce rapid translocation of novel PKC isoforms, such as PKCδ, to the cell membrane.[49] While syndecan-4 oligomerization promotes PKCα binding and activation, novel forms of PKC may induce the phosphorylation of Ser183 within the C1 domain of rat syndecan-4.[50,51] Of interest, phosphorylation of Ser183 abolishes phosphatidylinositol 4,5-biphosphate–dependent oligomerization of syndecan-4, which is required for mature adhesion complex formation. Thus, in addition to an absolute reduction in cell surface syndecan-4 molecules, inhibiting the oligomerization of those that remain could also contribute to the enhancement of cell motility that was observed early after the onset of strain.

It has been demonstrated that the net effect of physical forces on the arterial wall is to increase expression of a variety of growth-stimulating and proinflammatory gene products, albeit at force levels capable of inducing cell injury. In contrast, the repertoire of genes expressed in response to physiological levels of elevated mechanical strain is much more limited. The investigations reported herein serve to emphasize that changes in the local mechanical environment regulate syndecan-4 expression and shedding. As a result, alterations in related cell behavior may be an important contributing factor to those morphogenetic events that underlie stress-induced vascular wall remodeling processes.

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