Mechanical Stretch Inhibits Oxidized Low Density Lipoprotein-induced Apoptosis in Vascular Smooth Muscle Cells by Up-regulating Integrin αVβ3 and Stabilization of PINCH-1*

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To determine the mechanisms involved in regulating the balance between apoptosis and survival in vascular smooth muscle cells (VSMC), we studied anti-apoptotic stimuli that can counteract pro-apoptotic events in the process of early atherosclerotic lesions formation. Such a process involves VSMC accumulation even in the presence of oxidized low density lipoprotein (Ox-LDL). In the arch of the aorta, we find that integrin β3 is higher than in descending arteries. In the advanced atherosclerosis lesion, we found an inverse correlation between the level of integrin β3 and apoptosis (deoxynucleotidyltransferase-mediated dUTP nick end labeling-positive). We also found an increase in integrin αVβ3 (but not integrin β1) expression in VSMC that are subjected to cyclic stretch. VSMC subjected to stretch as well as VSMC with forced expression of αVβ3 were demonstrated to be resistant to Ox-LDL-induced cytoskeleton disruption and apoptosis. The anti-apoptotic effect of stretch was abolished by treatment of VSMC with small interfering RNA against integrin β3 as well as VSMC isolated from integrin β3 knock-out mice. Disruption of the cytoskeleton abolished the protective effect of stretch or αVβ3 overexpression on Ox-LDL-induced activation of Bax and apoptosis. We also demonstrated that stretch-mediated protection of Ox-LDL-induced apoptosis involved stabilization of PINCH-1; Ox-LDL decreased the level of PINCH-1, but the application of mechanical stretch or overexpression of either integrin β1 or integrin β3 prevented its down-regulation. In the arteries of integrin β3 null mice, there were lower levels of PINCH-1 and ILK-1. Moreover, deletion of integrin β3 in VSMC abolished the stretch protective effect on PINCH-1. Small interfering RNA-mediated knockdown of PINCH-1 disrupted the cytoskeleton and caused apoptosis of VSMC. These findings provided experimental evidence that mechanical stretch acted as a survival factor in the arches of aortas. Furthermore, mechanical stretch prevented VSMC from apoptosis via a mechanism that involves αVβ3 integrin expression, stabilization of PINCH-1, and remodeling of the cytoskeleton.

The atherosclerotic lesion is characterized by accumulation of vascular smooth muscle cells (VSMC), foam cells, and matrix protein and lipids in the intima (1). Atherosclerotic lesions occur preponderantly at arterial bifurcations and curvatures, suggesting a role for disturbed hemodynamic forces that produce a lower wall shear stress with increased mechanical stretch (2, 3). Disturbed hemodynamic forces stimulated inflammatory responses in the atherosclerosis-prone arch of aorta (1, 4) that led to a triggering recruitment of activated monocytes. Abnormal hemodynamics also increased the expression of the receptor for oxidized low density lipoprotein (Ox-LDL) in endothelial cells (5). The increase in these receptors leads to accumulation of Ox-LDL (6, 7). In addition, LDL trapped in the extracellular matrix of the vessel wall is oxidatively modified by monocytes in the neointima (6, 7).

Accumulation of Ox-LDL stimulates apoptosis, which should decrease the accumulation of VSMC. The pathway for Ox-LDL-induced apoptosis includes activation of a complex cascade of kinases, changes in cytoskeletons, changes in expression of Bcl-2 family genes, and activation of death effector sphingomyelinase and caspases; the pathway for Ox-LDL-induced apoptosis also included the activation of nuclear transcription factor-κB and other proinflammatory transcription factors (8, 9). In advanced atherosclerotic lesions, there was a significant correlation between Ox-LDL and VSMC apoptosis, but in the early atherosclerotic lesion, apoptotic cell death was almost absent (6, 7). One possible explanation is that anti-apoptotic mechanisms are present in early atherosclerotic lesion.

In the current study, we investigated how VSMC survive in the presence of Ox-LDL at this early stage. We uncovered a novel signaling mechanism by which expression of integrin αVβ3 integrin suppresses Ox-LDL-induced apoptosis. The novel pathway involves an integrin αVβ3-dependent stabilization of PINCH-1, a survival protein that is linked with integrin and the cytoskeleton (10–12). We also established that stabilization of PINCH-1 prevented disruption of the cytoskeleton and ultimately suppressed the activation of Bax.

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2 The abbreviations used are: VSMC, vascular smooth muscle cells; LDL, low density lipoprotein; Ox-LDL, oxidized LDL; TUNEL, deoxynucleotidyltransferase-mediated dUTP nick end labeling; siRNA, small interfering RNA; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; ELISA, enzyme-linked immunosorbent assay.
MATERIAL AND METHODS

Animals—ApoE$^{-/-}$ mice (C57Bl/6 background, 3 months old) were obtained from Jackson Laboratory (Bar Harbor, ME). The IACUC of Baylor College of Medicine approved our animal studies. Twelve ApoE$^{-/-}$ male mice were randomly fed a normal chow up to 45 weeks. The animals were kept under standardized conditions with free access to water and chow. Serum from tail vein blood was obtained by centrifugation for 10 min at 10,000 × g at 4 °C, and serum cholesterol was measured.

Materials—Antibodies against activated Bax (6A7) and PINCH-1 were from Sigma-Aldrich. Antibodies against α-tubulin, total Bax, and β3 integrin and its blocking peptide were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against αvβ3 (LM609), and αv antibodies were from Chemicon (Temecula, CA). Rabbit anti-α-smooth muscle actin was from Spring Bioscience (Fremont, CA). Cytochalasin D and nocodazole were from Calbiochem. The cytoskeleton labeling antibodies (phalloidin-FITC-conjugated antibody), monclonal mouse anti-α-tubulin antibody, and fluorescent secondary antibodies were obtained from Molecular Probe Inc. (Eugene, OR). Elastomer-bottomed plates were purchased from Flexcell International (McKeesport, PA).

Plasmids and Transfection—The pBabe-β3 retroviral plasmid was a gift of Dr. J. Marshal (The Institute of Cancer and Cancer Research UK Clinical Centre, London, UK) (13), and the plasmid pZEOSV2-β1 was a gift of Dr. R. Zent (Vanderbilt University School of Medicine, Nashville, TN). PINCH-1 expression plasmid was obtained from Open Biosystems (Eugene, OR). The retroviruses were generated using producer cells. These retroviruses were used to infect VSMC in the presence of 5 μg/ml polybrene (Sigma-Aldrich). Infected cells were selected in 3 μg/ml puromycin for integrin β3 or 200 μg/ml zeomycin for integrin β1 expressing cells (Invitrogen). The expressions of integrin β3 or β1 in VSMC/β3, VSMC/β1, or VSMC mock-transfected controls (wild type VSMC) were measured by Western blot. The integrin β3 promoter luciferase reporter p-575/+-21-β3-Luc was kindly provided by Dr. P. Bray (Thomas Jefferson University Hospital).

siRNA Preparation and Transfection—Rat PINCH-1 siRNAs (set 1, AAGTTGACGTGGTCTCTGCCC, and set 2, AAATA-CATCTGCGAGAATGCG) corresponding to the coding sequence were synthesized by Dharmacon (Chicago, IL). The siRNA Smart Pool was purchased from Dharmacon, Inc. VSMC were transfected with these siRNAs or plasmid using Nucleofector (AMAXA, program U-23) according to the manufacturer’s protocol. The siRNA against luciferase (CTGACGCGGAATACTTCGA) was used as a control.

Cell Culture and Cyclic Stretch—VSMC from aortas of mouse (both wild type and integrin β3 knock-out mice were obtained by digestion of freshly isolated aortae in collagenase and elastase) and kept in a humidified 95% air, 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C. For stretch experiments, VSMC were plated on silicone elastomer-bottomed and collagen-coated plates. Those cells that achieved 90% confluence were subjected to cyclic stretch (60 cycles/min, 15% elongation) with the computer-controlled mechanical strain unit (Flexcell 4000) (14).

Immunostaining—Immunofluorescence staining was used to determine F-actin and microtubule organization in VSMC. Briefly, VSMC were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. The cells were permeabilized using a PBS containing 0.1% Triton X-100 solution and blocked with 5% bovine serum albumin for 30 min. The fixed cells were washed with PBS and incubated for 20 min with FITC-conjugated phalloidin. For microtubulin staining, the cells were incubated with mouse monoclonal, anti-α-tubulin antibody for 20 min. After washing three times, the cells were stained with an anti-mouse IgG antibody conjugated to Alexa Fluor 568 dye. The images were obtained using a Zeiss LSM-510 inverted laser scanning confocal microscope with a Zeiss Plan Apochromat 63 ×, 1.4 N.A. objective lens.

Lipoprotein and Ox-LDL Preparation—LDL (density, 1.019 – 1.063 g/ml) was separated from freshly drawn, normal human plasma as described (15). Briefly, native LDL was isolated by sequential ultracentrifugation at 290,000 × g for 4 h in a NaBr gradient. After purification, LDL was oxidized by incubating with Cu$^{2+}$ (5 μmol/liter CuSO$_4$, 4 h at 37 °C) as described (15). The extent of modification was assessed by measuring thiobarbituric acid-reactive substances and by determining its electrophoretic mobility on agarose gels in barbital buffer at pH 8.6. Ox-LDL that was used exhibited a thiobarbituric acid-reactive substance value of 16.3–25.4 nmol/mg of protein, whereas the unoxidized LDL showed no detectable thiobarbituric acid-reactive substances. To validate the observations made with copper-oxidized LDL, we performed additional experiments using L5, an electronegative and minimally oxidized LDL that circulates in patients with hypercholesterolemia. L1, the nonelectro-negative and nonoxidized subtraction, was used as a control (15).

TUNEL Assay—The TUNEL assay was performed with a Promega (Madison, WI) kit. VSMC were cultured on glass coverslips in a 12-well plate at a density of 2 × 10$^5$ cells/well. After Ox-LDL treatment, VSMC were washed with PBS and fixed in 4% paraformaldehyde for 10 min at room temperature. The fixed cells were then incubated for 2 min on ice in a permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate). The slides were rinsed with PBS before the rTdT incubation mixture was added. After incubation for 60 min at 37 °C, the slides were rinsed three times with 2× SSC, and the nuclei were counterstained with 4’,6’-diamino-2-phenylindole. The numbers of TUNEL-positive cells were counted in five randomly chosen fields (magnification, ×200), and the mean and S.E. were calculated.

Determination of Bax Conformational Change—VSMC were serum-starved and incubated with Ox-LDL for 6 h in the presence or absence of drugs that induce cytoskeletal damage (cytochalasin D, 2 μM or nocodazole, 10 μM). The cells were lysed in CHAPS lysis buffer consisting of 10 mM HEPES, pH 7.4, 150 mM NaCl, 1% CHAPS, and 1 μg/ml protease inhibitors. The lysates were centrifuged (15,000 × g, 4 °C for 10 min), and 500 μg of protein was incubated overnight at 4 °C with the monoclonal antibody, 6A7. The antibody only recognizes and interacts with the activated form of Bax. Then 30 μl of anti-mouse IgG1-conjugated agarose beads (Sigma-Aldrich) were added, and the mixture was incubated overnight at 4 °C. After washing
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extensively with CHAPS lysis buffer and boiling the mixture, the released proteins were separated by SDS-PAGE (12% gel) and transferred to a nitrocellulose membrane. The level of activated Bax was detected by Western blotting (15).

Cell Death ELISA—Histone-associated DNA fragments were quantitated by ELISA (Roche Applied Science). VSMC from each well were collected by trypsinization and pelleted (200 × g, 5 min), lysed, and subjected to the ELISA according to the manufacturer’s protocol. Each experiment was carried out in triplicate and repeated independently at least three times.

Histological Examination—Aortas from wild mice and ApoE−/− were collected. Cryostat aortic sections were fixed in 4% paraformaldehyde for 20 min, blocked the endogenous peroxidase activity with 3% H2O2 in methanol for 15 min, and washed in PBS, pH 7.4. Triple immunostaining was performed as described (16). First, we detected apoptotic cells using a TUNEL kit (Roche Applied Science) according to the manufacturer’s protocol. Then sections were incubated for 30 min with different monoclonal antibodies individually (goat anti-β3 integrin or rabbit anti-α-SM actin) followed by a rhodamin-conjugated secondary antibody. The slides were examined for specific immunofluorescence using fluorescent microscope.

Before the third immunostaining, the same slides were washed in PBS, incubated with the third primary antibody against integrin β3 (β3 integrin blocking peptide was used as a control for specific staining) or α-smooth muscle actin, and exposed to peroxidase-conjugated secondary antibody. NovoRed (Vector, Burlingame, CA) was used as a substrate for peroxidase.

Statistics—All of the data are presented as the means ± S.E. Comparison between groups were made using one-way analysis of variance. The values of p < 0.05 were considered statistically significant.

RESULTS

Integrin αVβ3 Expression Is Increased in the Arch of the Aorta—We examined the expression of integrin αVβ3 partly because it can prevent vascular cells from apoptosis (17). Integrin β3 expression in the aortic arch was substantially increased compared with results from thoracic descending arteries in wild type mice fed with a normal chow (Fig. 1A). Because the aortic arch is subjected to increased mechanical stretch whereas distal thoracic descending arteries have less turbulence and reduced mechanical stress, it is possible that mechanical stretch in vivo is responsible at least in part for the up-regulation of integrin β3. To determine whether abnormal hemodynamics play a role in modulating integrin β3 expression, we studied cultured VSMC that were subjected to mechanical stretch. Our data show significant increase in integrin β3 expression in response to mechanical stretch of VSMC. Curiously, this was not a uniform response in terms of other integrins. For example, integrin β1 protein expression did not increase in these cells (Fig. 1B). Furthermore, we found that mechanical stretch markedly increased the levels of integrin β3 mRNA (peaked at 2 h; Fig. 1C). In addition, mechanical stretch increased the integrin β3 promoter activity; 3.92 ± 0.37-fold increase compared with values in unstretched, control cells, p < 0.05; n = 3; Fig. 1D).

FIGURE 1. The expression level of integrin β3 is increased in the arch of aorta and VSMC treated with cyclic stretch. A, the entire aortas of wild type mice fed a normal chow were removed, the different portions of the aorta (arch and descending part) were lysed, and the level of integrin β3 was examined by immunostaining and Western blotting. B, integrin β3 expression is induced by cyclic stretch. The cells (5 × 10^5/well) were seeded in elastomer-bottomed plates, and after cells obtained 90% confluence, cyclic stretch was applied (15% elongation, 1 Hz) for 2 h. The cells were kept for another 24 h, and cell lysates were prepared for Western blot analysis. C, cyclic stretch increased integrin β3 mRNA. The cells were subjected to cyclic stretch as described. RNase protection assays were performed. D, cyclic stretch increased integrin β3 transcription activity. The cells were transfected with an integrin β3 promoter-reporter plasmid before being subjected to cyclic stretch as described. The luciferase assays were performed after 24 h (n = 3; *, p < 0.01, compared with control). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Mechanical Stretch of VSMC or Forced Expression of αVβ3 in VSMC Inhibits Ox-LDL-induced Apoptosis—After 45 weeks of a normal chow, there were advanced atherosclerosis lesions in ApoE−/− mice. We found that integrin αVβ3 expression was inversely associated with TUNEL-positive staining. Quantitatively, approximately less than 3% of integrin αVβ3 positive VSMC were also positive of TUNEL. These data indicate that in vivo, integrin αVβ3 expression is associated with survival in VSMC (Fig. 2, A–F).

Treatment of VSMC with 50 µg/ml of copper-oxidized Ox-LDL induced DNA fragmentation (Fig. 2G, left panel, TUNEL assay). To exclude nonspecific reactions to copper, we examined the effects of treating VSMC with the “oxidized” L5 fraction of LDL directly isolated from patients with familial dyslipidemia (15). VSMC underwent a similar apoptotic response (Fig. 2H). We then examined the relationship between mechanical stretch and VSMC apoptosis. After 24 h of mechanical stretch (2 h, 15% stretch), VSMC were exposed to Ox-LDL for 6 h, we found that TUNEL-positive (green) apoptotic cells were fewer in cells subjected to mechanical stretch and Ox-LDL compared with cells that had not been prestimulated by mechanical stretch (42.7 ± 6.3% versus 25 ± 3.1%; Fig. 2G).

To determine whether the anti-apoptosis effect of mechanical stretch is a specific cause-effect relationship with integrin β3, we transfected VSMC with a siRNA against integrin β3 and successfully knocked down integrin β3 (Fig. 2I, inset).
treatment significantly \((p < 0.05)\) impaired mechanical stretch-mediated protection from Ox-LDL-induced apoptosis (Fig. 2I). Using VSMC isolated from integrin \(\beta 3\) null mice, we confirmed that the protective effect of stretch against Ox-LDL-induced apoptosis is significantly \((p < 0.01)\) attenuated (Fig. 2J). To avoid possible long term genetic reprogramming effects, integrin \(\beta 3\) cDNA was re-expressed into \(\beta 3\) null cells, and the anti-apoptotic effect was regained in these cells (Fig. 2J). Taken together, these data demonstrated clearly that integrin \(\beta 3\) was responsible for the mechanical stretch-mediated protection of VSMC against the Ox-LDL-induced apoptosis.

**Integrin \(\beta 3\)-mediated Suppression of Ox-LDL-induced Apoptosis Involves the Cytoskeleton—**Integrin functions in modulating cytoskeleton mechanical stability by providing dynamic, bidirectional links between the extracellular matrix and the cytoskeleton (19). When we examined changes in the cytoskeleton in Ox-LDL-treated VSMC, we found changes in cell shape including rounding and retraction (data not shown). In contrast, when we subjected VSMC to mechanical stretch or when we forced the expression of integrin \(\beta 3\) in VSMC, we found that Ox-LDL did not cause similar morphologic changes. We then examined the relative distribution of cytoskeletal F-actin and microtubules in VSMC that had been treated with Ox-LDL. As shown in Fig. 3A, when VSMC were exposed to Ox-LDL, there was substantial rearrangement and disruption of F-actin and microtubules. Individual stress fibers disappeared and F-actin became clustered around the nucleus in these cells. These findings suggest a substantial redistribution of F-actin filaments. In contrast, we found that mechanical stretch or forced expression of integrin \(\beta 3\) preserved F-actin and microtubule patterns (Fig. 3A).

Our results raised the possibility that cytoskeletal disruption is a key process to the understanding of Ox-LDL-induced apoptosis. To test this possibility, we exposed VSMC to cytochalasin D (2 \(\mu\)M) or nocodazole (10 \(\mu\)M). These chemicals inhibited actin and microtubule polymerization but did not induce apoptosis (DNA fragmentation assay; Fig. 3B). We then applied mechanical stretch to wild type VSMC that were pretreated with cytochalasin D or nocodazole for 30 min before adding Ox-LDL. A cell death ELISA analysis showed that both reagents...
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significantly impaired the ability of stretch to protect VSMC from Ox-LDL-induced apoptosis. Similar results were obtained in studies of cells with forced expression of integrin β3 (Fig. 3C). We conclude that the anti-apoptotic response induced by

mechanical stretch or by increasing integrin αβ3 expression resulted from stabilization of the cytoskeleton.

The Downstream Event of Mechanical Stretch/Integrin β3/Cytoskeleton-mediated Suppression of Apoptosis Is Protection from Activation of Bax—Upon exposure to apoptotic stimuli, proapoptotic proteins such as Bim or Bmf are released from the cytoskeleton. These proteins activated Bax, which translocated to the mitochondrial outer membranes to cause a loss of the membrane potential and release of cytochrome c (20, 21). Cytochrome c release activated caspases, ultimately leading to apoptosis. In cultured VSMC, we found that Ox-LDL resulted in a conformational change in Bax (Fig. 4A, 3.7-fold increase compared with control, n = 3, p < 0.01). To test whether mechanical stretch inhibits this Bax activation, VSMC were subjected to stretch and then incubated with Ox-LDL. As shown in Fig. 4A, mechanical stretch prevented Ox-LDL-induced activation of Bax. Similarly, forced overexpression of integrin β3 blocked Ox-LDL-induced activation of Bax (Fig. 4B). However, either cytochalasin D or nocodazole abolished the ability of integrin β3 to prevent Bax activation (Fig. 4C).

Mechanical Stretch Protects VSMC from Apoptosis by Stabilizing PINCH-1—PINCH-1 is a “hub” protein that connects integrin to the cytoskeleton and stabilizes it (22, 23). When we treated VSMC with Ox-LDL, we found a significant decrease in the level of PINCH-1 (Fig. 5A; 60 ± 5%, p < 0.05). Either mechanical stretch or forced expression of integrin β3 prevented Ox-LDL-induced down-regulation of PINCH-1 (Fig. 5A). These results suggested that integrin β3 stabilized PINCH-1 to prevent Ox-LDL-induced apoptosis. To examine whether integrin β3 was necessary for mechanical stretch-mediated stabilization of PINCH-1, we studied VSMC isolated from integrin β3 null mice. We found that the loss of integrin β3 abolishes stretch-mediated stabilization of PINCH-1 in VSMC that was treated with Ox-LDL (Fig. 5B).

Because Ox-LDL disrupted the cytoskeleton and down-regulated PINCH-1, we investigated whether there was a cause-effect sequence between decreased PINCH-1 and cytoskeletal disruption/apoptosis. We used two independent sets of siRNA against PINCH-1 and found that both sets knocked down PINCH-1 levels. In both cases, there was cytoskeleton disruption and cell rounding (Fig. 5C) as well as apoptosis (Fig. 5D).

In addition, we found that down-regulation of PINCH-1 significantly enhanced the ability of Ox-LDL to cause apoptosis (Fig. 5E). Importantly, down-regulation of PINCH-1 also abolished integrin β3-mediated protection from Ox-LDL-activation of Bax in VSMC subjected to mechanical stretch (Fig. 5F). Thus, our studies
revealed a pathway initiated by Ox-LDL, which suppresses PINCH-1 expression, causing cytoskeletal disruption, BAX activation, and apoptosis. To further establish a causative link between integrin αVβ3 and PINCH-1-dependent regulation of apoptosis, we forced expression of PINCH-1 in integrin αVβ3 null cells and found that expression of PINCH-1 in αVβ3 null cells prevented Ox-LDL-induced Bax activation and apoptosis (Fig. 5, G and H). To provide a link between integrin β3 and PINCH-1 in vivo, we examined the expression of these proteins in aorta arch and descending arteries from both wild type and β3 null mice. As shown in Fig. 5I, when integrin β3 is deleted, both PINCH-1 and ILK-1 were also lower in the arteries from β3 null mice, whereas integrin β1 remained the same.

These results lead to the hypothesis that stabilization of PINCH-1 by integrins suppresses apoptosis. To test this hypothesis, we established a VSMC line with forced overexpression of integrin β3 (Fig. 6A). Our data show that stabilization of PINCH-1 by integrin β1 also protected the cells from Ox-LDL-induced apoptosis (Fig. 6B).

**DISCUSSION**

Accumulation of VSMC is a hallmark of neointimal formation, the “soil” of atherosclerosis (24). This occurs despite the activation of specific signaling pathways that should cause VSMC to undergo apoptosis (8, 9). We found that integrin αVβ3 was a major determinant of Ox-LDL-induced apoptosis. This was because an increase in integrin αVβ3 expression prevented Ox-LDL-induced apoptosis in VSMC. Our results identify a connection between this integrin β3-dependent pathway and atherosclerosis. First, we found that in the aortic arch with higher turbulence, there was an increased expression of integrin β3 (Fig. 1A). Second, we found an inverse correlation between the expression of integrin β3 in advanced atherosclerotic lesion (ApoE−/− mice) and apoptosis identified as TUNEL-positive staining (Fig. 2, A–D). The integrin αVβ3-dependent mechanism that prevents apoptosis acted by increasing PINCH-1 expression, which caused stabilization of the cytoskeleton. This is important because when the cytoskeleton is intact, Ox-LDL no longer activates Bax, and hence, apoptosis.

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**FIGURE 5.** Ox-LDL down-regulates PINCH-1, which mediated integrin β3 effects on cytoskeleton stabilization, Bax inactivation, and anti-apoptosis. A, Ox-LDL induces down-regulation of PINCH-1 in VSMC. Both increased expression of integrin β3 and mechanical stretch prevents Ox-LDL-induced down-regulation of PINCH-1. B, null deletion of integrin β3 in mouse VSMC abolished mechanical stretch stabilization effect on Ox-LDL-induced down-regulation of PINCH-1. C, PINCH-1 siRNA disrupts cytoskeleton and causes cell rounding. D, knockdown PINCH-1 triggers apoptosis in VSMC. VSMC were transfected with two sets of PINCH-1 siRNA (50 nM). The PINCH-1 expression level was detected by Western blot analysis after 48 h. E, PINCH-1 siRNA suppress integrin β3-mediated anti-apoptotic effects in Ox-LDL-treated cells. F, PINCH-1 siRNA abolishes integrin β3-mediated Bax inactivation in Ox-LDL-treated VSMC/β3 cells. The cells were treated as C, and the Bax activation was done as described in the legend to Fig. 4. Forced expression of PINCH-1 in integrin β3 null cells prevented Ox-LDL-induced apoptosis (G) and Bax activation (H). I, loss of integrin β3 is associated with reduced level of PINCH-1 and ILK-1 in vivo. The aorta arch and descending arteries were removed from both wild type (WT) and β3 null mice, and the levels of integrin β3, PINCH-1, ILK-1, and integrin β1 were examined by Western blotting. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.
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**FIGURE 6.** Forced expression of integrin β1 inhibits Ox-LDL-induced apoptosis through stabilization of PINCH-1 in VSMC. **A**, Ox-LDL-induced down-regulation of PINCH-1 is prevented by forced expression of integrin β1. **B**, forced expression of integrin β1 prevents Ox-LDL-induced apoptosis. WT, wild type; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

**FIGURE 7.** Accumulation of VSMC in the process of vascular remodeling. Ox-LDL-induced apoptosis is involved in the down-regulation of PINCH-1 that links integrin to the cytoskeleton and thereby triggers apoptotic events. Mechanical stretch increases integrin αVβ3 expression that inhibits Ox-LDL-induced down-regulation of PINCH-1 and apoptotic events, leading to accumulation of VSMC.

is suppressed (summarized in Fig. 7). Thus, the mechanical stretch-mediated increase in expression of integrin αVβ3 is pro-atherogenic in the early phase of atherosclerosis. This is because it contributes to an imbalance between VSMC survival and apoptosis leading to VSMC accumulation in the early neointimal lesion of atherosclerosis.

**What Is Responsible for Increased Expression of αVβ3 Integrin in the Artery?**—Atherosclerotic plaques exhibit a predictable distribution in the arterial tree, most often occurring in areas surrounding arterial bifurcations. This localization of atherosclerotic lesions is attributed to the generation of hemodynamic forces at the outer segments of bifurcations where shear stress is more variable (25). The role of hemodynamic forces in atherogenesis was supported by results from cultured vascular cells; cyclic mechanical stretch stimulated the prolonged expression of several vasoactive factors (26). In the arch of the aorta, we found that integrin β3 expression is high compared with results from descending thoracic aortic segments with much less turbulence and mechanical stress (Fig. 1A). Our study in cultured VSMC confirms that cyclic stretch induces the expression of integrin β3 (at both the transcriptional and protein level; Fig. 1, B–D).

Evidence for a specific cause-effect role of integrin β3 included: knockdown of integrin β3 using a siRNA against integrin β3 or studies of a null deletion of integrin β3 abolished mechanical stretch-mediated protection from Ox-LDL-induced apoptosis (Fig. 2, I and J). We found that mechanical stretch only increased the expression of integrin β3 in VSMC (Fig. 1B), suggesting that there was a physiological specificity for integrin β3. We concluded that stretch-induced expression of integrin β3 counteracts the Ox-LDL-induced apoptotic responses. These results are consistent with the report that null deletion of integrin β3 significantly reduces injury-induced neointima formation (17).

**How Does Increased Expression of Integrin αVβ3 Prevent Apoptosis?**—We found that Ox-LDL caused disorganization of the cytoskeleton (Fig. 3A). This observation is important because disorganization of the cytoskeleton has been shown to release members of the BH3 family of pro-apoptotic proteins (e.g. Bim and Bmf), which can activate the pro-apoptotic Bax (21). Our results showed that either mechanical stretch or forced expression of integrin αVβ3 prevented cytoskeletal disruption when the cells were treated with Ox-LDL. This was consistent with an important role of cytoskeleton stabilization in preventing the activation of Bax (21).

Our current study established a mechanism by which stretch prevents Ox-LDL-induced cytoskeletal disruption and apoptosis. We have uncovered a cellular pathway that influences the degree of VSMC responses to Ox-LDL. Specifically, Ox-LDL down-regulated PINCH-1 (Fig. 5A), a key hub protein linking integrin with the cytoskeleton (10). PINCH-1 is known to play an essential role in mediating integrin signaling through the cytoskeleton (10–12), and we found that knockdown PINCH-1 caused depolymerization of the cytoskeleton, VSMC rounding, and apoptosis (Fig. 5, C–E). We also found that mechanical stretch prevented Ox-LDL-induced down-regulation of PINCH-1 in wild type VSMC but not in β3−/− cells (Fig. 5, A and B). Consistent with this pathway, knockdown of PINCH-1 in VSMC with forced expression of integrin αVβ3 still destabilized the cytoskeleton, activated Bax, and caused apoptosis (Fig. 5, E and F). Moreover, forced expression of PINCH-1 in β3 null cells prevented Ox-LDL-induced activation of Bax and apoptosis (Fig. 5, G and H). Lastly, the level of integrin β3 was closely correlated with PINCH-1 and its downstream signal ILK-1. Furthermore, the loss of integrin β3 is associated with lower level of PINCH-1 and ILK-1 in the arch of aorta and descending arteries (Fig. 5I). These data established a functional/causeative link between integrin β3 expression and PINCH-1-dependent regulation of apoptosis. These data also indicated that stabilization of PINCH-1 in response to either mechanical stretch or via an increase in integrin αVβ3 is an important mechanism that protects VSMC from apoptosis.

Interestingly, we found that overexpression of integrin β1 in VSMC also protected the cells from Ox-LDL-induced down-regulation of PINCH-1, disruption of cytoskeleton, and apoptosis (Fig. 6). These data indicated that the protective effect of PINCH-1 stabilization on Ox-LDL-induced apoptosis was not specific to integrin β3. However, because mechanical stretch only increased the expression of integrin β3 in VSMC but not integrin β1 (Fig. 1B), the physiological role of stretch was to increase integrin β3.

As summarized in Fig. 7, our results uncovered a novel physiological and pathological role for mechanical stretch-induced integrin αVβ3 in promoting VSMC survival even when Ox-
LDL levels were high. The uncovered cell signaling pathway contributes to the inhibition of down-regulation of PINCH-1 and thereby stabilized the cytoskeleton to suppress activation of Bax. Our results clearly demonstrated that mechanical stretch acts as an early pro-atherogenic role to prevent VSMC apoptosis at an early phase leading to VSMC accumulation. In this way, integrin αVβ3 expression influences the balance between VSMC apoptosis and survival.

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