NEGATIVE REGULATION OF MONOCYTE ADHESION TO ARTERIAL ELASTIC LAMINAE BY SIGNAL-REGULATORY PROTEIN α AND SH2 DOMAIN-CONTAINING PROTEIN TYROSINE PHOSPHATASE-1

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Running title: Regulation of monocyte adhesion to elastic laminae

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Elastic laminae are extracellular matrix constituents that not only contribute to the stability and elasticity of arteries, but also play a role in regulating arterial morphogenesis and pathogenesis. We demonstrate here that an important function of arterial elastic laminae is to prevent monocyte adhesion, which is mediated by the inhibitory receptor signal-regulatory protein (SIRP) α and SH2 domain-containing protein tyrosine phosphatase (SHP)-1. In a matrix-based arterial reconstruction model in vivo, elastic laminae were resistant to leukocyte adhesion and transmigration compared with the collagen-dominant arterial adventitia. The density of leukocytes within the elastic lamina-dominant media was about 58 – 70-fold lower than that within the adventitia from 1 to 30 days. An in vitro assay confirmed the inhibitory effect of elastic laminae on monocyte adhesion. The exposure of monocytes to elastic laminae induced activation of SIRP α, which in turn activated SHP-1. Elastic lamina degradation peptides extracted from arterial specimens could also activate SIRP α and SHP-1. The knockdown of SIRP α and SHP-1 by specific small interfering RNA (siRNA) diminished the inhibitory effect of elastic laminae, resulting in a significant increase in monocyte adhesion. These observations suggest that SIRP α and SHP-1 potentially mediate the inhibitory effect of elastic laminae on monocyte adhesion.

Arterial elastic laminae have long been considered a structure that determines the strength and elasticity of blood vessels (1-6). Recent studies, however, have demonstrated that arterial elastic laminae also participate in the regulation of arterial morphogenesis and pathogenesis (7-12). An important contribution of elastic laminae is to confine smooth muscle cells (SMCs) to the arterial media by inhibiting SMC proliferation (8,9) and migration (10), thus preventing intimal hyperplasia under physiological conditions. Arterial elastic laminae also exhibit thrombosis-resistant properties. When implanted in an artery, elastic lamina scaffolds are associated with significantly lower leukocyte adhesion and thrombosis compared with collagen matrix scaffolds (10). These observations suggest an inhibitory role for elastic laminae relative to collagen matrix. While such a role is well documented, the mechanisms remain poorly understood.

Leukocytes are known to express the inhibitory receptor SIRP α (also known as Src homology 2 domain-containing tyrosine phosphatase substrate-1), a transmembrane glycoprotein receptor that exerts an inhibitory effect on cell mitogenic (13-18) and inflammatory (19,20) activities. Upon ligand binding, SIRP α transmits inhibitory signals through tyrosine phosphorylation of its intracellular immunoreceptor tyrosine-based inhibitory motif (ITIM) (15-18,21,22). The phosphorylation of the ITIM initiates the recruitment of Src homology 2

domain-containing protein tyrosine phosphatase (SHP)-1 to SIRP α, which is known as a substrate of SHP-1 (21,22). The recruitment of SHP-1 also localizes and activates SHP-1 (23), which in turn dephosphorylates protein kinases, possibly including receptor tyrosine kinases (23-25), the Src family protein tyrosine kinases (26), phosphatidylinositol 3-kinase (26), and the Janus family tyrosine kinases (27,28). These activities potentially suppress inflammatory and mitogenic responses (29-32). Since the inhibitory effect of elastic laminae coincides with the activity of the inhibitory receptor in leukocytes, it is conceivable that, upon contacting leukocytes, elastic laminae may interact with SIRP α and activate SHP-1, leading to the inhibition of leukocyte adhesion. In this study, we test the possibility that SIRP α and SHP-1 mediate the inhibitory effect of elastic laminae.

MATERIALS AND METHODS

Matrix-based aortic reconstruction. We have established a matrix-based aortic reconstruction model to test the inhibitory effect of elastic laminae on leukocyte transmigration relative to collagen matrix. Aortic substitutes were constructed with three types of aortic matrix scaffold: NaOH-treated matrix scaffolds with an elastic lamina blood-contacting surface, untreated matrix scaffolds with a basal lamina blood-contacting surface, and NaOH-treated matrix scaffolds with an adventitial blood-contacting surface.

The first type of aortic substitute was created by treating fresh rat aortic specimens with 0.1M NaOH at 20^{o} C for 2 hours, followed by washing in distilled water for 12 hours with vigorous agitation. Such a treatment removes the cellular components, basal lamina, and medial collagen matrix, leaving an aortic matrix scaffold with medial elastic laminae and collagen-dominant adventitia, as detected by immunohistochemistry (10).

The aortic substitutes with untreated matrix scaffolds and a basal lamina blood-contacting surface were constructed by freezing (–76^{o} C) and thawing (20^{o} C) fresh aortic specimens for 3 cycles, followed by washing in distilled water with vigorous agitation for 12 hours. Such a treatment removes endothelial cells and destroy other cell types, leaving a matrix scaffold with intact matrix and a basal lamina blood-contacting surface. The removal of endothelial cells was verified by immunohistochemistry with an anti-factor VIII antibody (10). The exposure of the basal lamina was verified by using an anti-collagen type IV antibody (10).

To create aortic substitutes with an adventitial blood-contacting surface, fresh rat aortic specimens were treated with 0.1M NaOH at 20^{o} C for 2 hours, followed by washing in distilled water for 12 hours with vigorous agitation, which removes cells and proteoglycans in the adventitia. The aortic specimens were then turned outside in, resulting in aortic substitutes with an adventitial blood-contacting surface. The presence of the collagen blood-contacting surface was verified by immunohistochemistry with an anti-collagen type III antibody (10).

To create an aortic reconstruction model, a rat (Sprague Dawley, male, 300-350 gm) was anesthetized by intraperitoneal injection of 50 mg/kg sodium pentobarbital. Two aortic substitutes, either a pair with elastic lamina (NaOH-treated) and basal lamina (untreated) blood-contacting surfaces or a pair with elastic lamina (NaOH-treated) and adventitial surfaces, were anastomosed together in a series and grafted into the host rat abdominal aorta by using a method established previously (33). Such an arrangement ensured that each matrix substitute within the pair was in contact with the host aorta at one end. It should be pointed out that it is possible to graft all three types of matrix substitutes in a series in each animal. With such an arrangement, however, one of the three substitutes will not be in contact with the host aorta. Since cell migration from the host aorta to the substitutes contribute to intimal hyperplasia (34), a model with a series of three aortic substitutes will result in conditions of inconsistent controls. Observations were carried out at 1, 5 10, 20, and 30 days with 5 rats at each observation time. Experimental procedures were approved by the Animal Care and Use Committee of Northwestern University.

Measurement of leukocyte transmigration in vivo. To measure leukocyte transmigration in matrix substitutes, a rat was anesthetized as described above at each observation time.

Specimens were collected from all types of arterial substitutes, fixed in 4% formaldehyde in PBS, cut into transverse cryo-sections of 10 μm in thickness, incubated with phycoerythrin-conjugated anti-CD 11 b/c antibody (Caltag) and Hoechst 33258 (for cell nucleus labeling), and observed by fluorescence microscopy. The density of CD 11 b/c-positive cells in the elastic lamina-dominant media and collagen-dominant adventitia was measured and compared between different specimens.

Measurement of monocyte adhesion to elastic lamina, basal lamina, and adventitia in vitro. We used an in vitro monocyte adhesion assay to demonstrate the mechanisms of the inhibitory effect of elastic laminae. To collect monocytes for the in vitro assay, rats were anesthetized as described above. A blood sample of ~10 ml was collected from the vena cava of each rat, and mixed with 20% acid-citrate-dextrose (120 mmol/L sodium citrate, 110 mmol/L glucose, and 80mmol/L citric acid). Monocytes were collected by using a monocyte enrichment kit (Stemcell Technologies, Vancouver) according to the manufacturer's instructions. Enriched monocytes were suspended in 15% FBS DMEM at a cell count ~6 x 10^6 cells/ml, supplemented with 100 μg/ml streptomycin and 100 U/ml penicillin.

Aortic matrix specimens were prepared with four different surfaces for interacting with monocytes: NaOH-treated elastic lamina, untreated elastic lamina, basal lamina, and adventitia. Specimens with NaOH-treated elastic lamina, basal lamina, and adventitial surfaces were prepared as described in the section "Matrix-based aortic reconstruction". Matrix specimens with an untreated elastic lamina surface were prepared as follows. Fresh aortic specimens were collected and separated between the media and adventitia with fine tweezers under a surgical microscope. The media was further separated between interior elastic laminae to expose the surface of elastic laminae. Separated medial specimens were frozen (–76^o C) and thawed (20^o C) for three cycles. The surface of exposed elastic laminae was scraped with a fine metal wire along the elastic fiber direction to remove cell debris and other matrix components, including medial collagen and proteoglycans. The medial specimens were washed in distilled water for 12 hrs with vigorous agitation. Medial specimens with an elastic lamina surface were selected by immunohistochemistry. All prepared elastic lamina specimens were incubated consecutively with four antibodies, including anti-collagen type III (Chemicon), anti-collagen type IV (Chemicon), anti-vascular proteoglycans (US Biological), and anti-SMC α actin (Chemicon) antibodies. Hoechst 33258 was used for labeling cell nuclei. Specimens without collagen type III, collagen type IV, proteoglycans, SMC α actin filaments, and cell nuclei at the elastic lamina surface were selected by fluorescence microscopy and used as untreated elastic laminae. Figure 1 shows the presence of elastic lamina and the absence of collagen matrix and SMCs at the surface of prepared specimens.

Enriched monocytes were incubated in culture media at 37^o C in the presence of the 4 types of matrix specimen for 3, 6, 12, and 24 hrs with gentle agitation. At each observation time, samples from the 4 types of matrix specimen were collected, fixed in 4% formaldehyde in PBS, incubated with an anti-CD 14 antibody and Hoechst 33258, and observed with a fluorescence microscope for measuring the density of monocytes adhered to the matrix specimens. Specimens from 5 rats were used for statistical analyses at each time points.

Detection of the role of elastic laminae in regulating the activity of SIRP α and SHP-1. To test whether the exposure of monocytes to elastic laminae induces activation of SIRP α and SHP-1, enriched monocytes were cultured in dishes coated with four types of matrix specimens: NaOH-treated elastic lamina, untreated elastic lamina, basal lamina, and adventitia, which were prepared as described above. At culture times 3, 6, and 12 hours, monocytes were collected from each of the four types of matrix coating and prepared for detecting the expression and phosphorylation of SIRP α and SHP-1. Collected monocytes were lysed in lysis buffer, containing 1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM sodium orthovanadate, 1 mM EDTA, and a protease inhibitor cocktail (1 μg/ml aprotinin, 1 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM PMSF). The lysates were measured for total protein concentration, precleaned with protein A-

conjugated agarose beads (10% packed beads, Upstate), immunoprecipitated with an anti-SIRP α antibody (4 μg/ml, Santa Cruz, sc-17803) at 4° C for 4 hrs, and incubated with protein A-agarose beads (10% packed beads) at 4° C for 4 hrs. The agarose beads were collected and treated with protein sample buffer at ~100° C for 5 min. Immunoprecipitates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with the anti-SIRP α antibody (0.5 μg/ml). The relative level of protein was examined by secondary peroxidase-IgG labeling and chemiluminescent detection of peroxidase activity (35). The nitrocellulose membrane was stripped and immunoblotted consecutively with an anti-SHP-1 antibody (Santa Cruz, sc-287) and N anti-phosphotyrosine antibody (Upstate, clone 4G10, 05-321).

Detection of the role of SIRP α and SHP-1 in regulating monocyte adhesion to elastic laminae. To test the role of SIRP α and SHP-1 in regulating monocyte adhesion to elastic laminae, small interfering RNA (siRNA) specific to SIRP α and SHP-1 mRNA was used to degrade these mRNAs and thus knockdown the expression of SIRP α and SHP-1, respectively. Briefly, SIRP α- and SHP-1-specific siRNAs were prepared according to the provider's instruction (Santa Cruz). Enriched monocytes were transfected with siRNA for SIRP α (0.05 μM) or siRNA for SHP-1 (0.05 μM) by the mediation of siRNA transfection reagent (0.5%, Santa Cruz) at 37^{o} C for 48 hours. Monocytes transfected with a non-targeting scrambled siRNA control, which does not degrade known mRNAs, were used as a control (36,37). Note that the transfection medium contains 8.8% fetal bovine serum without antibiotics. Following siRNA transfection, fetal bovine serum was added to the culture to make the final serum concentration 15%. Matrix specimens with NaOH-treated and untreated elastic lamina, basal lamina, and adventitia, prepared as described above, were applied to the culture with siRNA-transfected monocytes. Cells were subsequently cultured with the matrix specimens for 3, 6, 12, and 24 hours. At each time, five matrix specimens were collected and prepared for measuring the density of monocytes adhered to the matrix surface by using methods described above.

The effectiveness of siRNA treatment was tested by immunoprecipitation and immunoblotting for each type of siRNA. At 48 hours of culture with a siRNA, monocytes were collected and prepared for detecting the expression of SIRP α and SHP-1 by immunoprecipitation and immunoblotting with anti-SIRP α and SHP-1 antibodies, respectively, as described above (2 assays done for each siRNA). Furthermore, monocytes were transfected with a fluorescein-conjugated non-targeting siRNA and observed by fluorescence microscopy for the verification of positive siRNA transfection. In addition, cell viability was tested by estimating the total cell number in each culture dish before and after the siRNA treatment (4 samples tested for each siRNA and the control siRNA). Note that after 48 hrs of culture, a fraction of cells adhered to the culture base. The total cell number after the siRNA treatment was the sum of the adherent and suspended cells.

Detection of elastic lamina degradation peptide binding to SIRP α. To test the possibility that elastic laminae interact with SIRP α in monocytes, a reasonable approach is to detect whether components from elastic laminae bind to SIRP α. To achieve such a goal, we prepared elastic lamina degradation peptides from rat aortic specimens by using a method established previously (38). Briefly, aortic specimens were collected from rats. The media of the aorta was manually separated from the adventitia under a surgical microscope with a pair of fine surgical forceps. The media was collected, minced, and treated with 1 N KOH-ethanol (80:20, v/v) at 37^{o} C for 1 hr. The resulting mixture was centrifuged and the supernatant was discarded. Samples were randomly selected from the remaining insoluble fraction and used for verifying the removal of medial cells, collagen fibers, and proteoglycans by immunohistochemistry as described above. Note that the KOH-treatment removed all medial components except the elastic laminae. The insoluble elastic lamina fraction was treated with the KOH-ethanol mixture at 37^{o} C for 1 hr. The supernatant was collected and neutralized with perchloric acid to pH 7.4 and the resulting precipitate was discarded. Elastic lamina degradation peptides were collected from the remaining supernatant.

Monocytes were cultured in the presence of 10 μg/ml elastic lamina degradation peptides, collected at 0.5, 1, and 3 hrs, and lysed in lysis buffer as described above. Lysates were processed for immunoprecipitation with an anti-elastin antibody (Elastin Products), resolved by SDS-PAGE, and probed with an anti-SIRP α antibody (Santa Cruz, sc-17803) and an anti-phosphotyrosine antibody (Upstate, clone 4G10) as described above.

Detection of SIRP α and SHP-1 phosphorylation in the presence of elastic lamina degradation peptides. To detect whether the binding of elastic lamina degradation peptides induces SIRP α phosphorylation and whether SIRP α phosphorylation induces SHP-1 recruitment, monocytes were cultured in the presence and absence of elastic lamina degradation peptides (10 μg/ml), collected at 0.5, 1, 3, and 6 hrs, and lysed in lysis buffer. Lysates were processed for immunoprecipitation with an anti-SIRP α antibody (Santa Cruz, sc-17803) and for consecutive immunoblotting by using anti-SIRP α, anti-SHP-1 (Santa Cruz, sc-287), and anti-phosphotyrosine (Upstate, clone 4G10) antibodies with antibody stripping after each immunoblotting reaction.

Cytometry confirmation of elastic lamina degradation peptide binding to SIRP α. To confirm the binding of elastic lamina degradation peptides to SIRP α, the influence of an anti-SIRP α antibody (Santa Cruz, sc-17803), developed with the extracellular domain of SIRP α1 (1-300 amino acids) as an antigen, on the relative binding of elastic lamina degradation peptides was detected by flow cytometry. Monocytes were treated with 0, 5, and 10 μg/ml anti-SIRP α antibody separately at 37° C for 1 hr, and subsequently incubated with 10 μg/ml fluorescein-conjugated elastic lamina degradation peptides at 37° C for 1 hr. Monocytes incubated with an unrelated fluorescein-conjugated secondary antibody (10 μg/ml) without elastic lamina degradation peptides was used as a control. In addition, an anti-CD11b antibody (10 μg/ml, R&D Systems, MLDP5) was used as a control in the presence of 10 μg/ml elastic lamina degradation peptides. Monocytes were detected for fluorescent intensity by flow cytometry (Beckman Coulter Epics XL-MCL).

Statistical Analyses. Means and standard deviations were calculated for each measured parameter at each observation time. The Student t-test was used for difference comparisons between two groups. A difference is considered statistically significant at $p < 0.05$.

RESULTS

Role of elastic laminae in preventing leukocyte transmigration in vivo

We used an in vivo matrix-based arterial reconstruction model to observe the role of elastic laminae in preventing leukocyte transmigration through the arterial media. As shown in Fig. 2, many cells found in the matrix of aortic substitutes were CD11 b/c-positive leukocytes (predominantly monocytes/ macrophages and granulocytes), especially during the early period. While a large number of leukocytes migrated into the collagen-dominant adventitia, few leukocytes were found within the elastic lamina-dominant media of the matrix-based aortic substitutes. The density of leukocytes in the media was 58- to 70-fold lower than that in the adventitia from 1 to 30 days after surgery, while no significant difference was detected in the media between NaOH-treated (with an elastic lamina blood-contacting surface) and untreated matrix (with a basal lamina blood-contacting surface) substitutes (Fig. 2). At the end of the elastic lamina-dominant media, leukocytes were not able to migrate into the gaps between the elastic laminae, even though the gaps were apparently larger than the diameter of leukocytes (Fig. 2A Day 10*). However, at locations with aneurysm-like changes (induced possibly by excessive mechanical stretch due to surgical damage to the adventitia), leukocytes migrated into the medial wall, where elastic laminae were largely destroyed (Fig. 2A Day 10**). These observations demonstrate that intact and NaOH-treated elastic laminae exert an inhibitory effect on leukocyte transmigration relative to the adventitial collagen matrix.

Inhibitory effect of elastic laminae on monocyte adhesion in vitro

We carried out an in vitro monocyte adhesion assay to observe the inhibitory effect of elastic laminae on monocyte adhesion relative to that of the arterial basal lamina and adventitia. As shown in Fig. 3, the exposure of monocytes to NaOH-treated and untreated elastic laminae induced monocyte adhesion ranging from 9 to 20 cells/mm^2 and 13 to 16 cells/mm^2, respectively, from 3 to 24 hours. In contrast, exposure to the basal lamina and adventitia resulted in a more than 11- and 98-fold increase in monocyte adhesion, respectively, compared with elastic lamina. These observations verify the inhibitory role of elastic laminae on monocyte adhesion relative to collagen-containing matrix.

Activation of monocyte SIRP α and SHP-1 in response to exposure to elastic laminae in vitro

Tyrosine phosphorylation of SIRP α is required for its inhibitory effect and for the recruitment and activation of SHP-1. To test whether the interaction of monocytes with matrix components induces SIRP α phosphorylation and SHP-1 recruitment, we examined co-immunoprecipitation of SIRP α with SHP-1 and the relative phosphorylation of these molecules in monocytes reacted with NaOH-treated and untreated elastic laminae, basal lamina, and adventitia. As shown in Fig. 4A, the exposure of monocytes to NaOH-treated and untreated elastic laminae induced an apparent increase in the relative phosphorylation of SIRP α. Phosphorylated SIRP α was co-immunoprecipitated with SHP-1, demonstrating recruitment of SHP-1 to SIRP α. Recruited SHP-1 was also phosphorylated. In contrast, the relative phosphorylation of SIRP α in monocytes reacted with the basal lamina and adventitia was not as apparent as that observed in cells reacted with elastic laminae (Fig. 4B). Little SHP-1 recruitment was found in monocytes cultured on the basal lamina and adventitia. These observations suggest that the exposure of monocytes to elastic laminae stimulates the activation of SIRP α, which induces the recruitment and phosphorylation of SHP-1.

Role of SIRP α and SHP-1 in the negative regulation of monocyte adhesion to elastic laminae in vitro

We used a siRNA approach to knockdown the expression of SIRP α and SHP-1 and thus to demonstrate the role of these molecules in mediating the inhibitory effect of elastic laminae on monocyte adhesion. As shown in Fig. 5A, transfection with SIRP α-specific siRNA apparently reduced the expression of SIRP α in monocytes. Such a treatment diminished the inhibitory effect of elastic laminae on monocyte adhesion, resulting in a significant increase in the density of monocytes on the surface of NaOH-treated and untreated elastic laminae (Fig. 5B). However, such a treatment did not significantly influence monocyte adhesion to the basal lamina and adventitia (Fig. 5B). Similar results were observed for the transfection with SHP-1-specific siRNA. These observations suggest that SIRP α and SHP-1 serve as potential mediators for the inhibitory effect of elastic laminae on monocyte adhesion.

We have also tested the influence of siRNA transfection on the cell viability by estimating the total cell numbers before and after siRNA transfection. Among the 4 groups tested, including a control group with culture medium only, a group with siRNA transfection reagent in culture medium, and two groups with siRNA for SIRP α and SHP-1 in culture medium supplemented with transfection reagent. The total cell numbers (in 2 ml of culture medium used for each assay) for the four groups were $1.15x10^6$ +/- $1.83x10^5$ (culture medium only), $1.21x10^6$ +/- $2.13x10^5$ (transfection reagent), $1.20x10^6$ +/- $1.80x10^5$ (siRNA for SIRP α), and $1.19x10^6$ +/- $1.64x10^5$ (siRNA for SHP-1) before siRNA transfection (n = 4 for each group). After 48 hrs siRNA transfection, the total cell numbers (sum of suspended and adherent monocytes) for the same groups were $1.17x10^6$ +/- $1.14x10^5$ (culture medium only), $1.18x10^6$ +/- $2.2x10^5$ (transfection reagent), $1.18x10^6$ +/- $1.52x10^5$ (siRNA for SIRP α), and $1.14x10^6$ +/- $1.01x10^5$ (siRNA for SHP-1). Although the total cell numbers were reduced for the groups with the transfection reagent and siRNA after siRNA transfection, no statistical significance was detected.

Binding of elastic lamina degradation peptides to SIRP α in vitro

To test whether components from elastic laminae bind to SIRP α, we prepared elastic lamina degradation peptides and examined co-immunoprecipitation of elastic lamina degradation peptides with SIRP α. As shown in Fig. 6A, in monocytes reacted with elastic lamina degradation peptides (10 μg/ml), SIRP α could be co-immunoprecipitated with elastic lamina degradation peptides by using an anti-elastin antibody, which was shown to react with elastin peptides in an immunoblotting analysis. In a flow cytometry test, a treatment with an anti-SIRP α antibody (5 and 10 μg/ml), developed with the extracellular domain of SIRP α as an antigen, competitively reduced the binding of fluorescein-conjugated elastic lamina degradation peptides (10 μg/ml) to monocytes (Fig. 6B). The relative fluorescent intensity at the peak distribution of the tested monocytes, a relative index for the level of ligand binding, was reduced by 50 +/- 15% and 63 +/- 12% for 5 and 10 μg/ml anti-SIRP α antibody, respectively (n = 3). In contrast, an anti-CD11b antibody did not apparently influence the binding of the elastic lamina degradation peptides. These observations suggest that elastic lamina degradation peptides can bind to SIRP α in monocytes.

Activation of monocyte SIRP α and SHP-1 in the presence of elastic lamina degradation peptides

To test whether elastic lamina degradation peptides influence the activity of SIRP α and SHP-1, we examined the relative phosphorylation of SIRP α and SHP-1 in the presence of elastic lamina degradation peptides. As shown in Fig. 7, a treatment with elastic lamina degradation peptides (10 μg/ml) induced an increase in the relative level of SIRP α phosphorylation, and heavily phosphorylated SIRP α was associated with increased co-immunoprecipitation with SHP-1 in monocytes, suggesting that SIRP α phosphorylation enhanced SHP-1 recruitment. The recruitment of SHP-1 was associated with an apparent increase in the relative level of SHP-1 phosphorylation in the presence of elastic lamina degradation peptides. These observations suggest that elastic lamina degradation peptides exert an activating effect on SIRP α and SHP-1, which is similar to that of elastic laminae.

DISCUSSION

The inhibitory effect of arterial elastic laminae

Biological activities are often regulated by coordinated stimulatory and inhibitory signaling mechanisms, so that the activities can be controlled precisely as needed. Extracellular matrix components participate in the regulation of cellular processes, such as cell adhesion, proliferation, and migration. Collagen-containing matrix has long been known to stimulate these cellular processes. However, the inhibitory aspect of extracellular matrix has been poorly understood. Several recent studies have suggested a role for the arterial elastic laminae in the negative regulation of vascular SMC proliferation and migration (9,10,39,40). Such a role is supported by several lines of experimental evidence. Genetically induced deficiency of elastin in a mouse model is associated with enhanced SMC proliferation, intimal hyperplasia, and arterial stenosis during the fetal stage, resulting in animal death shortly after birth (9). Incomplete development of arterial elastic laminae in human genetic disorders, such as Williams syndrome and supravalvular aortic stenosis, is associated with similar pathological changes in large arteries (39,40). In a model of arterial implantation, decellularized elastic laminae prevent SMC migration into the elastic lamina-dominant media (10). These observations have demonstrated that elastic laminae exert an inhibitory effect on the mitogenic activities of vascular SMCs. Such an inhibitory effect may counterbalance the stimulatory effect of collagen matrix.

The present study demonstrates that arterial elastic laminae are resistant to leukocyte adhesion and transmigration compared with collagen dominant adventitia. It is interesting to note that the density of leukocytes within the elastic laminae was 58 to 70-fold lower than that within the collagen-dominant adventitia in the in vivo aortic reconstruction model. Furthermore, in vitro tests showed that the density of monocytes adhered to elastic laminae was about 11- and 98-fold lower than that adhered to the basal lamina

and adventitia, respectively. These observations suggest an inhibitory effect of elastic laminae on leukocyte adhesion relative to collagen-containing matrix. It is often observed that the arterial media is associated with a relatively low-level of inflammatory reaction compared with the intima and adventitia. The inhibitory effect of elastic laminae may contribute to such a phenomenon.

While elastic laminae are resistant to inflammatory activities, we have observed monocyte infiltration in the arterial media with aneurysm. A possible mechanism is that elastin degradation occurs in arterial aneurysm due to the upregulation of elastolytic matrix metalloproteinases (MMPs), such as MMP-2 and -9 (41), and the resulting elastin degradation peptides may serve as chemotactic factors, which induce monocyte infiltration into the arterial media (42). While elastin degradation peptides may contribute to monocyte infiltration in aneurysmatic arteries, it may also be possible that the destruction of elastic laminae diminishes the protective effect of elastic laminae, thus allowing monocyte transmigration. This possibility is supported by the present observation that monocytes are found in the media of aortic substitutes with apparently destroyed elastic laminae, but not in the media with NaOH-treated and untreated elastic laminae (Fig 2A Day 10**). It is important to note that leukocytes are capable of producing and releasing matrix metalloproteinases that degrade matrix components, a process facilitating leukocyte migration. The inhibition of leukocyte adhesion to elastic laminae potentially prevents the degradation of arterial medial matrix, thus protecting the media from further leukocyte infiltration.

The discovery of the inhibitory effect of arterial elastic laminae suggests that elastic laminae may be potentially used for constructing the blood-contacting surface of arterial substitutes. During the past decades, investigators have been searching for ideal arterial substitutes that possess the structural and mechanical features of a natural artery as well as inflammation-resistant properties. A number of polymeric and biological materials, including non-biodegradable and biodegradable polymers (43-48), collagen matrix (49-51), and fibrin matrix (52), have been characterized and tested in experimental or clinical studies. While each of these biomaterials exhibits characteristics suitable for the construction of arterial substitutes, the patency of biomaterial-based arterial substitutes remains problematic because of inflammation and thrombogenesis (53). Endothelial cell seeding of biomaterials has been proposed and used for reducing the thrombogenicity of biomaterials (54). However, difficulties in cell retention during and after arterial reconstruction hinder the application of such an approach (55). Although autogenous vein grafts offer satisfactory results (33,34,56), not all patients possess veins available for arterial reconstruction. Thus a pressing issue in arterial reconstruction is to develop arterial substitutes that are inflammation/thrombosis-resistant and possess the structural and mechanical properties of natural arteries. Given the natural features, inhibitory properties, inert immunogenicity, and availability, arterial elastic laminae may be used as a blood-contacting material for arterial reconstruction.

Potential mechanisms for the inhibitory effect of elastic laminae

Leukocyte activation is a critical process that contributes to thrombogenesis and atherogenesis. Two opposing mechanisms, protein tyrosine kinase-induced phosphorylation and protein tyrosine phosphatase-induced dephosphorylation, are potentially involved in the regulation of leukocyte activities (29-32). While the activation of certain protein tyrosine kinases may initiate and promote leukocyte activities, the activation of corresponding protein tyrosine phosphatases may exert an opposite effect. Protein tyrosine phosphatases thus play a critical role in the prevention of excessive inflammatory activities.

Leukocytes express the inhibitory receptor SIRP α (13-15), which can recruit and activate protein tyrosine phosphatase SHP-1 (21-23). Activated SHP-1 in turn dephosphorylates or deactivates substrate protein tyrosine kinases, thus inhibiting inflammatory activities (23-32). In SHP-1 deficient mice, profound activation of macrophages and neutrophils occurs in association with excessive inflammatory reactions in the lung, liver, joint, and dermis (57). These observations confirm the role of SHP-1 in

suppressing leukocyte activities. Thus, the SIRP α–SHP-1 signaling pathway provides a potential mechanism that may mediate the inhibitory effect of arterial elastic laminae on monocyte adhesion.

The present study demonstrates that the exposure of monocytes to arterial elastic laminae induces activation of SIRP α, which further recruits and activates SHP-1. These activities were consistent with the inhibitory effect of elastic laminae on monocyte adhesion relative to the basal lamina and adventitial collagen matrix. When the expression of SIRP α or SHP-1 was suppressed by transfection with specific siRNA, the inhibitory effect of elastic laminae was reduced, resulting in a significant increase in monocyte adhesion to elastic laminae. In contrast, SIRP α- or SHP-1-specific siRNA did not significantly influence monocyte adhesion to the basal lamina and adventitial specimens. These observations suggest that SIRP α and SHP-1 mediate the inhibitory effect of elastic laminae, but do not significantly influence the interaction of monocytes with collagen-dominant matrix.

Further investigations have shown that the degradation peptides of elastic laminae are capable of binding to SIRP α, suggesting interactions of SIRP α with elastic lamina components. Similar to the reaction of monocytes with elastic laminae, a treatment with elastic lamina degradation peptides activates SIRP α and subsequently SHP-1. These observations further support the role of SIRP α and SHP-1 in mediating the inhibitory effect of elastic laminae on monocyte adhesion.

Our flow cytometry tests have demonstrated that the blocking antibody for SIRP α only partially blocks the binding of elastic lamina degradation peptides to SIRP α. This observation suggests that components from elastic laminae may interact with not only SIRP α, but also other types of receptor. A possible candidate is the laminin/elastin receptor, to which elastin degradation peptides can bind (1,3,58). Another possibility is that the blocking antibody may not be able to completely block the interaction of elastic lamina degradation peptides with SIRP α.

As reported previously, elastin degradation peptides from human aneurysmatic arteries interact with and activate the laminin/elastin receptor of monocytes, stimulating monocyte chemotactic activities (42). Furthermore, the binding of elastin degradation peptides to the laminin/elastin receptor has been shown to activate mitogenic signaling mechanisms and promote the proliferation of cultured SMCs (58). However, other studies have demonstrated that elastin degradation peptides exert an inhibitory effect on SMC proliferation and migration (59). These observations suggest that stimulatory and inhibitory receptors may coexist for elastin degradation peptides and may coordinate in the regulation of cell activities. Elastin degradation peptides may selectively interact with these receptors, depending on the relative strength of the receptors. Various factors, such as the type and state of cells and experimental conditions may influence the relative receptor strength. It is important to note that, while experimental observations on elastin degradation peptides are controversial, the inhibitory effect of elastic laminae has been consistently observed in human and experimental investigations (9,10,12,39,40). These observations suggest that the inhibitory components may be dominant at the surface of the elastic laminae.

In summary, the present observations suggest that, compared with collagen-dominant matrix, arterial elastic laminae are resistant to monocyte adhesion. Such an effect is potentially mediated by the inhibitory receptor SIRP α and SHP-1. The interaction of elastic laminae with monocytes may activate SIRP α- and SHP-1-related signaling pathways that potentially suppress pro-adhesion mechanisms. The inhibitory effect of elastic laminae may potentially counterbalance the stimulatory effect of collagen matrix, contributing to coordinated regulation of inflammatory activities in the wall of arteries. The inhibitory feature renders elastic lamina a potential blood-contacting material for arterial reconstruction.

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ACKNOWLEDGMENT

This work was supported by the National Science Foundation (BES-9908591 and 0401781) and the American Heart Association (9940033N and 0550164Z).

FIGURE LEGENDS

Figure 1. Preparation of aortic matrix specimens with a surface of untreated elastic lamina. **A.** Fluorescent micrograph showing an *en face* aortic matrix specimen with a partial medial-adventitial (med-adv) interface and partial elastic lamina (EL) surface, demonstrating the feasibility of preparing specimens with an untreated elastic lamina surface. Scale: 100 μm. **B - E.** Fluorescent micrographs of transverse sections from aortic matrix scaffolds, showing the absence of collagen type III (panel B), collagen type IV (panel C), proteoglycans (panel D), and SMC α actin (panel E) on the surface of elastic lamina. For these panels, the green color represents elastic lamina, the blue represents cell nuclei, and the red represents a molecule as indicated above. Scale: 10 μm. Arrow: elastic lamina surface with which cells were interacting.

Figure 2. Leukocyte transmigration in the elastic lamina-dominant media and collagen-dominant adventitia of matrix-based aortic substitutes in vivo. (**A**) Transverse fluorescent micrographs showing the distribution of CD 11b/c-positive leukocytes in the media and adventitia of matrix-based aortic substitutes. Note that leukocytes did not migrate into the gaps between the elastic laminae at the end of the aortic matrix substitutes (Day 10*). However, leukocytes migrated into the media of aortic matrix substitutes when the elastic laminae were largely destroyed at locations with aneurysmatic changes (Day 10**). Red: antibody-labeled CD 11 b/c. Green: elastic laminae. Blue: Hoechst 33258-labeled cell nuclei. Solid arrow: blood-contacting surface of aortic matrix substitutes. Open arrow: aneurysmatic change. Scale: 100 μm. (**B**) Density of CD-11 b/c-positive cells within the elastic lamina-dominant media and collagen-dominant adventitia of NaOH-treated and untreated matrix scaffolds. Differences were significant ($p < 0.0001$) between elastic laminae and adventitia at all observation times except time 0, at which no CD 11 b/c-positive cells were found. No significant difference was detected between NaOH-treated and untreated elastic laminae at any observation time ($p > 0.05$). Means and standard deviations are presented ($n = 5$ for each group).

Figure 3. Monocyte adhesion to different matrix specimens in vitro. (**A**) En face fluorescent micrographs showing monocytes adhered to NaOH-treated and untreated elastic lamina, basal lamina, and adventitia. EL: elastic lamina. Scale: 100 μm. (**B**) Measurements of monocyte density on NaOH-treated and untreated elastic lamina, basal lamina, and adventitia. Differences were significant between elastic lamina and basal lamina as well as between elastic lamina and adventitia at all observations times ($p < 0.001$). Means and standard deviations are presented ($n = 5$ for each group).

Figure 4. Co-immunoprecipitation and relative phosphorylation of SIRP α and SHP-1 in monocytes exposed to NaOH-treated and untreated elastic laminae (panel A) as well as to basal lamina and adventitia (panel B). Two IP/IB tests were conducted for each matrix specimen. C: control without exposure to a matrix specimen. EL: elastic lamina. IP: immunoprecipitation. IB: immunoblotting. 4G10: anti-phosphotyrosine antibody.

Figure 5. Influence of SIRP α- and SHP-1-specific siRNAs on monocyte adhesion to elastic laminae. (**A**) Knockdown of the relative expression of SIRP α and SHP-1 by transfection with SIRP α- and SHP-1-specific siRNA, respectively. Two tests were conducted for each siRNA treatment. Control: cells without siRNA. S-siRNA: scrambled siRNA. (**B**) Measurements of monocyte density on NaOH-treated and untreated elastic lamina, basal lamina, and adventitia in the presence of scrambled siRNA and SIRP α- and SHP-1-specific siRNA. The transfection with a SIRP α- or SHP-1-specific siRNA induced a significant increase in monocyte adhesion to NaOH-treated and untreated elastic lamina at all observations times ($p < 0.001$). In contrast, such a treatment did not induce significant changes in monocyte adhesion to basal lamina and adventitia ($p > 0.05$ for all observation times). Means and standard deviations are presented ($n = 5$ for each group). EL: elastic lamina.

Figure 6. Binding of elastic lamina degradation peptides (ELDPs) to SIRP α. (**A**) Co-immunoprecipitation of ELDPs with SIRP α and phosphorylation of SIRP α. Two tests were conducted. IP: immunoprecipitation. IB: immunoblotting. (**B**) Influence of anti-SIRP α antibody on the binding of FITC-conjugated ELDPs to SIRP α. Curve 1: FITC-conjugated ELDPs 10 μg/ml. Curve 2: FITC-conjugated ELDPs 10 μg/ml with anti-CD11b antibody 10 μg/ml. Curve 3 and 4: FITC-conjugated ELDPs 10 μg/ml with anti-SIRP α antibody 5 and 10 μg/ml, respectively. Curve 5: control with an unrelated FITC-conjugated secondary antibody 10 μg/ml. Three cytometry tests were conducted.

Figure 7. Co-immunoprecipitation and relative phosphorylation of SIRP α and SHP-1 in the presence of elastic lamina degradation peptides (ELDPs). Two tests were conducted. C: control monocytes without treatment with ELDPs. IB: immunoblotting. IP: immunoprecipitation.

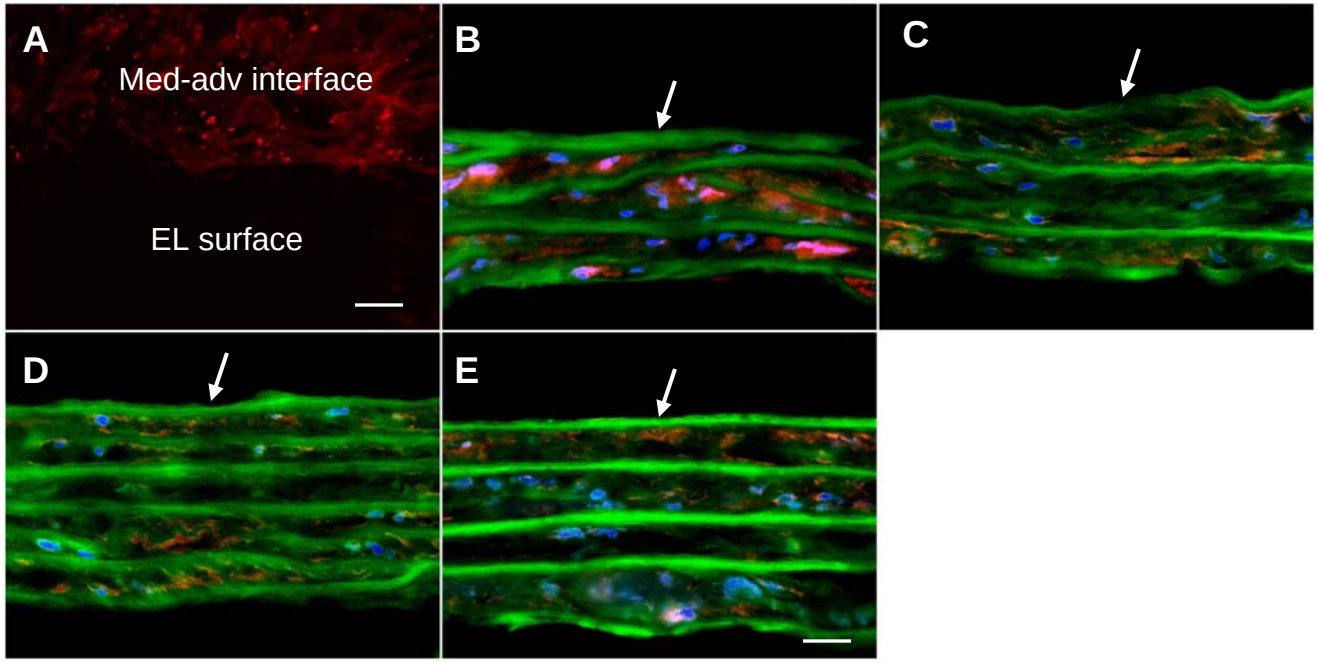

Figure 1

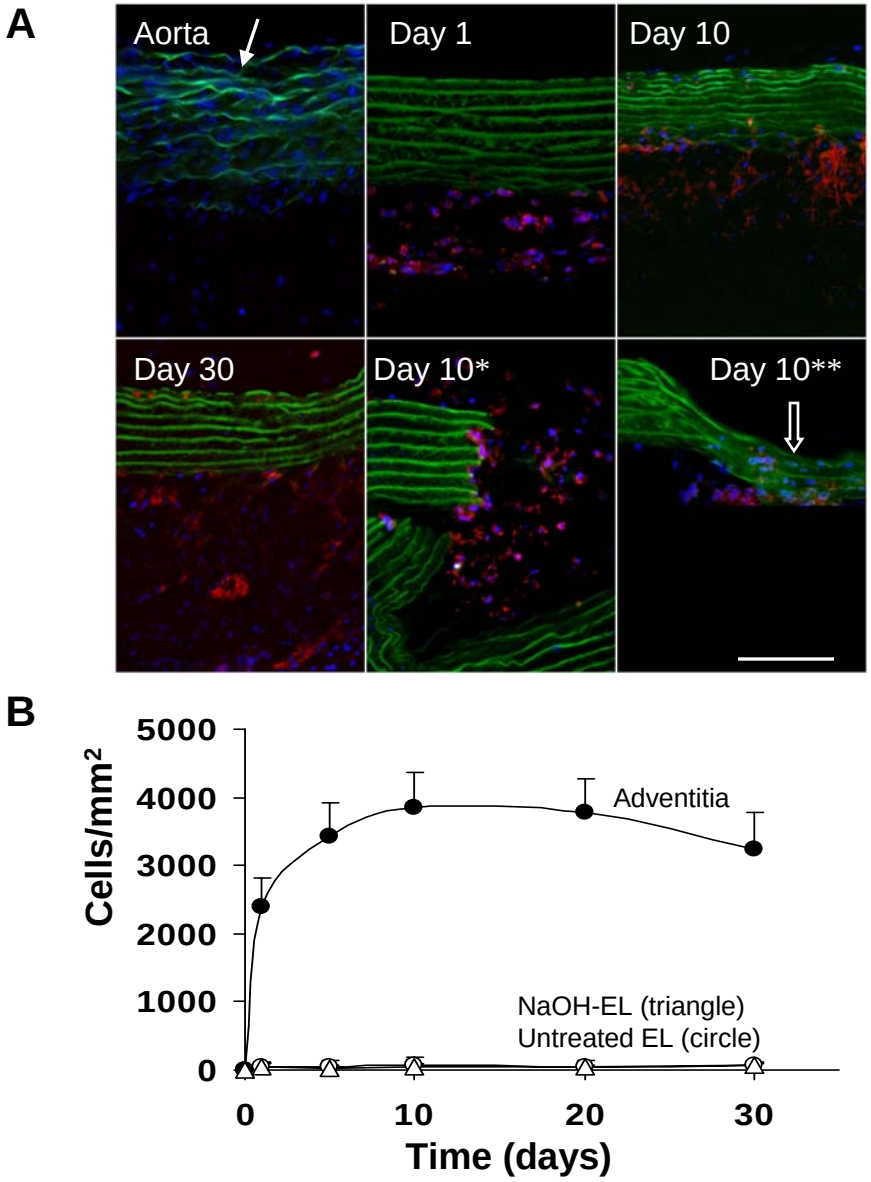
A
Aorta
Day 1
Day 10
Day 30
Day 10*
Day 10**
B
Cells/mm²
5000
4000
3000
2000
1000
0
Adventitia
NaOH-EL (triangle)
Untreated EL (circle)
0
10
20
30
Time (days)

Figure 2

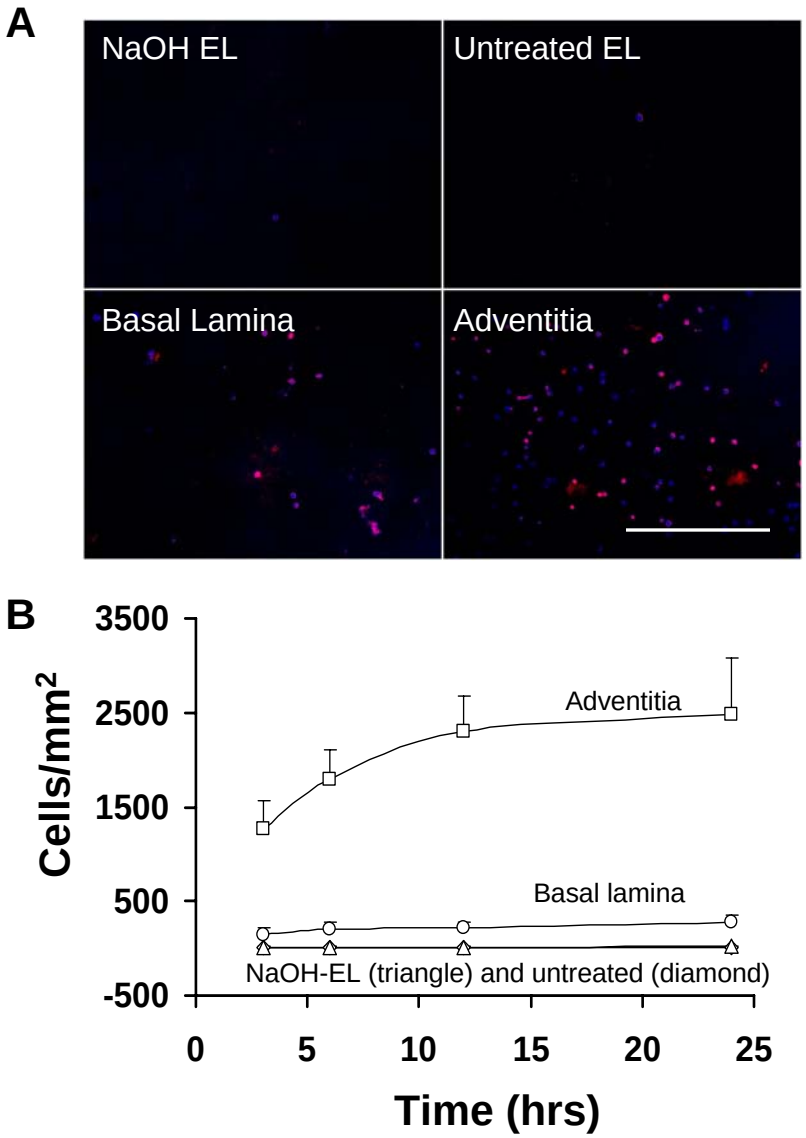
A
NaOH EL
Untreated EL
Basal Lamina
Adventitia
B
3500
2500
1500
500
-500
Cells/mm2
Adventitia
Basal lamina
NaOH-EL (triangle) and untreated (diamond)
0
5
10
15
20
25
Time (hrs)

Figure 3

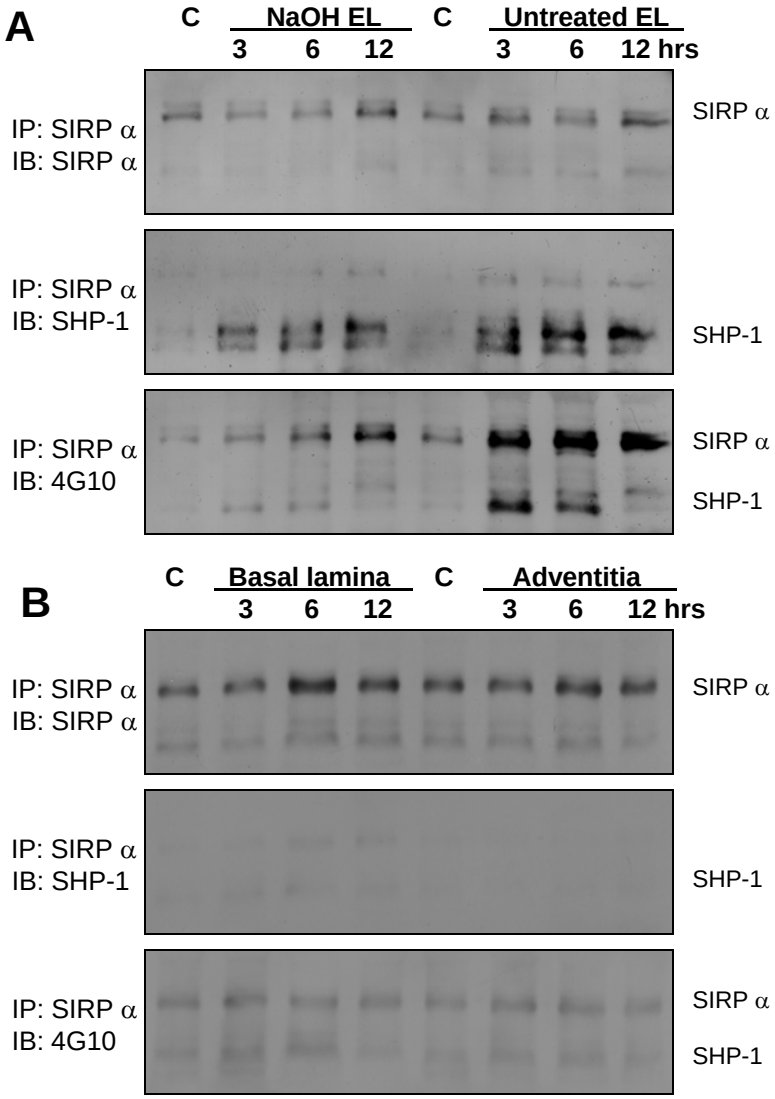
A
C
NaOH EL
C
Untreated EL
3
6
12
3
6
12 hrs
IP: SIRP α
IB: SIRP α
SIRP α
IP: SIRP α
IB: SHP-1
SHP-1
IP: SIRP α
IB: 4G10
SIRP α
SHP-1
B
C
Basal lamina
C
Adventitia
3
6
12
3
6
12 hrs
IP: SIRP α
IB: SIRP α
SIRP α
IP: SIRP α
IB: SHP-1
SHP-1
IP: SIRP α
IB: 4G10
SIRP α
SHP-1

Figure 4

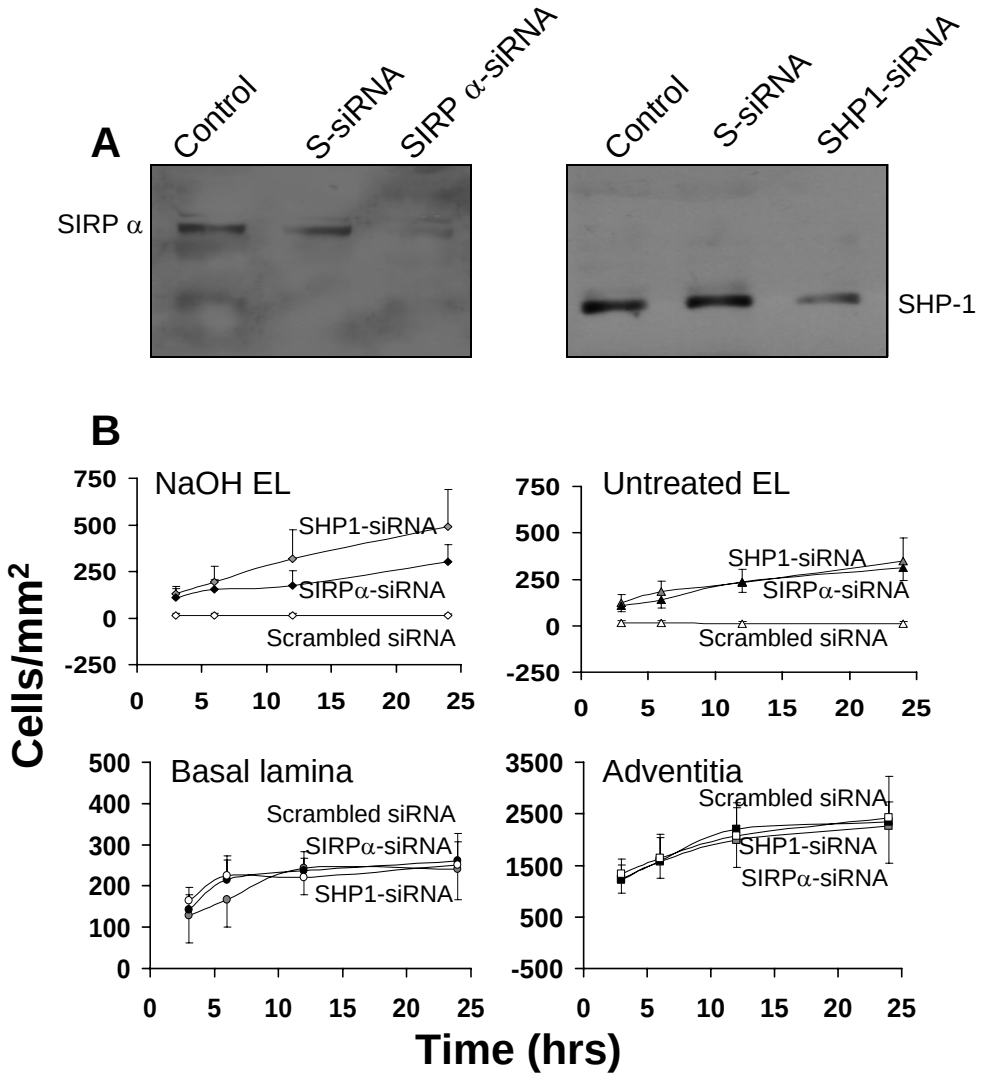
A
Control
S-siRNA
SIRP α-siRNA
Control
S-siRNA
SHP1-siRNA
SIRP α
SHP-1
B
NaOH EL
Untreated EL
Basal lamina
Adventitia
SHP1-siRNA
SIRPα-siRNA
Scrambled siRNA
Cells/mm2
Time (hrs)

Figure 5

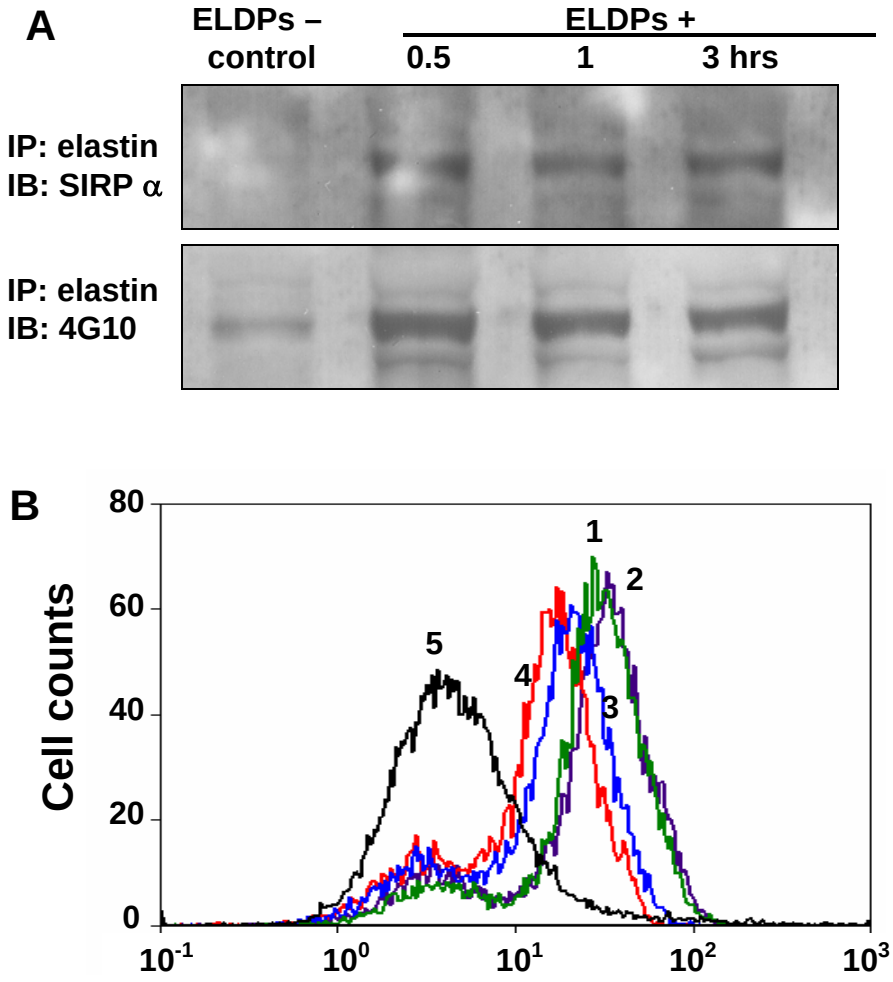
A
ELDPs –
control
ELDPs +
0.5
1
3 hrs
IP: elastin
IB: SIRP α
IP: elastin
IB: 4G10
B
Cell counts
80
60
40
20
0
1
2
3
4
5
10-1
100
101
102
103
Relative intensity

Figure 6

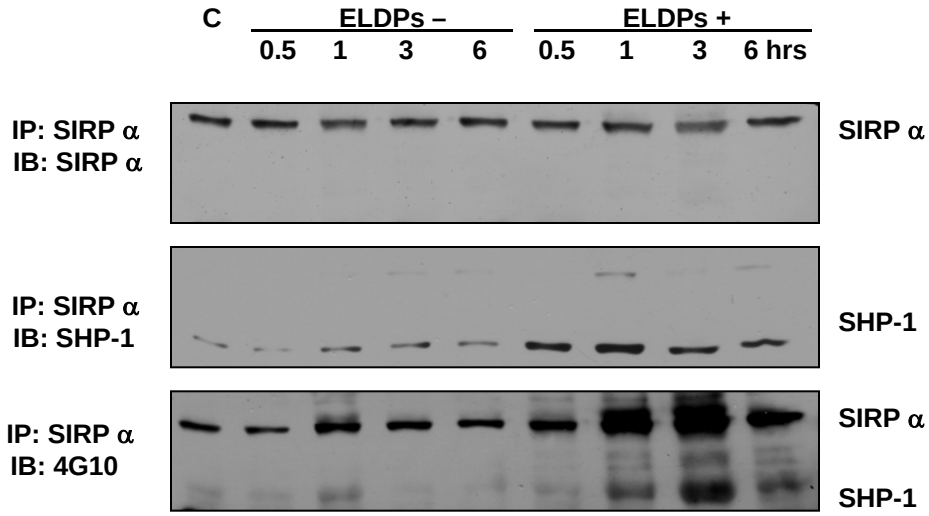
C
ELDPs –
ELDPs +
0.5
1
3
6
0.5
1
3
6 hrs
IP: SIRP α
IB: SIRP α
SIRP α
IP: SIRP α
IB: SHP-1
SHP-1
IP: SIRP α
IB: 4G10
SIRP α
SHP-1

Figure 7