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Journal Title: Arteriosclerosis, Thrombosis, and Vascular Biology
Volume: Volume 32, Number 2
Publisher: American Heart Association | 2012-02-01, Pages 386-U519
Type of Work: Article | Post-print: After Peer Review
Publisher DOI: 10.1161/ATVBAHA.111.242198
Permanent URL: https://pid.emory.edu/ark:/25593/trhvb

Final published version: http://dx.doi.org/10.1161/ATVBAHA.111.242198

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Accessed November 5, 2019 9:31 PM EST
Syndecan-1 Displays a Protective Role in Aortic Aneurysm Formation by Modulating T Cell-Mediated Responses

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Abstract

Objective—Chronic inflammation drives progressive and pathological remodeling inherent to formation of abdominal aortic aneurysm (AAA). Syndecan-1 (Sdc-1) is a cell surface heparan sulfate (HS) proteoglycan that displays the capacity to modulate inflammatory processes within the vascular wall. In the current investigation, the role of Sdc-1 in AAA formation was examined using two models of experimental aneurysm induction, angiotensin II infusion and elastase perfusion.

Methods and Results—Sdc-1 deficiency exacerbated AAA formation in both experimental models and was associated with increased degradation of elastin, greater protease activity, and enhanced inflammatory cell recruitment into the aortic wall. Bone marrow transplantation studies indicated that deficiency of Sdc-1 in marrow-derived cells significantly contributed to AAA severity. Immunostaining revealed augmented Sdc-1 expression in a subset of AAA localized macrophages. We specifically characterized a higher percentage of CD4+ T cells in Sdc-1 deficient AAA and antibody depletion studies established the active role of T cells in aneurysmal dilatation. Finally, we confirmed the ability of Sdc-1 macrophage to modulate the inflammatory chemokine environment.

Conclusions—These investigations identify crosstalk between Sdc-1 expressing macrophages and AAA-localized CD4+ T cells, with Sdc-1 providing an important counterbalance to T cell driven inflammation in the vascular wall.

Keywords
aneurysm; aorta; inflammation; glycoproteins; leukocytes
Abdominal aortic aneurysm (AAA) is a common and life-threatening clinical condition. In the United States, with approximately 15,000 deaths each year, AAA ranks as the 13th leading cause of death. Current treatment strategies for AAA are largely confined to endovascular or open surgical intervention or physician surveillance. Despite improvement in minimally invasive surgical techniques, the risk of periprocedural morbidity and mortality and subsequent secondary re-intervention remains significant. Non-surgical treatment options would be desirable. Indeed, a number of approaches have been proposed to prevent progression of aneurysmal disease during the period of aneurysm surveillance including hemodynamic control, as well as inhibition of inflammation and protease activity. However, a clinically effective pharmacotherapeutic that limits or reverses aortic dilatation has yet to be identified. Defining endogenous mediators that serve to dampen the inflammatory response within the setting of AAA may identify new avenues for medical therapy.

The syndecans are a family of four cell surface proteoglycans (Sdc-1, 2, 3, and 4) that display the capacity to modulate pro-inflammatory and proteolytic processes within the vascular wall. The biological function of syndecans is primarily exerted via pendant glycosaminoglycans (GAG), such as HS and chondroitin sulfate (CS), which sequester and regulate the activity of heparin-binding growth factors, pro-inflammatory chemokines, and proteases. Intact syndecan ectodomains can be released from the cell surface through proteolytic shedding. This process, which is upregulated within the context of inflammation, allows syndecans to exert a biological effect beyond the confines of the plasma membrane. Detailed reviews of syndecan biology can be found elsewhere. Within the syndecan family, syndecan-1 is emerging as an important regulator of inflammation. Several studies have documented the protective role of syndecan-1 in animal models with strong inflammatory components, such as nephritis, toxic shock, allergic lung inflammation, and myocardial infarction. Within these diverse investigations lies a common thread suggesting that in an environment of exaggerated inflammation, syndecan-1 can promote a dampened response by interfering with the inflammatory signaling cascade.

In adult tissue, syndecan-1 is found predominately expressed on epithelial cells and non-circulating plasma cells. Expression can also be induced in additional cell types, such as endothelial cells, smooth muscle cells, fibroblasts, and macrophages. Macrophage specific syndecan-1 expression is of particular interest as induction is governed through the cAMP/protein kinase A (PKA) signaling cascade. The recent availability of new cAMP analogs, which explicitly target PKA or guanine nucleotide exchange factor (EPAC), is generating increased interest in deciphering the specificity of these downstream effectors. Notably, cAMP/PKA specific activation in macrophages has been reported to be inhibitory toward the production of inflammatory mediators. Thus, it appears likely that syndecan-1 is induced on macrophages in the context of a broad signaling program designed to dampen an inflammatory response.

We have previously characterized the spatiotemporal expression for syndecan-1, -2, and -4 in a murine model of angiotensin II induced AAA. We observed that the expression of syndecan-1 is augmented during the course of aneurysm formation. In addition, syndecan-1 expression in the aneurysm wall seems restricted to a subset of macrophages. In this investigation, we sought to define the functional role of syndecan-1 in experimental AAA formation. We report that syndecan-1 has an important protective function in aneurysm formation that appears mediated, at least in part, through an effect on AAA-localized T cells.
METHODS

Elastase Perfusion Model

C57BL/6J mice (Jackson Laboratory) and Sdc-1\(^{-/-}\) mice (backcrossed 10 times onto a C57BL/6J background, provided by Dr. Pyong Woo Park, Baylor College of Medicine, Houston, TX) were studied. All experimental procedures were performed in male mice at maturity (8–12 weeks), according to a protocol approved by Emory University Institutional Animal Care and Use Committee. Elastase perfusion was performed as described previously.\(^{17}\) The percentage of increase in abluminal aortic diameter was calculated from the difference between the preperfusion and final aortic diameter, with AAA arbitrarily defined as the percentage of an increase in aortic diameter of more than 100%.

Generation of ApoE\(^{-/-}\)/Sdc-1\(^{-/-}\) Mice and Angiotensin II Infusion Model

ApoE\(^{-/-}\)/Sdc-1\(^{-/-}\) (C57BL/6J background) double knockout mice were generated by standard crossbreeding experiments. Sdc-1\(^{-/-}\) breeder males were mated with ApoE\(^{-/-}\) females (Jackson Laboratory), all mice were genotyped by PCR. Male mice were subsequently maintained on Paigen’s atherogenic diet (Research Diets) and received a subcutaneous infusion of angiotensin II (Ang II; 0.75 mg/kg/d) over a 2-week period by mini-osmotic pump (Alza Scientific Products). Systolic blood pressure before and after the implantation of miniosmotic pump was obtained from the mice using a noninvasive tail-cuff system (Visitech System). The incidence of AAA formation, fatal aortic rupture, and final aortic diameter at 2 weeks were determined. Total serum cholesterol was measured with Amplex Red (Molecular Probes).

Light Microscopy and Immunohistochemistry

Immunohistochemistry was performed as described previously.\(^{16}\) The following antibodies were used: Sdc-1 (N-18, Santa Cruz Biotechnology), neutrophil (NIMP-R14, Abcam), macrophage (Mac3, BD), CD4 (RM4-5, BD), CD8 (53-6.7, BD), Foxp3 (FJK-16s, eBioscience). Sections were incubated with biotinylated secondary antibodies (Vector Labs) followed by alkaline phosphatase streptavidin (Vector Labs). Negative controls with isotype IgG were prepared for each specimen. Spleen sections were used as a positive control tissue for identification of Foxp3 positive cells. Foxp3 positive cells were counted in each aortic section by a trained laboratory technician blinded to sample classification. At least four sections from each of three animals at each time point in both groups were examined. A mean value for positively stained cells was determined for each animal, and a mean for each animal group was then calculated. Acustain elastic stain kit (Sigma) was used for elastin degradation studies. Double fluorescent immunostaining was performed as described previously.\(^{16}\)

Flow Cytometry

Aortas (pooled from 3–6 individual mice) were excised from below the infrarenal arteries to just above the bifurcation after the blood content was flushed. Tissue was finely minced and shaken for 60 min at 37°C in 1 mL of RPMI-1640 supplemented with 10% FCS, 62.5 units/mL collagenase VII (Sigma), and 0.625 units/mL Dispase (BD); for Sdc-1 detection, Dispase was excluded. The isolated cells were passed through a 70-micron cell strainer to remove debris and then counted, followed by staining using a standardized protocol. The cell antibodies used included: anti-Gr1 (RB6-8C5), anti-Mac1\(\alpha\) (M1/70), anti-CD3 (145-2C11), anti-CD45 (30-F11), and anti-CD138 (281-2), all from BD. Cell suspensions were analyzed by flow cytometry (BD FACSort) and type-specific numbers of cells present in each sample were quantified and recorded.
Gelatin Zymography

Aortic tissue extract was prepared in 100 μL tissue homogenizing buffer (30 mM Tris-HCL pH 7.5, 150 mM NaCl, 10 mM CaCl₂, 10 μM E-64, 0.05% Brij35, 2 mM DMSF, 0.02% NaN₃ and 100 mM PMSF). Protein concentration was quantified using the BCA protein assay kit (Pierce). 5 μg of aortic tissue extract was run in 10% polyacrylamide containing 10% gelatin (Bio-Rad) under non-reducing conditions. The gel was developed (37°C, 3 days) and stained with 0.125% Coomassie blue. Gelatinolytic activity was quantified by densitometry (NIH Image J software).

Quantitative (Real-Time) RT-PCR

Messenger RNA levels within the aortic wall were analyzed with reverse transcriptase polymerase chain reaction (RT-PCR) using 18S rRNA as the internal control. All primers were obtained from Applied Biosystems. Four to five samples, each containing up to three pooled aortas, were obtained from each experimental time point. All PCR reactions were performed in triplicate with 10–25 ng of cDNA using the TaqMan PCR system (Applied Biosystems). Results were analyzed by comparing RNA level of samples with RNA obtained from untreated aortas using the comparative Cₜ method.

Antibody Depletion

Mice were made T lymphocytopenic by intraperitoneal injection of anti-CD3 antibody (50 μg, clone 17A2, Biolegend) at days −1, 4, and 9 after elastase perfusion. Time course of depletion protocol was verified with Thy-1⁺ (G7, Southern Biotech) staining in the spleen. Splenocytes were harvested and finely minced in RPMI-1640 medium supplemented with 5% FCS, tissue was passed through a 100 micron cell strainer, red blood cells were lysed and total cell count was recorded. Flow cytometry was used to analyze splenic T cell population.

Bone Marrow Transplantation

Sdc-1⁺/+ and Sdc-1⁻/⁻ recipients underwent lethal gamma irradiation (11 Gy) to eliminate endogenous bone marrow (BM) stem cells and circulating leukocytes. Using sterile procedures, BM cells were obtained by flushing femoral and tibial bones from Sdc-1⁺/+ and Sdc-1⁻/⁻ mice. Each irradiated mouse was injected intraorbitally with 10⁶ BM cells from designated donors. Three groups (8–10 mice/group) were studied: (1) Sdc-1⁺/+ BM cells transplanted to Sdc-1⁺/+; (2) Sdc-1⁻/⁻ BM cells transplanted to Sdc-1⁺/+; (3) Sdc-1⁺/+ BM cells transplanted to Sdc-1⁻/⁻ recipients. Mice were enrolled in the elastase perfusion model 8 weeks after transplantation.

Chemotaxis

CD4⁺ T cells were positively selected from splenocytes (Miltenyi Biotec), harvested 8–10 days post Listeria monocytogenes (10⁴ CFU/mouse) infection to produce the desired Th1 polarized response. Chemotaxis assays were performed using 4x10⁵ cells in 5 μm transwell plates (Corning) with RPMI, 0.1% BSA base media plus or minus chemokine. In some cases, HS (50 μg/mL) was included in the lower well. The migration response was quantified after 4 hours at 37°C in a humidified incubator.

Chemokine Profile

Day 4 thiglycollate elicited peritoneal macrophages were collected from C57BL/6J mice and plated in DMEM (10% FBS) at 20x10⁶ per 100x20 mm tissue culture dish. Classically activated M1 macrophage were generated through IFN-γ (100 U/mL)/LPS (10μg/mL) stimulation and syndecan-1 expression was induced with 6-Bnz-cAMP (100 μM). Syndecan-1 expression was verified with flow cytometry (anti-CD138, 281-2). Serum free
DMEM was added after initial stimulation and conditioned media collected after 12h. Adhered cells were exposed to a 1M NaCl wash to disrupt any surface electrostatic binding interactions. Conditioned media and wash fractions were combined, filtered (0.2 μm), subject to dialysis, as well as 4-fold concentration (Amicon Ultra 3000 NMWL) and analyzed for RANTES, MCP-1, MIP-1a, MIP-1b, IP-10, MIG, MDC, KC using the Mouse Common Chemokine ELISAArray (SA Biosciences). Three independent experiments were conducted.

**Statistical Analysis**

Mean and SEM were calculated for each parameter. All data were analyzed via two-tailed Student’s t-test, with the exception of frequency and mortality contingency tables in the angiotensin II AAA model (Fisher’s exact test). Values of \( P < 0.05 \) were considered statistically significant.

**RESULTS**

**Macrophage-Associated Syndecan-1 Expression Attenuates Experimental Aneurysm Formation**

Transient intraaortic perfusion with elastase is a common experimental model of AAA with consistent development of an infrarenal aortic aneurysm 14 days after initial perfusion. \(^{17}\) Immunohistochemistry was used to characterize syndecan-1 expression in concert with the neutrophil and macrophage infiltrate over the 14-day time course of aneurysm development. We observed discrete regions of neutrophil infiltration at 1, 4, and 7 days post elastase infusion; particularly, evident in both the intima and periadventitia (Supplemental Figure IA through ID). The 7 and 14 day inflammatory response was dominated by transmural infiltration of macrophages (Supplemental Figure IE through IH). Syndecan-1 expression was accentuated during the 14-day time course with most syndecan-1 positive cells localized to the adventitia (Supplemental Figure II through IL). Little to no expression of syndecan-1 was detectable in the native aorta (data not shown). Similar patterns of neutrophil and macrophage recruitment were observed in syndecan-1 deficient mice (Supplemental Figure IM through IT). Immunohistochemical analysis revealed an association between macrophage and syndecan-1 positive staining, which was confirmed by double immunofluorescence staining. As illustrated, syndecan-1 expression colocalized with Mac-3 positive cells, consistent with the notion that syndecan-1 expression is specific to infiltrating macrophages (Figure 1A).

To study the functional significance of syndecan-1 in the pathogenesis of AAA, we enrolled syndecan-1 deficient mice (Sdc-1\(^{-/-}\), C57BL/6 background) in the elastase perfusion model and results compared to wild-type C57BL/6 mice (Sdc-1\(^{+/+}\)). Syndecan-1 deficient mice have been previously characterized as healthy, with normal growth, reproduction, tissue morphology, hematologic profile, and serum chemistry parameters.\(^ {9, 21}\) There was moderate aortic dilation in both Sdc-1\(^{+/+}\) and Sdc-1\(^{-/-}\) mice up to 4 days after elastase perfusion. Increased dilation was noted by day 7, with AAA (Δ aortic diameter > 100%) in 34% of Sdc-1\(^{+/+}\) and 81% of Sdc-1\(^{-/-}\) mice. All mice formed aneurysms by day 14; however, the extent of aortic dilation was significantly greater among the Sdc-1\(^{-/-}\) group (\( P < 0.01 \); Figure 1B and Supplemental Table I) suggesting that endogenous syndecan-1 expression exerts a protective role in elastase induced AAA. Bone marrow transplantation experiments confirmed the protective source of syndecan-1 expression originates from the circulating leukocyte population during the development of AAA (\( P < 0.01 \); Figure 1C and 1D). Finally, to further characterize syndecan-1 expression in AAA tissue, we used flow cytometry (Mac-1/Sdc-1) to examine 14 day aortic tissue digests. Sdc-1\(^{+}\) cells were observed as a subset of Mac-1\(^{+}\) cells (Figure 1E); significantly, collective observations from double...
immunofluorescent staining, bone marrow transplantation, and flow cytometry all suggest macrophages provide a significant source of syndecan-1 expression in AAA.

To further test the hypothesis that macrophage syndecan-1 expression is protective in AAA, we used a second model of experimental aneurysm formation. The murine model of angiotensin-associated aortic aneurysm is produced by subcutaneous administration of angiotensin II into apolipoprotein E deficient mice (ApoE<sup>−/−</sup>).22, 23 Standard crossbreeding was used to generate ApoE<sup>−/−</sup>-Sdc-1<sup>−/−</sup> double knockout mice, which were enrolled in this model and responses compared to those of ApoE<sup>−/−</sup>-Sdc-1<sup>+/+</sup> mice. Animals were sacrificed at 3, 7, and 14 days and aortic tissue harvested for immunohistochemical examination, syndecan-1/macroage colocalization was confirmed by double immunofluorescence staining (Supplemental Figure IIA through IIP). Despite equivalent blood pressure responses and serum cholesterol levels (Supplemental Table II), significantly higher rates of AAA formation (>2-fold) and rupture (>6-fold) were observed in ApoE<sup>−/−</sup>-Sdc-1<sup>−/−</sup> mice (P<0.05; Figure 1F and 1G). This data suggests that the protective role of macrophage-associated syndecan-1 in AAA is independent of experimental animal model.

**Syndecan-1 Deficiency Increases Proteolytic Activity in the Aortic Wall**

Destruction of medial elastin is a hallmark of AAA pathogenesis. We stained aortic sections for elastic fibers and used a grading scheme to compare degradation of the medial elastic lamellae in Sdc-1<sup>+/+</sup> and Sdc-1<sup>−/−</sup> mice 7 and 14 days after elastase infusion (Figure 2A through 2D).24 We observed increased degradation in Sdc-1<sup>−/−</sup> mice at both time points (P<0.05; Figure 2E). MMP activity is abundant in AAA tissue and MMP-2 and MMP-9 have been causally linked to damage of structural matrix components in experimental models.25 Gelatin zymography revealed increased MMP-2 and -9 activity in aortic tissues harvested from Sdc-1<sup>−/−</sup> mice 7 days after elastase infusion (P<0.05; Figure 2F and 2G). These results suggest that syndecan-1 protects against AAA formation by dampening proteolytic activity.

**Syndecan-1 Deficiency Promotes Increased Inflammatory Cell Recruitment into the Aortic Wall**

Given the difficulty in quantifying the inflammatory response with standard immunohistochemistry, flow cytometry was used to compare neutrophil, macrophage, and T cell recruitment into the aortic tissue of Sdc-1<sup>+/+</sup> and Sdc-1<sup>−/−</sup> mice. To broadly characterize leukocyte infiltration, total CD45<sup>+</sup> cell count per aorta was analyzed 7 and 14 days after elastase infusion. A trend towards a greater inflammatory response was identified in Sdc-1<sup>−/−</sup> mice at day 7; by day 14, a significant increase (P<0.05) in CD45<sup>+</sup> cell count was observed in Sdc-1<sup>−/−</sup> AAA (Figure 3A through 3D). Macrophage, neutrophil, and T cell populations were analyzed after normalizing the data to total CD45<sup>+</sup> cell count within the harvested aortic tissue. As expected, the proportion of macrophages within the inflammatory infiltrate increased from day 7 to day 14 with a decline in the neutrophil population (Figure 3E, 3F, 3H, 3I and Supplemental Figure III), consistent with a shift from an acute to a chronic inflammatory response. The T cell population represented a smaller fraction of the overall inflammatory cell population, but the relative proportion of T cells was significantly higher in Sdc-1<sup>−/−</sup> aortas at both time points (P<0.01; Figure 3G and 3J). Given the increased severity of AAA in Sdc-1<sup>−/−</sup> mice, the observation of an augmented inflammatory response was not surprising. However, the enhanced T cell response was unanticipated and provides an additional mechanistic link in understanding the protective role of syndecan-1 in AAA.
T Cells Mediate Enhanced Severity of AAA in Syndecan-1 Deficient Mice

To confirm increased T cell localization in Sdc-1−/− AAA, CD4+ and CD8+ T cell populations were characterized using immunohistochemical staining. CD4+ and CD8+ T cells were readily detectable in the adventitia Sdc-1+/+ and Sdc-1−/− aneurysms at 14 days after elastase infusion (Figure 4A). Quantitative analysis confirmed a dominant CD4+ response in both Sdc-1+/+ and Sdc-1−/− AAA; in addition, there was a significant increase in CD4+ localized T cells in Sdc-1−/− over Sdc-1+/+ aneurysm tissue (P<0.05; Figure 4B). To explore the functional role of T cells in the enhanced inflammatory environment of Sdc-1−/− AAA, T cell depletion was performed. No detectable difference was observed in the T cell population (CD90+) assessed by flow cytometry in spleens harvested from Sdc-1+/+ and Sdc-1−/− mice (Figure 4C). Anti-CD3 treatment afforded 75% depletion in T cells at day 14 and resulted in a statistically significant reduction in final aortic diameter of Sdc-1−/− mice (P<0.01; Figure 4D and 4E), confirming the active role of T cells in aneurysmal dilatation.

Characterization of Inflammatory and Anti-inflammatory Mediators in Sdc-1+/+ and Sdc-1−/− AAA

To provide further insight into the inflammatory signaling cascade, quantitative real-time RT-PCR was used to measure mRNA levels of interferon (IFN)-γ, interleukin (IL)-4, IL-10, and Foxp3 (forkhead/winged helix transcription factor). IFN-γ and IL-4 have both been implicated in AAA; moreover, IFN-γ/IL-4 ratio is often characterized to describe the dominant T cell cytokine profile. In both Sdc-1+/+ and Sdc-1−/− AAA, IFN-γ was markedly upregulated (40-fold) while IL-4 expression was low (2-fold), with no significant difference between groups (Figure 5A and 5B). IL-10 expression was significantly upregulated (4-fold) in the Sdc-1+/+ mice, but not in Sdc-1−/− mice (Figure 5C). Although Foxp3 expression was upregulated in both wild-type and knockout mice, levels were significant higher in Sdc-1+/+ mice (34-fold vs. 16-fold; Figure 5D). We sought to confirm the presence of regulatory T cells in the aortic wall, given the increased levels of Foxp3 in Sdc-1+/+ mice. Regulatory T cells were readily detectable in the adventitia at both 7 and 14 days by immunohistochemical staining for Foxp3 (Figure 5E–5H). Quantitative analysis confirmed a greater number of regulatory T cells in the AAA tissue from Sdc-1+/+ mice (Figure 5I).

Collectively, these results illustrate the reduced capacity of Sdc-1−/− mice to limit vessel wall inflammation in AAA.

Macrophage Syndecan-1 Modulates Inflammatory Chemokine Profile

Based on our findings that syndecan-1 plays an active role in restricting the localized T cell population during aneurysm formation, we elected to investigate the potential for syndecan-1 to modulate AAA-driven T cell chemotaxis. Chemokines are small HS-binding proteins that direct infiltration of leukocytes into inflamed tissue and are recognized as critical components in sustaining chronic inflammation. GAG/chemokine binding interactions are well established and thought to be mediated, at least in part, by electrostatic interactions of basic chemokines with negatively charged HS. Such interactions provided motivation to test the capacity of HS, as a syndecan-1 mimetic, to competitively inhibit T cell chemotaxis to AAA relevant chemokines. In light of the CD4+/IFN-γ-dominant response that was characterized during the formation of AAA, we specifically investigated chemotaxis in Th1 polarized CD4+ T cells from Sdc-1+/+ mice. Quantitative real-time RT-PCR was used to identify chemokines of interest (potential to elicit Th1localization) in elastase induced aneurysm formation. In both Sdc-1+/+ and Sdc-1−/− AAA, there was significant expression of CCL2 (MCP-1), CCL3 (MIP-1a), CCL5 (RANTES), and CXCL9 (MIG); however, no difference in expression was noted between genotypes (data not shown). We tested the ability of HS to limit Th1 polarized CD4+ T cell chemotaxis to CCL2, CCL3, CCL5, and CXCL9 in a standard transwell migration assay. HS effectively
limited chemotaxis to CCL2, CCL3, and CXCL9 without influencing basal migration, we did not observe an impact on CCL5-driven chemotaxis (P<0.05; Figure 5). Of note, initial studies were performed to ensure equivalent chemotactic response in Sdc-1\(^{+/+}\) and Sdc-1\(^{-/-}\)CD4\(^{+}\) T cells, no inherent differences were observed; in addition, both naïve and activated CD4\(^{+}\) T cells from Sdc-1\(^{+/+}\) mice were examined for syndecan-1 expression using flow cytometry, there was no evidence of syndecan-1 expression on CD4\(^{+}\) T cells (data not shown). These studies suggest that macrophage syndecan-1 may modulate the T cell inflammatory response through HS-chemokine binding interactions.

Alternatively, syndecan-1 expressing macrophages may represent a regulatory or M2 macrophage population displaying a unique chemokine profile with dampened potential to incite inflammatory cell chemotaxis. We examined the chemokine profile of syndecan-1 expressing macrophages against classically activated (M1) inflammatory macrophages. Syndecan-1 induction was achieved using cAMP, the only reported stimulant to drive expression,\(^{12, 13}\) while IFN-\(\gamma\)/LPS induction was used to induce classically activated macrophages.\(^{20}\) Significantly, we observed a dramatic difference in chemokine profile between syndecan-1 expressing and M1 macrophages, with dampened detection of RANTES, MCP-1, MIP-1\(\alpha\), IP-10, MIG, MDC, and KC in the syndecan-1 induced population (Supplemental Figure IVA). Notably, we confirmed absence of syndecan-1 expression on M1 macrophages (Supplemental Figure IVB and C), lending plausibility to the idea that syndecan-1 expressing macrophages represent an alternatively activated regulatory population in the vessel wall.

**DISCUSSION**

Abdominal aortic aneurysm is a complex multifactorial disease. Little is understood about the mechanisms that initiate AAA; however, chronic inflammation plays a key role in driving the progressive and pathological remodeling of the aorta.\(^{31−33}\) This dysregulated process of inflammation arises from an imbalance of pro- and anti-inflammatory mediators in the vascular wall. Our investigations identify macrophage-expressed syndecan-1 as an endogenous modulator of the inflammatory response in AAA. Significantly, we provide evidence for the presence of a macrophage counterbalance to T cell driven inflammation in the vascular wall.

We used Sdc-1\(^{-/-}\) mice, in concert with both the elastase perfusion and angiotensin II induced experimental models of AAA, to explore the functional relevance of syndecan-1 in aneurysm pathology. During the course of aneurysm formation in both experimental models, the expression of macrophage-associated syndecan-1 became increasingly prominent within the aortic wall. As evident by the significant increase in aneurysm diameter in the elastase perfusion model and an increase in the incidence of aneurysm formation and frequency of aortic rupture in the angiotensin II infusion model, macrophage expressed syndecan-1 appears to play a protective role. Bone marrow transplantation studies confirmed deficiency of Sdc-1 in marrow-derived cells significantly contributed to AAA severity. Medial elastin degradation and MMP analysis revealed greater proteolytic activity within the aortic wall in Sdc-1\(^{-/-}\) mice. Gel zymography alone does not allow us to attribute the increase in Sdc-1\(^{-/-}\) proteolytic activity to a specific cell type within the aortic wall. However, several reports have now demonstrated that within aortic aneurysm tissue, macrophages and smooth muscle cells are the primary source of MMP-9 and MMP-2, respectively.\(^{17, 25}\) Flow cytometry of digested AAA tissue confirmed increased inflammatory cell recruitment in Sdc-1\(^{-/-}\) samples; accordingly, by absolute number, Sdc-1\(^{-/-}\) aneurysms contained a greater number of neutrophils, macrophages, and T cells. We chose to focus further efforts on T cell infiltration after subsequent analysis of individual neutrophil, macrophage, and T cell populations as a proportion of total CD45\(^{+}\) signal revealed a significantly higher percentage
of T cells in Sdc-1−/− mice at both 7 and 14 days. We characterized a CD4+IFN-γ dominant response in our elastase induced model of AAA and used depletion studies to confirm the role of T cells in aneurysmal dilatation. Furthermore, in reporting dampened expression of IL-10 and Foxp3, we provide evidence that Sdc-1−/− mice display a reduced capacity to limit inflammation within the aortic wall and, in vitro, we demonstrate the capacity of syndecan-1 expressing macrophages to control the local chemokine environment. Thus, we conclude that syndecan-1 expressing macrophages serve as a critical component in the vessel wall anti-inflammatory response. Notably, regulatory macrophages would exert influence through multiple pathways within aneurysm tissue; however, the current report directs focus towards the specific modulation of the T cell response.

T lymphocytes have long been recognized as a significant infiltrate in AAA yet the specificity of adaptive immunity in aneurysm pathogenesis remains controversial and undefined. T cell receptor gene expression has been probed in human tissue to provide evidence for selective, antigen specific activation. Yen et al analyzed 5 human explants, reporting polyclonal or nonrestricted diversity, while Platsoucas et al examined 10 human explants and reported evidence for oligoclonal T cell populations, suggesting antigen specific expansion. Many immunological triggers have been considered, including microbial infection, molecular mimicry, or products of vessel wall proteolysis. Infiltrating T cells provide a powerful vehicle for mediating pro-inflammatory cytokine release by macrophages. Thus, many studies have sought to characterize the dominant cytokine profile during the course of AAA formation. Xiong et al reported the critical role of CD4+ T cells and IFN-γ in a mouse model of CaCl2-induced aneurysm formation. The predominant presence of CD4+ T cells with high levels of IFN-γ or Th1-type transcripts have also been reported in human AAA. In contrast, using a murine model of aortic transplantation, Shimizu et al reported an IL-4 or Th2 dominant response was critical to aneurysm formation and King et al reported a protective role for IFN-γ in angiotensin-II induced AAA, both reports suggest a Th1 response may limit aneurysm formation. In effect, the literature addressing the role of T lymphocytes in AAA is conflicting and highlights the need for continued investigation. Moreover, the current paradigm broadens the realm of T cell subsets and cautions against simple definitions that rely upon terminal commitment of Th1 or Th2 responses.

HS-chemokines binding interactions are well established. Thus, it is reasonable to hypothesize that syndecan-1 interferes with T cell infiltration through HS-mediated sequestration of T cell specific chemokines. Furthermore, augmented T cell responses have been previously reported in Sdc-1−/− mice and syndecan-1 sequestration of T cell-specific chemokines has been shown to inhibit T cell migration. We tested the ability of HS to interfere with effector CD4+ T cell migration to chemokines specifically upregulated in our elastase induced model of aneurysm formation. HS significantly inhibited chemotaxis to CCL2, CCL3, and CXCL9, all potentially important chemokines in driving T cell migration in AAA formation. We did not observe a significant impact on CCL5 migration; however, RANTES has been reported to be more discriminatory in GAG binding. Adventital localization and sequential infiltration of macrophage and T cell populations is presumed to be critical in the specific inhibition of the T cell population by syndecan-1. Macrophage syndecan-1 expression is firmly established in the adventitia as the T cell population emerges during aneurysm formation; such spatiotemporal expression is believed to impart some specificity to heparan sulfate proteoglycan (HSPG)-chemokine binding interactions. In addition to chemokine sequestration, it is also plausible that syndecan-1 expressing macrophages modulate the chemokine environment in a more direct manner; we chose to characterize the chemokine expression profile of syndecan-1 macrophages against classically activated M1 inflammatory macrophages. Syndecan-1 expressing macrophages display a significantly dampened chemokine profile when compared to M1 macrophages.
such reduced expression of inflammatory chemokines supports the notion that syndecan-1 expressing macrophages function to regulate the inflammatory response during AAA. Notably, we observed no evidence of syndecan-1 expression on macrophages driven to an M1 phenotype; continued investigations within our laboratory are directed at characterizing the specific subtype of syndecan-1 expressing macrophages. Collectively, these findings define a protective role for syndecan-1 expression during the formation of AAA. By modulating the chemokine environment, syndecan-1 expressing macrophages can influence the participation of T lymphocytes and dampen the magnitude of the inflammatory response. However, an important caveat of our study is that T lymphocyte participation in AAA may be governed through multiple pathways, including trafficking, survival, and proliferation.

Although many reports have documented the significance of chemokine driven lymphocyte trafficking to chronically inflamed aortic tissue, alternate hypotheses warrant further study.

Our in vivo data supports the notion that syndecan-1 expression may be an important component in a series of programmed events designed to downregulate the inflammatory response within the aortic wall. Indeed, IL-10 was upregulated only in Sdc-1+/− mice and expression of the regulatory T cell marker, Foxp3, was much lower in the absence of Sdc-1. Standard immunohistochemistry confirmed the localization of regulatory T cells to aneurysm tissue and subsequent quantification revealed a significantly greater presence of regulatory T cells in Sdc-1−/− AAA. Regulatory T cells can specifically home to inflamed tissue and effectively suppress both innate and adaptive immune responses. Significantly, regulatory T cells orchestrate suppression of innate immunity through release of anti-inflammatory cytokines such as IL-10 and TGF-β and they have been implicated in dampening the inflammatory potential of neighboring macrophages. Indeed, regulatory T cells may contribute to elevated IL-10 transcripts observed in Sdc-1+/+ AAA. Given reported macrophage/regulatory T cell crosstalk, it is tempting to speculate that polarized macrophage populations influence localization of regulatory T cells to sites of inflammation. However, further study is warranted to assess the mediators, which govern both trafficking and suppressor function of regulatory T cells in aneurysm tissue.

Our study was designed to examine the role of macrophage-expressed syndecan-1 in AAA. Because syndecans have been reported to both augment and inhibit inflammatory signaling events, predicting the role of syndecan-1 in AAA was not intuitive. Moreover, studying the functional significance of syndecan-1 in macrophages, a cell population fundamentally regarded as inflammatory in chronic disease, complicated interpretation of results. The significance of our findings lies in the seemingly contradictory observation that an endogenous anti-inflammatory counterbalance emanates from an AAA localized population of macrophages. However, the presence of a counterbalance is expected within the current paradigm of the inflammatory cascade and macrophages are recognized as key components in the switch to inflammatory resolution. Microenvironmentally-derived signals give rise to macrophages that are polarized with specific functional properties, including: classically activated, pro-inflammatory M1 macrophages, and alternatively activated, potentially anti-inflammatory, M2 macrophages. Inherent negative feedback control in macrophage activation and functional plasticity would be expected to result in an overlapping M1/M2 presence in chronic inflammation. In both human and mouse AAA, syndecan-1 expression is augmented in a subset of localized macrophages; herein, we present evidence that syndecan-1 is induced on regulatory macrophages in the context of a programmed response to dampen inflammation. Indeed, it has been suggested that chemokine modulation is a critical functional attribute in alternatively activated macrophages. However, the effectiveness of such negative regulation is most likely limited in a pathological setting. By the very nature of chronic inflammation, the native processes that serve to restore homeostasis can be overpowered by the exaggerated degree of the inflammatory insult.
Within this paradox lie new targets for therapeutic intervention. Characterizing the molecular mechanisms that regulate the intrinsic counterbalance is an important step towards the development of new approaches to promote the resolution of chronic inflammation in the vascular wall.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

SOURCES OF FUNDING

This work was funded by NIH grant HL060903 (E.L. Chaikof) and fellowships from the American College of Surgeons (S.V. Smith) and American Heart Association (J. Angsana).

References


Arterioscler Thromb Vasc Biol. Author manuscript; available in PMC 2013 February 01.


Figure 1.
Macrophage expressed syndecan-1 attenuates experimental aneurysm formation. A, Immunofluorescence staining of the aortic adventitia of Sdc-1+/+ mice 14 days after elastase perfusion. Macrophage, Mac-3 (top middle, Alexa 594, red). Syndecan-1 (bottom middle, fluorescein, green). DAPI nuclear stain, blue. B, Aneurysmal dilation after elastase perfusion. Enlargement of aortic diameter at various intervals after elastase perfusion (n = 3, 3, 32, and 32 at day 1, 4, 7, and 14 respectively, for each group). **P < 0.01. C and D, Aneurysmal dilation after elastase perfusion in bone marrow transplanted mice. C, The extent of immediate dilation was calculated as the percentage increase between AD Pre and AD Post. D, The extent of overall dilatation was expressed as the percentage increase between AD Pre and AD Final. **P < 0.01. E, Aortic cell suspensions (14 days post elastase perfusion) were stained for Mac-1 and Syndecan-1 and analyzed by flow cytometry. F,G, Angiotensin II induced AAA in ApoE−/−Sdc-1+/+ (n = 20) and ApoE−/−Sdc-1−/− (n = 30) mice. F, Frequency of AAA formation. G, Fatal aortic rupture. *P < 0.05. Scale bars, 100 μm. Data are mean ± SEM.
Figure 2.
Syndecan-1 deficiency increases medial elastin degradation and MMP activity in the aortic wall during AAA formation. A through D, Medial elastin degradation during the course of elastase-induced aneurysm formation, elastin degradation-grading keys: A, grade I; B, grade II; C, grade III; D, grade IV. E, Histological sections were scored for the integrity of aortic elastin on a 4-point scale according to the elastin degradation-grading keys (n=3). *P < 0.05. F, Gelatin substrate zymogram analysis of MMP-2 and MMP-9 in the aortic wall during elastase induced AAA formation (n=3 for Sdc-1+/+ and Sdc-1-/- mice; 5 μg/well). G, Densitometry was used to quantify gel zymogram, results presented as relative density units. *P < 0.05. Scale bar, 100 μm. Data represent mean ± SEM.
Figure 3.
Syndecan-1 deficiency promotes increased inflammatory cell recruitment into aneurysmal tissue. A through D, FACS analysis of aortic tissue for total cell number and percentage of CD45+ leukocytes. A and B, Number of CD45+ cells in Sdc-1+/+ and Sdc-1−/− AAA tissue at 7 and 14 days post elastase perfusion, n=5 for each group. *P < 0.05. C and D, Representative CD45+ staining at 14 days post elastase perfusion. E through J, Percentage neutrophils, macrophages, T cells with respect to the total number of CD45+ leukocytes at day 7 and day 14, n=5 for each group. **P < 0.01. All data represent the mean ± SEM.
Figure 4.
T cells promote AAA in syndecan-1 deficient mice. A and B. Characterization of CD4 and CD8 populations in Sdc-1^{+/+} and Sdc-1^{−/−} AAA at 14 days post elastase perfusion. A, Representative immunohistochemical staining in adventitial AAA sections. B, CD4 and CD8 cell counts in aortic wall per high powered field, n=3 for each group. *P < 0.05, **P < 0.01. C through E, Effects of T cell depletion on AAA formation in Sdc-1^{−/−} mice. C, The number of T cells (Thy-1^{+}) in native spleens and 14 days after anti-CD3 antibody treatment (n=3 in both Sdc-1^{+/+} and Sdc-1^{−/−} control group, n=8 in T lymphocytopenic Sdc-1^{−/−} group). **P < 0.01. D and E, Aortic diameter measurements were obtained before (Pre) and immediately after (Post) elastase perfusion, with final measurement obtained at 14 days after surgery (Final). D, The extent of immediate dilatation was calculated as the percentage increase between AD Pre and AD Post. E, The extent of overall dilatation was expressed as the percentage increase between AD Pre and AD Final. **P < 0.01. Scale bars, 100 μm. All data represent the mean ± SEM.
Figure 5.
Expression of pro- and anti-inflammatory mediators within aneurysmal tissue of Sdc-1\textsuperscript{+/+} and Sdc-1\textsuperscript{−/−} mice. A through D, Quantitative mRNA expression in aortic tissue, n=5. A, IFN-γ. B, IL-4. C, IL-10. D, Foxp3. **P<0.01. E through I, Localization of Treg cells within aneurysmal adventitia. E, Sdc-1\textsuperscript{+/+} mice day 7; F, Sdc-1\textsuperscript{−/−} mice day 7; G, Sdc-1\textsuperscript{+/+} day 14; H, Sdc-1\textsuperscript{−/−} day 14. I, Foxp3 positive cell counts, n=3, *P<0.05, **P<0.01. Scale bar 100μm. All data represent the mean ± SEM.
Figure 6.
HS-mediated inhibition of CD4 T cell chemotaxis to AAA expressed chemokines. Migration of CD4 cells to CXCL9, CCL2, CCL3, CCL5 +/- HS (50 ug/mL) in a transwell migration assay. Chemotactic index was calculated using basal migration media (RPMI + 0.1% BSA) as a control, there was no difference observed for background movement in basal media +/- HS, n=3. *P < 0.05. Data represent the mean ± SEM.