Syndecan-1 Promotes Staphylococcus aureus Corneal Infection by Counteracting Neutrophil-mediated Host Defense*

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Many microbial pathogens subvert cell surface heparan sulfate proteoglycans (HSPGs) to infect host cells in vitro. The significance of HSPG-pathogen interactions in vivo, however, remains to be determined. In this study, we examined the role of syndecan-1, a major surface HSPG of epithelial cells, in Staphylococcus aureus corneal infection. We found that syndecan-1 null (Sdc1−/−) mice significantly resist S. aureus corneal infection compared with wild type (WT) mice that express abundant syndecan-1 in their corneal epithelium. However, syndecan-1 did not bind to S. aureus, and syndecan-1 was not required for the colonization of cultured corneal epithelial cells by S. aureus, suggesting that syndecan-1 does not mediate S. aureus attachment to corneal tissues in vivo. Instead, S. aureus induced the shedding of syndecan-1 ectodomains from the surface of corneal epithelial cells. Topical administration of purified syndecan-1 ectodomains or heparan sulfate (HS) significantly increased, whereas inhibition of syndecan-1 shedding significantly decreased the bacterial burden in corneal tissues. Furthermore, depletion of neutrophils in the resistant Sdc1−/− mice increased the corneal bacterial burden to that of the susceptible WT mice, suggesting that syndecan-1 moderates neutrophils to promote infection. We found that syndecan-1 does not affect the infiltration of neutrophils into the infected cornea but that purified syndecan-1 ectodomain and HS significantly inhibit neutrophil-mediated killing of S. aureus. These data suggest a previously unknown bacterial subversion mechanism where S. aureus exploits the capacity of syndecan-1 ectodomains to inhibit neutrophil-mediated bacterial killing mechanisms in an HS-dependent manner to promote its pathogenesis in the cornea.

Microbial pathogens express a multitude of factors that interact with host components. Pathogens use these host-pathogen interactions to their advantage to survive in the host environment. Studies during the last several decades have proposed that many viral, bacterial, and parasitic pathogens bind to cell surface HSPGs to facilitate their initial attachment and subsequent invasion of host cells (1–3). Evidence that the HSPG interaction is biologically important is provided by the finding that HS-binding pathogens show markedly attenuated attachment or invasion of host cells whose HS expression has been reduced by enzymatic treatment or mutation. Furthermore, exogenous HS or heparin (pharmaceutical functional mimic of HS) inhibits pathogen attachment and entry. Furthermore, where examined, mutant strains lacking the HSPG adhesin are viable and show normal growth rates, suggesting that the ability to bind to HSPGs is strictly a virulence activity. However, the significance of HSPG-pathogen interactions in infectious diseases has yet to be clearly established in vivo.

Syndecans comprise a major family of cell surface HSPGs (1, 4). Syndecans are type I transmembrane HSPGs composed of four members in mammals. At the cell surface, syndecans function primarily as a co-receptor for various HS-binding ligands and regulate cellular processes, such as adhesion, proliferation, migration, and differentiation. Although all syndecans harbor the ligand-binding HS chains in their extracellular domains, dramatic pathological phenotypes emerge when the single syndecan null mice are challenged with infectious or inflammatory stimuli (5–12), indicating that certain post-developmental functions of each syndecan are specific and cannot be compensated by another syndecan or other HSPGs. How this is accomplished is incompletely understood, but syndecans likely perform specific functions in vivo because they are expressed on different cell types and locations at different levels and times (1, 13). For example, in adult tissues, syndecan-1 is abundantly expressed by both simple and stratified epithelial cells and expressed to a lesser degree by other cell types (e.g. fibroblasts) (1, 13, 14).

The role of syndecans in microbial infections is currently an active area of research. For instance, Neisseria gonorrhoeae binds to the HS moiety of syndecan-1 and -4 through the Opa protein, and this interaction mediates both bacterial attachment and invasion (15). The intact syndecan cytoplasmic domain is essential in gonococcal invasion as N. gonorrhoeae.

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The abbreviations used are: HSPG, heparan sulfate proteoglycan; HBP, heparin-binding protein (azurocidin/CAP37); HS, heparan sulfate; KC, keratinocyte-derived chemokine (CXCL1); NET, neutrophil extracellular trap; NMuMG, normal murine mammary gland; RANTES, regulated upon activation normal T cell expressed and secreted; TSB, tryptic soy broth; cfu, colony-forming unit; HBSS, Hank’s balanced salt solution.

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Syndecan-1 Promotes S. aureus Keratitis

**Materials**—281-2 rat anti-mouse syndecan-1 ectodomain monoclonal antibodies and Ky8.2 rat anti-mouse syndecan-4 monoclonal antibodies were purchased from BD Biosciences. Rat anti-mouse GR1 and rat anti-mouse Mac3 monoclonal antibodies were obtained from Biolegend (San Diego). Alexa 594 donkey anti-rat antibodies, EpiLife culture medium, human corneal growth supplement, Superscript one-step RT-PCR kit, ViraPower adenovirus expression system, AccuPrime Pfx DNA polymerase, pENTR/SD/D-TOPO cloning kit, pENTR/U6 vector, pAd/CMV/V5-DEST vector, pAd/Block IT-DEST vector, LR clonase II, and S. aureus BioParticles opsonizing reagent were obtained from Invitrogen. N-Acetylcysteine was from Sigma. CNBr-activated Sepharose 4B beads were from GE Healthcare. Immobilon Ny+ (cationic nylon membrane) and GM6001 were from Millipore (Billerica, MA). Bolton-Hunter iodination reagent was from Pierce. Porcine mucosa HS was from Neoparin (Alameda, CA). RNase mini kit was from Qiagen (Valencia, CA). Oligonucleotide PCR primers were purchased from Integrated DNA Technologies (Coralville, IA). All other materials were purchased from either Thermo Fisher Scientific (Waltham, MA) or VWR (Westchester, PA).

**Bacteria**—S. aureus strains 8325-4 (20), 12598 (34), Woods (34), and P1 (35) were from our culture collection. S. aureus strains were grown to late log growth phase in tryptic soy broth (TSB), and the bacterial concentration was approximated by measuring absorbance at 600 nm. After washing, the concentration was adjusted to ~3–5 × 10^6 cfu/5 μl of TSB with or without the indicated test agents. The exact bacterial concentration in the inoculum was determined by immediately plating out dilutions of the initial inoculum onto TSB agar plates.

**Mice**—Unchallenged Sdc1^−/−^ mice on both the BALB/c and C57BL/6J backgrounds are healthy with normal growth, reproduction, tissue morphology, complete blood cell counts, and serum chemistry parameters (9–11). Both female and male Sdc1^−/−^ mice and corresponding littermate WT mice on the BALB/c or C57BL/6J background (backcrossed 10 times) were used at an age of 5–8 weeks. Mice were maintained in microisolator cages under specific pathogen-free conditions in a 12-h light/dark cycle and fed a basal rodent chow ad libitum. All animal experiments were approved by the Institutional Animal Care and Use Committee of Children’s Hospital, Boston, and complied with federal and Association for Research in Vision and Ophthalmology guidelines for research with experimental animals.

S. aureus Corneal Infection—A single vertical scratch was made with a 29-gauge needle in one of the corneas of each anesthetized mouse without penetrating beyond the superficial stroma. The other eye served as an uninjured control.

**EXPERIMENTAL PROCEDURES**

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S. aureus Corneal Infection—A single vertical scratch was made with a 29-gauge needle in one of the corneas of each anesthetized mouse without penetrating beyond the superficial stroma. The other eye served as an uninjured control. A
5-μl suspension of various doses of *S. aureus* with or without test agents was applied topically to injured or uninjured corneas. At various times post-infection, mice were euthanized, and the bacterial burden in whole enucleated eyes or isolated corneas was determined. Whole eyes or isolated corneas were homogenized in TSB containing 0.1% (v/v) Triton X-100, and serial dilutions of homogenates were plated onto TSB agar plates. The bacterial burden was consistently similar between isolated whole eyes and isolated corneas, indicating that *S. aureus* almost exclusively infects injured corneas when applied topically to the ocular surface.

*S. aureus* Binding to Syndecan-1—Syndecan-1 ectodomain was purified from the conditioned medium of normal murine mammary gland (NMuMG) epithelial cells by QAE chromatography, CsCl density centrifugation, and 281-2 immunoaffinity chromatography (8, 36), and 3 μg of purified syndecan-1 ectodomain was radiiodinated with 0.5 mCi of Na$^{125}$I using the Bolton-Hunter iodination reagent. Unincorporated radioactivity was separated from the radiolabeled syndecan-1 by PD-10 gel chromatography. Approximately 60 ng (200,000 cpm) of radiiodinated syndecan-1 ectodomains were incubated with 281-2 anti-syndecan-1 antibodies conjugated to Sepharose 4B beads or 2 × 10⁸ cfu of *S. aureus* strain 8325-4, 12598, or P1 for 1 h at room temperature in 200 μl of TSB. After washing, bound syndecan-1 was quantified by measuring bead- or bacteria-associated radioactivity in a gamma counter.

*S. aureus* Colonization of Epithelial Cells—Mouse A6(1) corneal epithelial cells, derived from the corneal epithelium of 14-day-old Immorto-Mouse (Charles River Laboratories), were obtained from Dr. Joram Piatigorsky (NEI, National Institutes of Health) (37). Confluent A6(1) cells were grown for 1–2 days in 96-well plates at the nonpermissive temperature of 37 °C in EpLife culture medium supplemented with 20% FBS and human corneal growth supplement. Alternatively, confluent A6(1) cells were infected with adenovirus harboring shRNA knockdown constructs of U6 lamin or mouse syndecan-1 at a multiplicity of infection of 10 and incubated for 1 day with virus and for 2 more days after virus removal in EpLife culture medium with FBS and human corneal growth supplement. NMuMG epithelial cells were from our culture collection (21) and were used at confluency or infected with adenovirus shRNA constructs and used at confluency. Briefly, to knock down syndecan-1 expression, the top strand CAC CCG TCT ACT TTA GAC AAC TTC GAA AAG TGG TCT AAA GTA GAC C and bottom strand AAA AGG TCT ACT TTA GAC AAC TTT TCG AAG TTG TCT AAA GTA GAC C targeting mouse syndecan-1 were annealed and ligated into linearized pENTR/U6 by T4 ligase. The shRNA region was transferred into pAd/BlockIT/DEST by LR-clonase II, linearized by Pacl digestion, and transfected into 293A virus packaging cells. Virus particles were collected, propagated in 293A cells, and stored at −80 °C until use. A6(1) corneal epithelial cells or NMuMG cells transduced with or without adenovirus harboring shRNA constructs were incubated with various doses of *S. aureus* in 100 μl of culture medium for 2 h at 37 °C, washed, and lysed with 0.1% (v/v) Triton X-100 in TSB.

The bacterial colonization was quantified by plating serial dilutions of lysates onto TSB agar plates.

Syndecan-1 Shedding at the Ocular Surface—The corneas of anesthetized mice were scratched once with a 29-gauge needle, and 5 μl of TSB or TSB with *S. aureus* (3 × 10⁸ cfu) was topically applied. At 3, 7, and 16 h post-infection, mice were euthanized, and their ocular surface was incubated with 5 μl of 1% N-acetylcysteine in PBS for 5 min to break the mucus layer of tear film and facilitate recovery of ocular surface fluids (38). The ocular surface was then rinsed with 5 μl of 1% N-acetylcysteine and 10 μl of PBS, and the ocular surface fluids were collected from the inferior fornix. The recovered ocular surface fluids were combined and spun down, and the concentration of syndecan-1 and -4 ectodomains was determined by dot immunoblotting (20, 21).

Neutrophil-mediated *S. aureus* Killing—Neutrophils were isolated from bone marrows of WT or *Sdc1−/−* mice by Percoll density gradient centrifugation. Briefly, femurs and tibias were isolated from mice under anesthesia, cleaned, and flushed with Hanks’ balanced salt solution (HBSS) containing 10 mM HEPES, pH 7.4 (HBSS/HEPES). Bone marrow cells were centrifuged at 300 × g for 10 min, resuspended in 45% Percoll solution, layered on top of a 62 and 81% Percoll gradient solution, and centrifuged at 1500 × g for 30 min. The neutrophil cell layer between 62 and 81% Percoll was collected, washed, resuspended in HBSS/HEPES, and counted. Neutrophils (10⁶ cells) were incubated with *S. aureus* Woods strain (2–3 × 10⁶ cfu) pre-opsonized with *S. aureus* BioParticles® opsonizing reagent in HBSS/HEPES containing 5% mouse serum for 2 h at 37 °C. Bacterial killing was enumerated by incubating test samples with 0.1% Triton X-100 in HBSS/HEPES for 30 min and plating serial dilutions onto TSB agar plates.

RT-PCR—Total RNA isolated from four corneas at 0 and 7 h post-infection was pooled and reverse-transcribed into cDNA and amplified using the Superscript one-step RT-PCR kit. The primers used were as follows: 5′-ATG AGA CGC GCC GGC CTC TG-3′ (sense) and 5′-CTG ATT GGC AGT TCC ATC CT-3′ (antisense) for syndecan-1; 5′-TTG TCT GTG CAG CGC TGC TG-3′ (sense) and 5′-GGG GCC TCA GGC AA-3′ (antisense) for syndecan-4; 5′-TCA CCA TCA TCC TCA CTG-3′ (sense) and 5′-TTA GCC TTG CCT TGG TTC ATG ATC-3′ (antisense) for RANTES; and 5′-GGG GCC TCA GGC CAA AA-3′ (sense) and 5′-CTC TTT GAT GAT TCG CAC GAC GAT TTC-3′ (antisense) for β-actin. Samples were separated on 1.5 or 2% agarose gels and visualized by ethidium bromide staining.

Histopathology—Eyes were enucleated at various times post-infection, fixed in 4% paraformaldehyde/PBS for 4 h at room temperature, embedded in paraffin, and sectioned horizontally. Eye sections (5 μm) were stained with hematoxylin and eosin or immunostained with anti-mouse GR1, anti-mouse Mac3, 281-2 anti-mouse syndecan-1 or Ky8.2 anti-mouse syndecan-4 monoclonal antibodies, and Alexa 594 donkey anti-rat secondary antibodies. Stained tissue sections were visualized with the Zeiss Axiovert 40 CFL microscope,
and pictures were taken with the AxiosCam MRm high resolution camera. Adobe Photoshop CS4 was used to process the acquired images.

Statistical Analyses—All data are expressed as means ± S.E. Differences between experimental and respective control groups were examined by Student’s t test, and p values of less than 0.05 were considered statistically significant.

RESULTS

Sdc1−/− Corneas Significantly Resist S. aureus Infection Compared with WT Corneas—To determine whether syndecan-1 is an important host determinant for S. aureus corneal infection, we first compared the virulence of S. aureus in injured corneas of WT and Sdc1−/− mice on the BALB/c background. The avascular cornea is remarkably resistant to bacterial infections, but when the corneal epithelial barrier is breached, it becomes susceptible to infection by several opportunistic bacterial pathogens. S. aureus (strain 8325-4) was inoculated topically onto WT and Sdc1−/− corneas injured by a single scratch with a 29-gauge needle. The corneal bacterial burden was determined between 1 and 24 h post-infection by homogenizing the isolated corneas and plating out serial dilutions of the homogenates. The bacterial burden was similar between WT and Sdc1−/− corneas at 1 h post-infection (Fig. 1A). However, the bacterial burden was significantly reduced by 5-fold in Sdc1−/− corneas compared with WT corneas at 7 h (Fig. 1A) and reduced by 3-fold at 24 h after infection (data not shown), indicating that deletion of syndecan-1 is a gain of function mutation that enables mice to rapidly clear S. aureus in the cornea. Similar results were obtained with WT and Sdc1−/− mice on the C57BL/6J background (Fig. 1B) and also by using a different strain of S. aureus (strain P1) with WT and Sdc1−/− mice on the BALB/c background (Fig. 1C), indicating that the ability of Sdc1−/− mice to resist S. aureus corneal infection is not specific to mouse background or bacterial strain. Furthermore, uninfected corneas of WT and Sdc1−/− mice on both the BALB/c and C57BL/6J backgrounds rapidly cleared the S. aureus inoculum within 1 h post-infection (data not shown), consistent with the feature that the intact ocular surface is remarkably resistant to bacterial infections. In addition, Sdc1−/− mice show delayed corneal wound repair (12), and impairment of corneal re-epithelialization is a prominent risk factor for bacterial keratitis, but Sdc1−/− corneas nonetheless rapidly and significantly cleared S. aureus relative to WT corneas. These data suggest that potential epithelial cell defects are not contributing to the resistance seen in Sdc1−/− mice. Together, these findings indicate that either bacterial or host defense mechanisms are significantly altered in Sdc1−/− corneal tissues and suggest that syndecan-1 is an important host factor that promotes S. aureus corneal infection.

Syndecan-1 Is Not Essential in the Colonization of Corneal Epithelial Cells by S. aureus—To determine how Sdc1−/− mice resist S. aureus corneal infection, we examined if S. aureus binds to syndecan-1 and if cell surface syndecan-1 facilitates the colonization of corneal epithelial cells by S. aureus. We unexpectedly found that all three strains of S. aureus examined do not bind avidly to radiolabeled purified syndecan-1 (Fig. 2A). Compared with the binding of radiolabeled syndecan-1 to affinity beads conjugated with anti-syndecan-1 antibodies, binding to S. aureus strains was only slightly above background levels (Fig. 2A), and the low level of binding observed was not inhibited by addition of excess unlabeled syndecan-1 ectodomain or HS (data not shown). Furthermore, shRNA-mediated knockdown of syndecan-1 had no effect on S. aureus colonization of cultured mouse corneal (A6(1)) or mammary gland (NMuMG) epithelial cells (Fig. 2B). The knockdown efficiency was ~40 and 50% in A6(1) and NMuMG cells, respectively, as determined by measurement of cell surface syndecan-1 by mild trypsin digestion and dot immunoblotting of trypsinates (21, 39). Furthermore, consistent with the lack of direct interaction, purified ectodomains or HS did not affect the growth rate of S. aureus (data not shown). These data suggest that cell surface syndecan-1 does not mediate the initial attachment of S. aureus to injured corneas and are consistent with the similar bacterial burden in WT and Sdc1−/− corneas at earlier times post-infection.

S. aureus Infection Induces Syndecan-1 Shedding in Corneal Epithelial Cells—We next tested if S. aureus infection induces syndecan-1 shedding from the surface of corneal epithelial cells, and syndecan-1 ectodo-
Upon infection, the signal for cell surface syndecan-1 in corneal epithelial cells, especially in the apical compartment, was markedly diminished across the entire corneal epithelium (Fig. 3A). Steady-state syndecan-1 mRNA levels were not decreased in the cornea by *S. aureus* infection (data not shown), suggesting that syndecan-1 shedding caused the observed reduction in cell surface syndecan-1 levels. Indeed, levels of syndecan-1 ectodomains in ocular surface fluids increased rapidly and significantly upon *S. aureus* infection (Fig. 3B). Syndecan-1 ectodomains in ocular surface fluids were increased by over 10-fold at both 3 and 7 h post-treatment in groups whose corneas were injured and infected with *S. aureus* compared with those that were only injured (Fig. 3B), indicating that infection, and not injury, induces syndecan-1 shedding in corneal epithelial cells. Furthermore, the increase in syndecan-1 ectodomains was transient as it returned to near basal levels by 24 h post-infection (Fig. 3B). Notably, the kinetics of syndecan-1 shedding was associated closely with the clearance of *S. aureus* in mouse corneas. The bacterial burden was similar in WT and *Sdc1−/−* corneas at 1 h post-infection where there was minimal syndecan-1 shedding, whereas it was significantly reduced in *Sdc1−/−* corneas at 7 h post-infection where there was maximal shedding in WT corneas. Syndecan-4 ectodomain levels in ocular surface fluids also increased, but not as rapidly or significantly as syndecan-1 ectodomains (data not shown). Collectively, these observations indicate that *S. aureus* specifically induces syndecan-1 shedding in corneal epithelial cells and suggest that the lack of this mechanism enables *Sdc1−/−* mice to efficiently clear *S. aureus* in their corneas.

**Syndecan-1 Shedding Enhances *S. aureus* Virulence in Injured Corneas**—To determine the physiological relevance of syndecan-1 shedding in *S. aureus* corneal infection, we assessed the effects of administering purified syndecan-1 ectodomains and inhibiting syndecan-1 shedding. If *Sdc1−/−* mice indeed resist *S. aureus* corneal infection because they cannot shed their syndecan-1 ectodomains, then administering purified ectodomains should promote and inhibiting shedding should attenuate pathogenesis. To evaluate this hypothesis, injured *Sdc1−/−* corneas were infected with *S. aureus* or infected with *S. aureus* and topically administered 170 ng of purified syndecan-1 ectodomains or HS at 3.5 h after infection, and the bacterial burden was measured at 10 h post-infection. Syndecan-1 ectodomains and HS were applied in a delayed manner to simulate the kinetics of syndecan-1 shedding (Fig. 3B). Administration of purified ectodomains or HS significantly increased the corneal bacterial burden by 3.5-fold compared with those that were infected by *S. aureus* only and restored the wild type response to infection with *S. aureus* (Fig. 3A). Similar increases in the bacterial burden (3.5-fold) were seen when syndecan-1 ectodomains were given at an earlier time (1 h post-infection). Along with the observations that syndecan-1 does not bind to *S. aureus* and shRNA-mediated gene knockdown of syndecan-1 does not inhibit *S. aureus* colonization of cultured corneal epithelial cells, these data suggest that syndecan-1 ectodomains enhance *S. aureus* survival at the ocular surface by affecting mechanisms of the host and not those of the bacteria.
Syndecan-1 Promotes S. aureus Keratitis

We next examined the effects of inhibiting syndecan-1 shedding with GM6001, a broadly acting metalloproteinase sheddase inhibitor, on S. aureus corneal infection. Injured WT corneas were infected with S. aureus or co-infected with S. aureus and GM6001. GM6001 significantly reduced levels of syndecan-1 ectodomains in ocular surface fluids of mice co-infected with S. aureus and GM6001 compared with those infected with S. aureus only (Fig. 4B), indicating that GM6001 inhibits corneal syndecan-1 shedding in vivo. Moreover, the bacterial burden was significantly decreased by ~2-fold in mice co-infected with S. aureus and GM6001 compared with those that were infected with S. aureus only (Fig. 4C). At the dose tested, GM6001 did not affect the growth rate of S. aureus in vitro, the overall corneal morphology, or mRNA levels of syndecan-1 in the cornea (data not shown). On the basis of these findings, we propose that S. aureus subverts syndecan-1 shedding to promote its pathogenesis in injured corneas.

Neutrophil Depletion Increases S. aureus Virulence in the Resistant Sdc1−/− Corneas—To determine a potential cellular basis for the improved outcomes in Sdc1−/− mice, we next examined the effects of immunodepleting neutrophils in these mice. Neutrophils are rapidly recruited to the site of infection in bacterial keratitis and they play a key role in the clearance of bacterial pathogens (40, 41), but exaggerated or prolonged neutrophil responses can contribute to the pathogenesis of bacterial keratitis by causing excessive damage to corneal tissues. Sdc1−/− mice were injected intraperitoneally with anti-GR1 antibodies to immunodeplete neutrophils (9), and injured corneas were infected with S. aureus at 24 h after anti-GR1 injection. Consistent with previous results, the majority of S. aureus was cleared by 7 h post-infection in the corneas of Sdc1−/− mice that were pretreated with vehicle (Fig. 5A). However, the corneal bacterial burden was significantly increased in neutropenic Sdc1−/− mice to the level of WT mice at 7 h post-infection, and the high bacterial burden was sustained at 14 h post-infection (Fig. 5A). These data indicate that neutrophils are essential for Sdc1−/− mice to rapidly clear S. aureus in their corneas and to resist S. aureus corneal infection.

We next examined if neutrophil infiltration was altered in Sdc1−/− corneas. CXC chemokines, such as KC (CXCL1), MIP-2 (CXCL2), lipopolysaccharide-induced CXC chemokine (CXCL5) (42), and complement component C5a (43), are the major chemotactic factors that induce neutrophil infiltration. Syndecan-1 shedding has been shown to moderate neutrophil infiltration in injured corneas. CXC chemokines, such as KC (CXCL1), MIP-2 (CXCL2), lipopolysaccharide-induced CXC chemokine (CXCL5) (42), and complement component C5a (43), are the major chemotactic factors that induce neutrophil infiltration. Syndecan-1 shedding has been shown to moderate neutrophil infiltration in injured corneas.
Syndecan-1 Promotes *S. aureus* Keratitis

Addition of purified syndecan-1 ectodomain or HS significantly inhibited bacterial killing by 97 and 83%, respectively, whereas ectodomain devoid of HS, and bacterial killing was assessed by counting live *S. aureus* recovered from the neutrophil and *S. aureus* mixture at the end of the experiment. *Sdc1−/−* neutrophils killed a slightly higher proportion of the bacterial inoculum compared with WT neutrophils (30 versus 25%), but the difference did not reach significance (Fig. 6A), indicating that *Sdc1−/−* neutrophils do not have an inherent defect in their ability to kill *S. aureus*. These findings are consistent with previous observations that neutrophils do not express syndecan-1; WT and *Sdc1−/−* neutrophils are similar in size, granularity, and pattern of GR1 staining; *Sdc1−/−* neutrophils do not have an inherent defect in their ability to migrate; and *Sdc1−/−* mice contain normal numbers of circulating neutrophils (7, 11).

Based on these results, we next explored if syndecan-1 ectodomains enhance the survival of *S. aureus* by inhibiting the bacterial killing activity of neutrophils. Isolated neutrophils were incubated with pre-opsonized *S. aureus*, and bacterial killing was assessed by counting live *S. aureus* recovered from the neutrophil and *S. aureus* mixture at the end of the experiment. *Sdc1−/−* neutrophils killed a slightly higher proportion of the bacterial inoculum compared with WT neutrophils (30 versus 25%), but the difference did not reach significance (Fig. 6A), indicating that *Sdc1−/−* neutrophils do not have an inherent defect in their ability to kill *S. aureus*. These findings are consistent with previous observations that neutrophils do not express syndecan-1; WT and *Sdc1−/−* neutrophils are similar in size, granularity, and pattern of GR1 staining; *Sdc1−/−* neutrophils do not have an inherent defect in their ability to migrate; and *Sdc1−/−* mice contain normal numbers of circulating neutrophils (7, 11).

Based on these observations, we assessed whether chemokine expression and neutrophil infiltration were altered in infected *Sdc1−/−* corneas. Scarified WT and *Sdc1−/−* corneas were inoculated with *S. aureus*, and steady-state mRNA levels of chemokines in isolated corneas were measured. KC and MIP-2 mRNA levels were similarly induced at 7 h post-infection in both WT and *Sdc1−/−* corneas (Fig. 5B), although KC mRNA levels were slightly decreased in infected *Sdc1−/−* corneas relative to WT corneas. RANTES, a ligand of CCR5 and mediator of T cell recruitment, was slightly induced by infection but not as significantly as KC or MIP-2. These data indicate that the deletion of syndecan-1 does not increase the expression of CXC chemokines in response to *S. aureus* corneal infection.

Consistent with these findings, we found that a similar number of neutrophils are recruited to infected WT and *Sdc1−/−* corneal tissues. Eye sections prepared from WT and *Sdc1−/−* mice at 7 h after *S. aureus* infection were stained with hematoxylin and eosin or immunostained with anti-GR1 or -Mac3 antibodies. In both WT and *Sdc1−/−* mice, abundant accumulation of neutrophils and minor edema were seen mostly in the upper half of the corneal stroma toward the basal epithelial aspect (Fig. 5C). Little to no accumulation of macrophages was observed (data not shown). These results indicate that syndecan-1 does not modulate the influx of neutrophils into *S. aureus*-infected corneas.

**Syndecan-1 Ectodomain Inhibits Neutrophil-mediated Killing of *S. aureus* in an HS-dependent Manner**—Increased susceptibility to *S. aureus* infections has been linked to a decrease in neutrophil infiltration or function. Because our data indicated that syndecan-1 does not affect neutrophil infiltration, we next examined if syndecan-1 regulates neutrophil function. Isolated WT and *Sdc1−/−* neutrophils were incubated with pre-opsonized *S. aureus*, and bacterial killing was assessed by counting live *S. aureus* recovered from the neutrophil and *S. aureus* mixture at the end of the experiment. *Sdc1−/−* neutrophils killed a slightly higher proportion of the bacterial inoculum compared with WT neutrophils (30 versus 25%), but the difference did not reach significance (Fig. 6A), indicating that *Sdc1−/−* neutrophils do not have an inherent defect in their ability to kill *S. aureus*. These findings are consistent with previous observations that neutrophils do not express syndecan-1; WT and *Sdc1−/−* neutrophils are similar in size, granularity, and pattern of GR1 staining; *Sdc1−/−* neutrophils do not have an inherent defect in their ability to migrate; and *Sdc1−/−* mice contain normal numbers of circulating neutrophils (7, 11).

Based on these data, we next explored if syndecan-1 ectodomains enhance the survival of *S. aureus* by inhibiting the bacterial killing activity of neutrophils. Isolated neutrophils were incubated with pre-opsonized *S. aureus* in the absence or presence of purified syndecan-1 ectodomain, HS, or ectodomain devoid of HS, and bacterial killing was assessed. Addition of purified syndecan-1 ectodomain or HS significantly inhibited bacterial killing by 97 and 83%, respectively, whereas ectodomain devoid of HS chains had no inhibitory
One of the surprising observations from our studies was that syndecan-1 is not essential for the initial attachment of \textit{S. aureus} to the injured corneal epithelium, suggesting that other HSPGs or other matrix components mediate \textit{S. aureus} attachment to corneal tissues. Indeed, the collagen-binding adhesin has been shown to be a virulence factor in a rabbit model of soft contact lens-associated \textit{S. aureus} keratitis (48), and deletion of \textit{S. aureus} fibronectin-binding proteins A and B has been shown to reduce \textit{S. aureus} attachment and invasion of human corneal epithelial cells by 99% (49). These observations suggest that collagen and/or fibronectin, and not HSPGs, are the key host determinants that mediate the initial attachment of \textit{S. aureus} to injured corneal tissues.

We also conclude that \textit{S. aureus} takes advantage of the capacity of syndecan-1 ectodomains to inhibit neutrophil-mediated bacterial killing mechanisms by inducing syndecan-1 shedding from the surface of corneal epithelial cells. How \textit{S. aureus} induces syndecan-1 shedding in corneal epithelial cells is not understood. However, we previously reported that \textit{S. aureus} α- and β-toxin induce syndecan-1 shedding in lung epithelial and mammary gland epithelial cells (20). Furthermore, studies of \textit{S. aureus} keratitis in rabbit and mouse models showed that α-toxin mediates the majority of the virulence activities (33, 50, 51), whereas β-toxin is also important but contributes less to virulence in the cornea (33). Together, these observations suggest that \textit{S. aureus} induces syndecan-1 shedding in the cornea through α- and β-toxin, and the capacity to enhance syndecan-1 shedding and generate soluble ectodomains is an important virulence activity of these exotoxin virulence factors.

The pro-pathogenic effects of HS on \textit{S. aureus} corneal infection suggest that other HSPGs may function similarly to syndecan-1. However, because other syndecans and HSPGs are intact in \textit{Sdc1}−/− mice, the significant difference in the capacity of WT and \textit{Sdc1}−/− corneas to clear \textit{S. aureus} suggests that syndecan-1 ectodomains function specifically to increase bacterial survival. Precisely how syndecan-1 ectodomains act in this manner is not known, but previous studies have also shown that loss of syndecan-1 alone results in dramatic pathological phenotypes in animal models of various infectious and inflammatory diseases (6–11), suggesting that other HSPGs cannot compensate for the loss of syndecan-1 in \textit{vivo}. In the corneal epithelium, both syndecan-1 and -4 are expressed, but syndecan-4 is expressed at a lower level than syndecan-1. Furthermore, although syndecan-4 shedding was also induced during \textit{S. aureus} corneal infection, syndecan-4 ectodomain levels did not increase as rapidly or significantly

**FIGURE 6. Syndecan-1 ectodomain inhibits the killing of \textit{S. aureus} by neutrophils.** A, WT and \textit{Sdc1}−/− neutrophils (10⁶) were incubated with 3 × 10⁵ cfu of \textit{pre-opsonized S. aureus} Woods strain for 2 h at 37 °C. Percent bacterial killing was enumerated by plating serial dilutions of detergent lysates onto TSB agar plates (n = 6). B, WT neutrophils (10⁶) were incubated with 2 × 10⁵ cfu of \textit{pre-opsonized S. aureus} Woods strain in the absence or presence of 1 μg/ml purified syndecan-1 ectodomain, HS, or ectodomain devoid of HS for 2 h at 37 °C, and bacterial killing was determined (n = 5–6).
as those of syndecan-1. These observations suggest that the specificity of syndecan-1 functions in S. aureus corneal infection may be a reflection of its abundant expression and prominent shedding in corneal epithelial cells. Alternatively, because syndecan-1 acts in an HS-dependent manner, the potential unique structural features of syndecan-1 HS chains may mediate its specific functions in S. aureus corneal infection. However, the structure and function of syndecan-1 and -4 isolated from mammary gland epithelial cells are indistinguishable, and syndecan HS chains are considered to be cell type-specific and not core protein-specific (36), suggesting that the structure and function of syndecan-1 and -4 are also similar in the corneal epithelium. Nonetheless, these observations imply that despite similar cellular distribution and HS structures, corneal syndecan-1 and -4 have nonredundant roles in vivo. Future studies determining the response of Sdc4−/− mice in S. aureus corneal infection and analyzing the fine structure of syndecan-1 and -4 HS chains should address these issues.

Both passive and active defense mechanisms effectively protect the cornea from infections. The mechanical action of the eyelid physically removes pathogen; the outermost lipid layer of the tear film serves as a physical barrier, and the washing effects of tears also remove pathogens (41). Furthermore, the aqueous tear fluid contains a wide array of antimi-

phagocytosis-potentiating activities, although it is not known if HBP affects bacterial phagocytosis by neutrophils.

The underlying mechanisms of how syndecan-1 ectodomains inhibit the capacity of neutrophils to kill S. aureus in an HS-dependent manner remain unknown. Because syndecan-1 ectodomain does not bind to S. aureus and does not affect the viability of isolated neutrophils, ectodomains must be inhibiting key processes of neutrophils that kill bacteria. Furthermore, because our preliminary data suggest that sulfated domains in HS interfere with the killing of S. aureus by neutrophils, cationic defense factors and their mechanisms are presumably inhibited by syndecan-1 ectodomains. However, given that cell surface receptors for syndecan-1 or HS on neutrophils have not been reported and syndecan-1 ectodomains are not membrane-permeable, syndecan-1 ectodomains are likely acting on extracellular killing mechanisms of neutrophils. Activated neutrophils secrete a highly cationic heparin-binding protein (HBP, azurocidin/CAP37) that has antimicrobial activities against several bacterial pathogens, including S. aureus (54). HBP has also been shown to increase the phagocytosis of S. aureus by monocytes and macrophages (55). These observations suggest that syndecan-1 HS chains may bind to HBP and interfere with its bacterial killing and phagocytosis-potentiating activities, although it is not known if HBP affects bacterial phagocytosis by neutrophils.

Syndecan-1 Promotes S. aureus Keratitis

The underlying mechanisms of how syndecan-1 ectodomains inhibit the capacity of neutrophils to kill S. aureus in an HS-dependent manner remain unknown. Because syndecan-1 ectodomain does not bind to S. aureus and does not affect the viability of isolated neutrophils, ectodomains must be inhibiting key processes of neutrophils that kill bacteria. Furthermore, because our preliminary data suggest that sulfated domains in HS interfere with the killing of S. aureus by neutrophils, cationic defense factors and their mechanisms are presumably inhibited by syndecan-1 ectodomains. However, given that cell surface receptors for syndecan-1 or HS on neutrophils have not been reported and syndecan-1 ectodomains are not membrane-permeable, syndecan-1 ectodomains are likely acting on extracellular killing mechanisms of neutrophils. Activated neutrophils secrete a highly cationic heparin-binding protein (HBP, azurocidin/CAP37) that has antimicrobial activities against several bacterial pathogens, including S. aureus (54). HBP has also been shown to increase the phagocytosis of S. aureus by monocytes and macrophages (55). These observations suggest that syndecan-1 HS chains may bind to HBP and interfere with its bacterial killing and phagocytosis-potentiating activities, although it is not known if HBP affects bacterial phagocytosis by neutrophils.

Alternatively, syndecan-1 ectodomains may inhibit a recently described extracellular killing mechanism of neutrophils called neutrophil extracellular traps (NETs) (56). NETs are made by activated neutrophils, but not naive neutrophils, and consist of negatively charged, decondensed chromatin fibers with cationic antimicrobial factors, such as HBP, cathe-

licins, defensins, and elastase, embedded in them. NETs trap pathogens and facilitate killing by bringing the pathogens and neutrophil-derived antimicrobial factors to proximity. Importantly, impaired NET formation in vivo predisposes the host to bacterial infections (57, 58). Because functional NETs are formed by the ionic interactions between anionic chromatin fibers and cationic antimicrobial factors, the highly anionic HS chains of syndecan-1 ectodomains may bind to and displace the cationic antimicrobial factors from anionic NET fibers and inhibit NET-mediated killing of S. aureus. Altogether, these observations raise the possibility that syndecan-1 ectodomains may inhibit one of more bacterial killing mechanisms of neutrophils. Further studies will be required to elucidate the impact of syndecan-1 ectodomains on these host defense mechanisms of neutrophils in corneal tissues.

In summary, our findings extend the biological functions of syndecan-1 from that of a cell surface proteoglycan that regulates cell adhesion, proliferation, and migration to that of a soluble HSPG ectodomain capable of modulating neutrophil-mediated host defense mechanisms crucial to the clearance of S. aureus in injured corneas. Interestingly, the majority of bacterial pathogens that can induce syndecan-1 shedding in vitro are major etiological agents of bacterial keratitis in vivo (e.g. S. aureus, P. aeruginosa, and S. pneumoniae), suggesting that subversion of the capacity of syndecan-1 ectodomains to counteract neutrophil-mediated bacterial killing is a general virulence mechanism of various ocular surface pathogens. Although the normal functions of syndecan-1 in the resting cornea remain to be defined, our findings suggest a possible
beneficial role of inhibiting syndecan-1 shedding or neutralizing syndecan-1 ectodomains in treating bacterial keratitis.

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REFERENCES

1. Bernfeld, M., Götte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincecum, J., and Zako, M. (1999) Annu. Rev. Biochem. 68, 729–777
2. Rostand, K. S., and Esco, J. D. (1997) Infect. Immun. 65, 1–8
3. Bartlett, A. H., and Park, P. W. (2010) Expert Rev. Mol. Med. 12, e5
4. Park, P. W., Reizes, O., and Bernfeld, M. (2000) J. Biol. Chem. 275, 29923–29926
5. Echtermeyer, F., Streit, M., Wilcox-Adelman, S., Saoncella, S., Denhaz, F., Detmar, M., and Goetinck, P. (2001) J. Clin. Invest. 107, R9–R14
6. Haynes, A., 3rd., Ruda, F., Oliver, J., Hamood, A. N., Griswold, J. A., Rosenbloom, J. D., and Esko, J. D. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 15359–15364
7. Henry-Stanley, M. J., Hess, J. R., Montes-Rodriguez, A., Kao, W. W., and Zako, M. (1999) Invest. Ophthalmol. Vis. Sci. 40, 44–50
8. Kao, W. W., and Zako, M. (1998) J. Biol. Chem. 273, 8977–8982
9. Sanderson, R. D. (2001) Semin. Cell Dev. Biol. 12, 89–98
10. Park, P. W., Foster, T. J., Nishii, E., Duncan, S. J., Klagsbrun, M., and Chen, Y. (2004) J. Biol. Chem. 279, 251–258
11. Park, P. W., Pier, G. B., Preston, M. J., Goldberg, O., Fitzgerald, M. L., and Bernfeld, M. (2000) J. Biol. Chem. 275, 3057–3064
12. Chen, Y., Götte, M., Bennett, A. E., Hollingshead, S. K., and Park, P. W. (2007) J. Biol. Chem. 282, 159–167
13. Popova, T. G., Mills, B., Bradburne, C., Nazarenko, S., Bailey, C., Chandhoke, V., and Popov, S. G. (2006) BMC Microbiol. 6, 8–24
14. Bourcier, T., Thomas, F., Borderie, V., Chaumeil, C., and Laroche, L. (2003) Br. J. Ophthalmol. 87, 834–838
15. Limberg, M. B. (1991) Annu. J. Ophthalmol. 112, 25–95
16. Binnert, M., and Gilmore, M. S. (2002) DNA Cell Biol. 21, 397–404
17. Green, M., Apel, A., and Stapleton, F. (2008) Cornea 27, 22–27
18. Schaefer, F., Bruttin, O., Zografos, L., and Guex-Crosier, Y. (2001) Br. J. Ophthalmol. 85, 842–847
19. Ly, C. N., Pham, J. N., Badenoch, P. R., Bell, S. M., Hawkins, G., Rafferty, D. L., and McClellan, K. A. (2006) Clin. Exp. Ophthalmol. 34, 44–50
20. Li, P., Li, M., Lindberg, M. R., Kennett, M. J., Xiong, N., and Wang, Y. (2010) J. Exp. Med. 207, 1853–1862