Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Enoxaparin Improves the Course of Dextran Sodium Sulfate-Induced Colitis in Syndecan-1-Deficient Mice

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Syndecan-1 (Sdc1) plays a major role in wound healing and modulates inflammatory responses. Sdc1 expression is reduced in lesions of patients with ulcerative colitis. The aim of this study was to investigate the role of Sdc1 in murine dextran sodium sulfate (DSS)-induced colitis. DSS colitis was induced in *Sdc1*-deficient (knockout (KO)) and wild-type mice by oral administration of 3% DSS. KO mice exhibited a significantly increased lethality as compared with wild-type controls (61 versus 5%, $P < 0.05$). Impaired mucosal healing and prolonged recruitment of inflammatory cells in KO mice were accompanied by significant up-regulation of tumor necrosis factor- α , CC chemokine ligand 3/macrophage inflammatory protein-1 α , and vascular cell adhesion molecule-1, as determined by histological correlation between 0 and 15 days after colitis induction, TaqMan low-density array analysis, and quantitative real-time PCR. Treatment from days 7 through 14 with enoxaparin, a functional analogue of the Sdc1 heparan sulfate chains, significantly reduced lethality of KO mice due to DSS-induced colitis, which was correlated with improved mucosal healing. *In vitro*, Sdc1-deficient polymorphonuclear cells displayed increased adhesion to endothelial cells and intercellular adhesion molecule-1, and enoxaparin reverted adhesion to wild-type levels. Small interfering RNA-mediated knockdown of Sdc1 expression resulted in reduced basic fibroblast growth factor-mediated mitogen-activated protein kinase signaling and reduced Caco-2 cell proliferation. We conclude that Sdc1 has a protective effect during experimental colitis. The modification of missing Sdc1 function by heparin analogues may emerge as a

promising anti-inflammatory approach. (Am J Pathol 2010; 176:146–157; DOI: 10.2353/ajpath.2010.080639)

Syndecan-1 (Sdc1) is the most important representative of the heparan sulfate proteoglycans (HSPGs) covering epithelial cell surfaces.¹ It serves multiple biological roles, such as cell-matrix interactions, modulation of inflammatory responses, tumorigenesis, and wound healing.^{2–4} The highly conserved cytoplasmic domains of Sdc1 interact with scaffolding proteins and participate in integrin-mediated signaling events, thus providing a physical and functional link to the cytoskeleton. In addition, most of the extracellular-binding interactions are mediated by the heparan sulfate chains, which are structurally and functionally related to heparin, an extensively sulfated and epimerized derivative of heparan sulfate.¹ Sdc1 serves as a coreceptor for several tyrosine kinase receptors. For example, it increases the activity of the complex of basic fibroblast growth factor (bFGF) and the FGF receptor and, therefore, contributes to improved wound healing via stimulation of keratinocyte proliferation.^{1,5} A role for Sdc1 in wound repair *in vivo* has been demonstrated in Sdc1-deficient (*Sdc1*-knockout (KO)) mice, which show delayed skin and corneal wound healing⁵ and functionally adverse repair following experimental myocardial infarction due to dysregulation of chemokine expression and matrix metalloproteinase-mediated tissue remodeling.⁶ Sdc1 forms chemotactic gradients due to binding of chemokines on heparan sulfate chains of the molecule. Therefore, Sdc1 is able to act as coreceptor for chemokine signaling.^{7,8} In addition, endothelial leukocyte recruitment and extravasation is modulated by Sdc1, possibly via interference with heparin-binding adhesion molecule function.^{9–12}

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Day et al¹³ described in 1999 a reduced expression of Sdc1 in patients with ulcerative colitis, which was linked to disrupted healing of colonic ulcers. In addition, this group demonstrated the benefit of the Sdc1 ectodomain for the FGF-induced proliferation of intestinal epithelial cell lines *in vitro*. The function of Sdc1 could be restored with heparin, representing a highly sulfated and epimerized form of heparan sulfate, the major functional constituent of the Sdc1 ectodomain.

Heparin sees widespread use as anticoagulant drug based on its antithrombin III-activating properties. Enoxaparin, is a low molecular weight heparin with similar features *in vitro* and *in vivo* like heparin; however, it exhibits a more favorable pharmacological side effect profile. Both low molecular weight heparins (enoxaparin) as well as heparin were recently found to possess anti-inflammatory properties.¹⁴

The hypothesis of Sdc1 being involved in the pathogenesis of ulcerative colitis is underlined by multiple clinical observations of patients who have been treated with heparins for different reasons.¹⁵ In a number of cases, this treatment has lead to an improved course of disease. A limited number of uncontrolled clinical trials with heparins in the treatment of low to medium active ulcerative colitis showed a variable outcome,^{16–18} which may be explained by variations in treatment regimes that may have failed to include the optimal dose, class of heparin, and mode of delivery. For example, most studies have involved either i.v. or s.c. delivery of heparin, whereas a more appropriate mode of delivery for stimulating mucosal healing might be the topical application or microsphere-mediated delivery of heparin.^{14,15} Furthermore, the outcome of heparin therapy may depend on the degree to which Sdc1 expression is reduced in inflammatory bowel disease (IBD) patients.¹⁹

Moreover, the expression of Sdc1 and the proinflammatory cytokine tumor necrosis factor- α (TNF- α) are inversely correlated in the colonic mucosa of patients with Crohn's disease,²⁰ and a reduction of Sdc1 expression has been shown to result in increased TNF- α signaling in an *in vitro* model of protein-losing enteropathy,^{19,21} further suggesting a regulatory role for Sdc1 in proinflammatory cytokine signaling.

In this study, our goal was to characterize the impact of a Sdc1 deficiency on the dextran sodium sulfate (DSS)-induced colitis of the mouse. Furthermore, the efficacy of low molecular weight heparin to restore altered wound healing was studied *in vivo*. In addition, *in vitro* trials were performed to study the role of Sdc1 deficiency in the adhesion and transmigration of leukocytes under inflammatory conditions.

Materials and Methods

DSS Colitis and Enoxaparin Treatment

Sdc1-deficient (*Sdc1* KO) mice on a C57BL/6 background^{5,12} and control wild-type mice were obtained from the clinical research laboratory of the Department of Gynecology, University of Muenster. Mice were bred, housed, and handled according to the guidelines of the local animal ethics committee (number A101/2005). Twelve-week-old male mice were placed in the Central

Animal Facility of the University Hospital of Muenster with a 12-hour day/night light cycle and standard chow and water *ad libitum*. After 3 weeks of adaptation, the trials were performed as follows: starting on day 1 and continued to day 6 an oral administration of 3% DSS in drinking water was administered. On day 7, the water was switched to normal drinking water. Of 92 mice used in this study, 10 male wild-type mice and 10 *Sdc1* KO mice were used to study the course of disease. The course of disease was monitored by daily weight measurement and a daily assessment of blood in stool with a commercially available stool test (Hemoccult; Beckman Coulter, Fullerton, CA).

Thirty-six wild-type mice and 36 *Sdc1* KO mice were used for histological evaluation and for real-time PCR analysis of colon tissue divided in a treatment group of 18 mice and a control group of another 18 mice. The treatment group received an i.p. injection of enoxaparin (500 IU/kg/body weight) from days 7 to 14 (control group: 0.9% NaCl) once per day. Three animals out of each group were sacrificed for further histological examination on days 0, 3, 6, 9, 12, and 15.

Histological Examination

After euthanasia of animals, the proximal, medial, and distal colon was resected after laparotomy. The colon was flushed with PBS and covered with optimal cutting temperature Tissue-Tek (Sakura). The tissue was snap frozen in liquid nitrogen and stored at -80°C until use. Seven-micrometer frozen sections were cut, and standard H&E staining was performed as described previously.²² Severity of the colitis was assessed by an established scoring system as published before.²³ For immunohistochemical analysis, the following primary antibodies were used: Sdc1 (rat anti-mouse Sdc1 mAb clone 281-2, diluted 1/100 in PBS with 1% BSA; BD Pharmingen, Heidelberg, Germany), Sdc2 (rabbit-anti-mouse, diluted 1/50; Santa Cruz Biotechnology, Santa Cruz, CA), Sdc3 (rat-anti-mouse mAb clone 312607, diluted 1/50; R&D Systems, Wiesbaden, Germany), Sdc4 (rat-anti-mouse clone KY/8.2, diluted 1/50; BD Pharmingen), F4/80 (rat-anti-mouse, clone BM8; eBioscience, San Diego, CA), CD4, CD8, and B220 (rat anti-mouse, clone C3T4 (CD4), clone 53-6.7 (CD8), and clone RA3-6B2 (B220), diluted 1/100; BD Pharmingen). M cells were stained with the UEA1 lectin method (Sigma-Aldrich, Deisenheim, Germany) as described elsewhere.²³

Briefly, the sections were blocked with 10% goat serum or 1% BSA (Dako, Glostrup, Denmark) in PBS for 30 minutes. Incubation with the primary antibody was performed in a humid chamber overnight at 4°C . After three washes with PBS (5 minutes), the secondary antibody (dilution 1/1000 in 10% goat serum/PBS) was incubated for 1 hour at room temperature. For immunofluorescence microscopy, Alexa Fluor-labeled secondary antibodies were used (Invitrogen, Karlsruhe, Germany), while for conventional immunohistochemistry, the Vectastain ABC system (Vector Laboratories, Burlingame, CA) or the Dako EnVision system (Dako) were used, depending on the primary antibody used. For immunofluorescence microscopy, sections were washed again in PBS for 5 min-

utes, air-dried, and treated with prolong antifade kit (Invitrogen). For conventional immunohistochemistry, 3-amino-9-ethylcarbazole substrate (Dako) was used for color development, followed by Mayer's Hemalum counterstaining (Merck, Darmstadt, Germany). Pictures were taken with a Leica or Zeiss Axioscope microscope, and digital cameras directly connected to a PC system. Images were processed with Adobe Photoshop version 7.0 and Zeiss Axiovision software.

Flow Cytometry

To study the composition of intestinal lymphoid subpopulations, flow cytometry was performed.²⁴ Briefly, Peyer's patches (PPs) and lamina propria lymphocytes from the colon were extracted and analyzed by flow cytometry. All PPs were counted and resected from the intestine. PPs were homogenized mechanically with a Falcon cell mesh (pore size, 70 μ m; BD Pharmingen) and mixed with ice-cold RPMI 1640 medium and 10% fetal calf serum. After centrifugation at $10,000 \times g$ for 5 minutes, the supernatant was discarded, and the pellet was washed with ice-cold PBS. This washing/centrifugation step was performed 3 times. The pellet was resuspended in ice-cold PBS and added to Ficoll (Genaxxon Bioscience, Biberach, Germany). After centrifugation at $5000 \times g$ for 15 minutes, the layer containing the leukocytes was retrieved and placed into a fluorescence-activated cell sorting tube. After a washing step with PBS and centrifugation, the pellet was incubated with an antibody for surface markers CD4 (direct fluorescein isothiocyanate-conjugated mAb, clone C3T4; BD Pharmingen), CD8 (direct phycoerythrin-conjugated mAb, clone 53-6.7; BD Pharmingen), and B220 (direct allophycocyanin-conjugated mAb, clone RA3-6B2; BD Pharmingen). Furthermore, in lamina propria lymphocyte populations, the markers CD3 (clone 145-2C11), CD11c (clone HC3), CD25 (clone 7D4), CD127 (clone A7R34), and Gr-1 (clone RB6-8C5) (all antibodies purchased from BD Pharmingen) were examined. Fluorescence-activated cell sorting analysis was performed with a FACSCalibur flow cytometer from BD Biosciences (Heidelberg, Germany).

Analysis of Cell Proliferation and bFGF-Dependent Signal Transduction in Sdc1 Silenced Caco-2 Cells

The human colon carcinoma cell line Caco-2 (German Collection of Microorganisms and Cell Cultures, Department of Human and Animal Cell Cultures, Braunschweig, Germany) was cultured in minimal essential medium (Invitrogen) containing 10% fetal calf serum, 1% penicillin/streptomycin and 1 mm of L-glutamine. Small interfering RNA (siRNA)-mediated knockdown of Sdc1 expression was performed exactly as previously described,²⁵ using siRNA numbers 12527 and 12432 (Ambion, Cambridgeshire, UK) targeting the coding region of Sdc1 and a negative control siRNA (number 301698; Qiagen, Hilden, Germany) at 40 nmol/L and Dharmafect reagent (Dharmacon, Lafayette, CO). Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bro-

mid assay essentially as previously described,²⁶ using 5000 Caco-2 cells/well plated 2 days after Sdc1 silencing for an incubation period of 3 days. bFGF-mediated mitogen-activated protein kinase (MAPK) activation was studied 72 hours after Sdc1 knockdown. Silenced serum-starved Caco-2 cells were stimulated with 20 nmol/L bFGF (R&D Systems, Wiesbaden, Germany) for 0, 10, or 20 minutes, respectively, and cell extracts were subjected to SDS-PAGE and Western blotting for phosphorylated and total p44/42 MAPK using rabbit polyclonal antibodies (Cell Signaling Technology, Beverly, MA) exactly as described previously.²⁵ All experiments were performed at least three times.

Static Leukocyte-Endothelial Cell Adhesion Assay

Polymorphonuclear (PMN) cells were purified from the bone marrow of Sdc1-deficient and wild-type mice by Histopaque (Sigma-Aldrich) gradient centrifugation as previously described,¹¹ and leukocyte adhesion to the murine bEnd.3 endothelial cell line, derived from viral oncogene-immortalized brain endothelial cells²⁷ was measured under static conditions. Briefly, PMN cells were fluorescently labeled with 2,7-bis-(2 carboxyethyl)-5-carboxyfluorescein acetoxymethyl ester (Invitrogen), washed, and incubated in sextuplets (2×10^6 cells/ml, 50 μ l/well) with confluent bEnd.3 monolayers in 96-well plates (10 minutes, 37°C). For inhibition experiments, PMN cells were preincubated for 30 minutes with 10 or 25 μ g/ml enoxaparin, followed by incubation in the presence of the drug as described above. After three washes with PBS, adhering cells were lysed, and the fluorescence signal was quantified in a Spectramax fluorimeter (excitation, 485 nm; emission, 535 nm). Statistical analysis was performed using Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

Intercellular Adhesion Molecule-1 Adhesion Assay

Adhesion of Sdc1-deficient and wild-type mouse PMN cells to recombinant Intercellular adhesion molecule-1 (ICAM-1) was performed essentially as described.²⁸ Briefly, 10 μ g/ml recombinant murine ICAM-1 protein in PBS/1% fetal calf serum was coated onto 96-well flat-bottom plates (Maxisorp; Nunc, Wiesbaden, Germany). Murine PMN cells, prepared as described above, were added and incubated at a concentration of 3×10^5 cells/well for 45 minutes at 4°C to allow for cell-ICAM-1 interactions. For inhibition experiments, PMN cells were preincubated for 30 minutes with 10 or 25 μ g/ml enoxaparin, followed by incubation in the presence of the drug as described above. Plates were washed four times with PBS, and adherent cells were fixed using 1% paraformaldehyde (w/v) in PBS. Adhering cells were observed at 32-fold magnification using a Zeiss Axioskop microscope (Zeiss, Göttingen, Germany) equipped with a charge-coupled device camera. Cell numbers were quantified

and expressed as cells/mm² with the assistance of Zeiss Axiovision imaging software. Statistical analysis was performed using Student's *t*-test. A value of *P* < 0.05 was considered statistically significant.

Selectin-Binding Assay

Granulocytes were incubated with 10 µg/ml E- and P-selectin-Fc proteins consisting of the extracellular parts of the murine selectins and the Fc portion of human IgG essentially as described previously.^{29,30} Human IgG1 (Sigma-Aldrich) served as negative control. Before protein incubation, unspecific binding was blocked by incubation with 10 µg/ml Fc block 2.4G2 (BD Pharmingen). Phycoerythrin-conjugated donkey-anti-human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) served to visualize binding using a FACSCanto flow cytometer and FACSDiva software (BD Biosciences).

Quantitative Real-Time PCR and Low-Density TaqMan Microarray Analysis

Animals were sacrificed, and the medial to distal colon was resected. Colon tissue was immediately placed in RNAlater solution (Ambion, Huntington, UK), followed by preparation of total RNA using the RNeasy kit (Qiagen). Five hundred nanograms of total RNA was transcribed using the high-capacity cDNA synthesis kit (Applied Biosystems). cDNA corresponding to 0.5 ng of total RNA was used as a template in the PCR consisting of ABI MasterMix (Applied Biosystems, Darmstadt, Germany) and predesigned TaqMan gene expression systems (Applied Biosystems) or the TaqMan Low-Density Mouse Immune Panel (ABI number 4367786), according to the manufacturers instructions. PCR was performed using 7900HT Fast and 7300 Real-Time PCR Systems (Applied Biosystems). For detection of *Sdc1* to *Sdc4*, P-Selectin, TNF-α, vascular cell adhesion molecule-1 (VCAM-1), ICAM-1, CC chemokine ligand (CCL)3/macrophage inflammatory protein-1α (MIP-1α), CCL2/ monocyte chemoattractant protein-1, interleukin-6 mRNA, primers Mm00448918_m1 (*Sdc1* exon 2–3), Mm00484718_m1 (*Sdc2* exon 4–5), Mm01179831_m1 (*Sdc3* exon 2–3), Mm00488527_m1 (*Sdc4* exon 4–5), Mm 00441295_m1 (P-Selectin exon 8–9), Mm00443258_m1 (TNF α exon 1–2), Mm00449197_m1 (VCAM-1 exon 5–6), Mm00516023_m1 (ICAM-1 exon 2–3), Mm00441242_m1 (CCL2 exon 1–2), Mm00441258_m1 (CCL3 exon 1–2), and Mm00446190_m1 (interleukin-6 exon 2–3) were used and normalized to the expression of mammalian 18S rRNA (Hs99999901_s1, all primers from Applied Biosystems). Quantitative real-time PCR was performed using the ABI PRISM 7300 Sequence Detection System (Applied Biosystems) by using the default thermal cycling conditions (10 minutes at 95°C and then 40 cycles of 15 seconds at 95°C plus 1 minute at 60°C). Relative quantification was performed using the comparative cycle threshold method. For low-density array analysis 2 (*Sdc1* KO DSS), or 3 (*Sdc1* KO control, wild-type control, DSS) biological replicates were used. For quantitative real-time PCR, 5–9 biological replicates were used. For statistical analysis

of ΔCt values, Student's *t*-test was used. A value of *P* < 0.05 was considered statistically significant.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling Assay

The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay was used on frozen sections of the colon according to the manufacturers manual (TdT-fragEL; Calbiochem, San Diego, CA). The apoptotic cells were visualized by using the horseradish peroxidase reaction with diaminobenzidine. Hematoxylin was used as counterstaining. For negative controls, distilled H₂O was substituted for the TdT during the labeling step.

In Vivo BrdU labeling

5-Bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich, Munich, Germany) was used to visualize DNA replication in epithelial cells. BrdU (50 mg/kg body weight) was given i.p. 24 hours before resection of the colon. The resected colon was embedded in Tissue-Tek optimal cutting temperature compound (Sakura-Fintek, Torrance, CA) and immediately frozen in liquid nitrogen. BrdU was visualized on cryosections by a monoclonal antibody against BrdU conjugated with biotin, followed by incubation with streptavidin-horseradish peroxidase and 3-amino-9-ethylcarbazole substrate (BrdU *in situ* detection kit; BD Pharmingen). Sections were counterstained with hematoxylin before microscopy. Quantitative analysis of BrdU labeling was performed by determining the number of BrdU-positive cells per well-defined crypt, excluding cross-sectioned crypts from the analysis. A minimum of 200 crypts from three independent experimental series were analyzed per treatment group.

Statistical Analysis

For statistical analysis of body weight, average body weight and SD were determined for each day. To test for significance of the results, Student's *t*-test was applied when data were normally distributed; otherwise, the Mann-Whitney *U*-test was applied using Sigma Stat 3.1 statistical software. Survival of animals was evaluated with the log-rank test. Values of *P* < 0.05 were considered significant.

Results

Effects of Enoxaparin in DSS Colitis of *Sdc1*-Deficient Mice

Murine colitis was induced by application of 3% DSS in drinking water for 6 days. The average body weight of the animals was 22 g (+/– 1 g) at the beginning of the experiment before starting DSS. Weight loss was observed, starting on days 4 to 5 of DSS application. Enoxaparin or 0.9% NaCl as placebo was used in a therapeutic setting starting on day 7 when DSS treatment was stopped.

Figure 1. **A:** Course of DSS colitis indexed as weight loss in wild-type (WT) mice receiving either 0.9% saline or enoxaparin (500 IE/kg/body weight/once per day). **B:** Course of DSS colitis in *Sdc1* KO mice receiving either 0.9% saline or enoxaparin (500 IE/kg/body weight/once per day). **C:** Survival rates of wild-type mice with DSS colitis. **D:** Survival rates of KO mice with DSS colitis. * $P < 0.05$. Arrows indicate the start of enoxaparin application.

We compared four groups of animals in terms of weight loss, survival, as well as blood loss via the stool as parameters of disease progression. At first we evaluated the effect of enoxaparin treatment on the course of colitis in wild-type mice. Both groups showed similar weight loss without significant changes in the weight loss curves between enoxaparin-treated and placebo-treated wild-type groups (Figure 1A). The onset of hematochezia was seen on day 3 and persisted until day 10 after colitis induction with DSS in wild-type mice. Enoxaparin-treated wild-type mice suffered from hematochezia until day 12 after DSS application. The onset of hematochezia in *Sdc1* KO mice DSS colitis started on day 3 and lasted until day 12 after DSS induction. Enoxaparin-treated *Sdc1* KO mice did not show a prolonged rectal bleeding past day 12 (data not shown).

On day 15, 95% of wild-type mice treated with 0.9% NaCl survived colitis, whereas 80% of wild-type mice receiving enoxaparin survived the course of disease (Figure 1C). In summary, there was no statistical significance regarding survival rates between these two groups, but a tendency toward a poorer outcome of enoxaparin-treated animals.

Second, we induced DSS colitis in *Sdc1* KO mice in the same way as it was performed in wild-type mice. Weight loss started around 24 hours earlier as compared with wild-type mice. Because of the bias of higher lethality, the weight curve is similar to the weight curve of wild-type mice without significant changes (Figure 1B). Interestingly, the onset of regaining weight was seen earlier in enoxaparin-treated *Sdc1* KO mice compared with

placebo-treated *Sdc1* KO mice. Statistically significant benefit for *Sdc1* KO mice treated with enoxaparin was seen for survival rates (Figure 1D). *Sdc1* KO mice treated with 0.9% NaCl had a poor outcome with only 40% survival on day 15, whereas *Sdc1* KO animals treated with enoxaparin had a survival rate of ~80% ($P < 0.05$ by log-rank test). *Sdc1* KO mice weight data interfered with the accuracy of the weight data due to the high lethality of *Sdc1* KO mice and the increased survival of wild-type mice during DSS colitis. In summary, enoxaparin treatment significantly improves the outcome of DSS colitis in *Sdc1* KO mice mainly due to an improved recovery phase with little difference in the induction phase.

Enoxaparin Treatment Improves Intestinal Wound Healing in *Sdc1*-Deficient Mice during DSS-Induced Colitis

To evaluate the mechanism of the observed effect of enoxaparin in *Sdc1* KO mice, a histology-based analysis of the colon was performed. Animals from all four groups were analyzed after DSS exposure and sacrificed on days 3, 6, 9, 12, and 15, respectively. The colon was removed, and specimens from the proximal, middle, and distal colon were examined.

To characterize the inflammatory process we used a histology scoring system in a blinded fashion.²³ Briefly, inflammatory activity was characterized by the severity of inflammatory damage and destruction of cytoarchitecture of the gut (loss of epithelial cells (grade 1)

Figure 2. A: Representative histology of KO mice with placebo (first row) and enoxaparin treatment (third row) compared with normal wild-type (WT) DSS colitis (second row) and wild-type treated with enoxaparin (fourth row). H&E staining, magnification, $\times 200$. **B:** Inflammatory changes indexed as histology score. **C:** Leukocyte infiltration score. * $P < 0.05$.

versus large ulcerations penetrating deep muscular layers (grade 4)) as well as the amount of leukocyte infiltration (eg, epithelial infiltration (grade 1) versus transmural infiltration (grade 4)).

On day 9, placebo-treated *Sdc1* KO mice showed a strong epithelial disintegration with ulcerations, edema, muscular thickening, and wide areas of epithelial denudation; nearly no intact mucosa was seen (Figure 2A). Colonic tissue from placebo and enoxaparin-treated wild-type mice had less severe signs of epithelial damage with ulcerations and edema; epithelial healing on day 9 was detectable. On day 15, histological changes in placebo-treated *Sdc1* KO mice persisted, whereas wild-type mice (both placebo- and enoxaparin-treated wild-type mice) and *Sdc1* KO mice treated with enoxaparin almost completely recovered (Figure 2B). We also specifically evaluated the amount of leukocyte infiltration (Figure 2C); however, no significant changes were seen among the four groups with one exception: *Sdc1* KO mice treated with placebo showed a persistent leukocyte infiltration on day 15. Data suggest that enoxaparin might substitute for the loss of some of the functions induced by *Sdc1* KO regarding the epithelial integrity and leukocyte infiltration.

BrdU labeling of *Sdc1* KO mice undergoing DSS colitis confirmed a lack of regeneration on day 6 compared with wild-type mice undergoing DSS colitis (Figure 3A). In contrast, epithelial repair was considerably delayed in *Sdc1* KO mice, taking place at day 15, when repair was complete in wild-type mice (Figure 3B). The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay (Figure 3C) showed that apoptosis in the area of the epithelium persisted in *Sdc1* KO mice on day 15 compared with wild-type mice during DSS colitis. No

differences were seen between both groups on days 1 and 6.

Expression Analysis of *Sdc1* and Inflammation-Related Genes in DSS Colitis

We next investigated the expression of *Sdc1* mRNA in colon samples of wild-type control and DSS-treated mice. Quantitative real-time PCR analysis revealed a slight reduction of *Sdc1* expression during DSS colitis (not significant, $P = 0.61$) (Figure 4, A and B; also Supplemental Table 1, see <http://ajp.amjpathol.org>). At the mRNA level, *Sdc1* was the most abundantly expressed member of the syndecan family in unchallenged wild-type colon tissue (Figure 4A). Immunohistochemistry confirmed a primarily epithelial staining of *Sdc1* in wild-type mice during DSS colitis (Supplemental Figure S1, see <http://ajp.amjpathol.org>). No increase in *Sdc2* to *Sdc4* immunostaining and mRNA expression was observed in *Sdc1* KO compared with wild-type mice, suggesting the absence of a potential compensatory up-regulation of other syndecan family members. To further characterize the influence of *Sdc1* on intestinal inflammation, we also studied mRNA expression of 93 inflammation-related genes using TaqMan microfluidic card low-density PCR array technology. About 25% of the investigated genes were either not expressed, or at the limit of detection, whereas ~50% of the genes were not consistently differentially expressed between *Sdc1* KO and wild-type mice. However, a number of relevant proinflammatory genes were up-regulated in colon tissue of DSS-treated *Sdc1*-deficient compared with wild-type mice (Figure 5, A–D; also

Figure 3. Altered cell proliferation and apoptosis in Sdc1 KO mice subjected to DSS colitis. **A:** BrdU and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. A delay in epithelial repair is observed in Sdc1 KO mice versus wild-type (WT) mice. Prolonged epithelial apoptosis is detectable in KO mice until day 15. Representative immunohistochemical stainings of BrdU-positive cells at day 1, day 6, and day 15, $\times 400$. **B:** Quantitative analysis of BrdU-positive cells per well-defined colonic crypt. ** $P < 0.01$. **C:** Positive and negative controls of the TUNEL staining.

Supplemental Table 1, see <http://ajp.amjpathol.org>). Among these genes the expression of six identified relevant targets (P-selectin, TNF- α , VCAM-1, macrophage inflammatory protein-1 α (CCL3/MIP-1 α), monocyte chemoattractant protein-1 (CCL2/MCP-1), and interleukin-6) was further investigated in a larger sample collective ($n = 9$) by real-time PCR. Compared with wild-type controls the expression of the proinflammatory cytokine TNF- α , the chemokine CCL3/MIP-1 α , and of the leukocyte adhesion molecules P-selectin and VCAM-1 was up-regulated 3- to 60-fold in DSS-treated Sdc1-deficient colon tissue compared with wild-type con-

trols ($P < 0.05$) suggesting that syndecan might also influence intestinal leukocyte trafficking. A differential regulation of monocyte chemoattractant protein-1, IL-6, and of the adhesion molecule ICAM-1 could not be independently confirmed by quantitative PCR (Supplemental Table 1, see <http://ajp.amjpathol.org>, and results not shown).

Enoxaparin Inhibits Increased Adhesion of Sdc1-Deficient PMN Cells to Endothelial Cells and to the Adhesion Molecule ICAM-1

Since leukocyte infiltration during DSS colitis was increased in Sdc1-deficient compared with wild-type mice, we next investigated leukocyte-endothelial interactions of PMN cells from these mice in a static adhesion assay. Adhesion of Sdc1-deficient PMN cells to the murine bEnd.3 endothelial cell line was increased by 25% ($P < 0.05$) compared with wild-type control PMNs (Figure 6A). Although treatment of wild-type PMN cells with enoxaparin (10 to 25 $\mu\text{g/ml}$) did not alter leukocyte-endothelial interactions (Figure 6B), enoxaparin treatment reduced endothelial adhesion of Sdc1 KO PMN cells by $\sim 35\%$ ($P < 0.05$), indicating a role for the heparan sulfate chains of Sdc1 in the adhesion process (Figure 6C). We next aimed at identifying the target molecule that may mediate increased ligand binding and leukocyte adhesion in Sdc1-deficient PMN cells. Previous data from our laboratory suggested that ICAM-1 may be a prime candidate in this process,^{2,9,12} and pharmacological inhibi-

Figure 4. Expression of Sdc1 to Sdc4 in wild-type (WT) and Sdc1 KO mice subjected to DSS colitis. **A** and **B:** Quantitative real-time PCR analysis of Sdc1 to Sdc4. Sdc1 KO and WT mice were treated with saline (control) or DSS for 7 days, respectively. mRNA was prepared from colon samples of five to nine mice, transcribed into cDNA, and subjected to quantitative real-time PCR analysis. The fold change of the mean C_t values, as calculated by the $2^{-\Delta\Delta C_t}$ method, is shown (Supplemental Table S1, see <http://ajp.amjpathol.org> for C_t values and SD). No significant differences in the expression of Sdc2 to Sdc4 were observed between WT and Sdc1 KO mice (Supplemental Figure S1, see <http://ajp.amjpathol.org> for immunohistochemical investigation of Sdc1 to Sdc4).

Figure 5. Quantitative real-time PCR analysis of P-selectin, TNF- α , MIP-1 α /CCL3, and VCAM-1 expression. Sdc1 KO and wild-type (WT) mice were treated with saline (control) or DSS for 7 days, respectively, mRNA was prepared from colon samples of five to nine mice, transcribed into cDNA, and subjected to quantitative real-time PCR analysis. **A:** Expression of P-selectin. **B:** Expression of the proinflammatory cytokine TNF- α . **C:** Expression of the chemokine MIP-1 α /CCL3. **D:** Expression of the vascular adhesion molecule VCAM-1. The fold change of the mean C_t values, as calculated by the $2^{-(\Delta\Delta C_t)}$ method, is shown (Supplemental Table 1, see <http://ajp.amjpatbol.org> for C_t values and SD). * $P < 0.05$ versus WT control.

tion of ICAM-1 has been shown to ameliorate DSS colitis.³¹ Thus, we performed *in vitro* adhesion assays of Sdc1 KO and wild-type PMN cells to recombinant murine ICAM-1 (Figure 6D). Compared with controls, Sdc1 KO PMN cells displayed ~45% increased adhesion to ICAM-1. Of note, similar to our PMN-endothelial adhesion assays, enoxaparin was able to decrease ICAM-1 adhesion of Sdc1-deficient PMN cells to wild-type levels (Figure 6D). Because the altered ability of granulocytes to bind to endothelium may also be caused by changes in interactions with endothelial selectins, we analyzed binding of soluble E- and P-selectin proteins to granulocytes. Wild-type and Sdc1-deficient granulocytes bound to E-selectin and P-selectin with similar efficiency (Figure 6E). Moreover, fluorescence-activated cell sorting analysis revealed no difference in the expression of the integrin

subunits CD11a, CD11b, and CD49, and of the selectin ligand P-selectin glycoprotein ligand-1 between Sdc1 KO and wild-type neutrophils (results not shown).

Normal Gut-Associated Lymphatic Tissue Composition in Sdc1-Deficient Mice

To test whether Sdc1-deficient mice exhibit an overall disturbed gut-associated lymphatic tissue, we examined the complete small bowel, colon, PPs, and spleen by H&E staining of frozen sections. No obvious changes could be detected. Moreover, we analyzed lymphocyte subsets within the lamina propria and within PPs as well as within the spleen of wild-type and Sdc1 KO mice. Flow cytometric analysis showed similar amounts of B cells,

Figure 6. *In vitro* leukocyte adhesion assays and Selectin binding assays. **A–D:** PMNs were purified from the bone marrow of Sdc1-deficient and wild-type (WT) mice and subjected to static adhesion assays to bEnd3 endothelial cells (**A–C**) or immobilized ICAM-1 (**D**) as described in *Materials and Methods*. Sdc1-deficient PMNs display increased adhesion to endothelial cells and ICAM-1, which can be decreased to WT levels by enoxaparin. Enx = enoxaparin treatment. **E:** Selectin-binding assay. WT and Sdc1-deficient (Sdc1 KO) granulocytes were incubated with E- and P-selectin Fc chimeric proteins, respectively. Fluorescence obtained with a secondary anti-Fc antibody was measured on a flow cytometer. * $P < 0.05$ (**A–C**).

CD4 T cells, and CD8 T cells (Supplemental Figure S2, see <http://ajp.amjpathol.org>). Furthermore, no differences for Gr1-positive granulocytes, CD11c dendritic cells and CD25 or CD127-positive T cells were found. In addition, we were not able to find differences in the architecture of PPs between Sdc1 KO and wild-type animals. For example, the localization of B cells and T cells within the PP was not affected. The appearance of M cells in the follicle-associated epithelium of Sdc1 KO mice was comparable with that in wild-type animals. Immunohistochemical staining of macrophages in both Sdc1 KO and wild-type colon did not show differences (Supplemental Figure S2, see <http://ajp.amjpathol.org>).

siRNA-Mediated Knockdown of Sdc1 in Caco-2 Cells Results in Decreased bFGF-Mediated MAPK Signaling and Reduced Proliferation

Since the coreceptor function of Sdc1 for receptor tyrosine kinases^{1,25} may have contributed to the epithelial repair phenotype of Sdc1 KO mice, we investigated the role of Sdc1 in bFGF-mediated MAPK signal transduction and in colonic epithelial cell proliferation *in vitro*. siRNA-mediated silencing of Sdc1 in Caco-2 cells resulted in an >50% reduction of Sdc1 mRNA expression (Figure 7A). Low Sdc1 expression was associated with a significant reduction of cell proliferation (Figure 7B). Of note, bFGF-mediated activation of p44/42 MAPK signaling was induced in control siRNA-treated Caco-2 cells already 10 minutes after bFGF stimulation, whereas no activation was observed in Sdc1-silenced cells, confirming a coreceptor role for Sdc1 in bFGF-mediated activation of colonic epithelial cell proliferation.

Discussion

Sdc1 and DSS Colitis

The DSS colitis of the mouse is the most established and understood model of intestinal inflammation.³² In this well-studied model, a chemical disruption of the epithelial barrier leads to alterations of the crypts, ulcerations and neutrophil infiltration in a highly reproducible setting. TNF- α plays a crucial role in this model as demonstrated by elevated levels of TNF- α ³³ and by the benefit of TNF- α blockage in animal and human disease models.^{34,35} Furthermore, disruption of the epithelial barrier leads to the production of cytokines and migration of inflammatory cells via activated endothelial cells. ICAM-1 plays a vital role in this inflammatory setting.³⁶ Our data demonstrate differentially increased TNF- α expression in *Sdc1* KO mice during DSS colitis. Stimulation of epithelial cells with the proinflammatory cytokine TNF- α induces Sdc1 down-regulation and results in its shedding after cleavage by matrix metalloproteinases, thereby enhancing cellular migration.³⁷ Reduced Sdc1 expression is correlating with increased TNF- α levels and activity.^{19–21} Increased recruitment of TNF- α -producing leukocytes in *Sdc1* KO mice emerges as a major mechanistic aspect of this

Figure 7. siRNA knockdown results in decreased Caco-2 cell proliferation and reduced bFGF-dependent MAPK signaling. Caco-2 cells were subjected to siRNA mediated knockdown of syndecan-1 mRNA expression as described in the Methods section. **A:** Quantitative real-time PCR analysis confirms successful knockdown of Sdc1 expression by >50% ($n = 3$, $P < 0.01$). **B:** Cell proliferation is significantly reduced in Sdc1-silenced Caco-2 cells ($n = 3$, $P < 0.001$). **C:** Western blot analysis of bFGF-mediated p44/42 MAPK activation in Sdc1-silenced Caco-2 cells. Caco-2 cells were transfected with a control siRNA or a Sdc1 siRNA construct and serum-starved for 24 hours after transfection. Cells were treated ± 20 nmol/L bFGF for 0, 10, or 20 minutes, lysed, and analyzed by Western blotting for phospho-p44/42 MAPK and total p44/42 MAPK.

phenotype, since Sdc1 modulates leukocyte recruitment and the inflammatory response in experimental models: in *Sdc1* KO mice subjected to contact allergies, an increased expression of ICAM-1 relative to wild-type mice was found.^{2,12} In addition, a differentially increased leukocyte-endothelial interaction was observed *in vitro* and *in vivo* following i.p. TNF- α stimulation.^{9,10} Our data suggest that persistent leukocyte infiltration may have been induced by increased adhesion of Sdc1-deficient PMNs to endothelial cells and ICAM-1, concomitant with an alteration of chemokine, proinflammatory cytokine, and vascular adhesion molecule expression, such as TNF- α , P-selectin, VCAM-1, and CCL3. The migration of leukocyte subsets within the intestine was not significantly affected under noninflammatory conditions. CCL3 has been shown to be up-regulated in the acute inflammation phase of DSS colitis in C57BL/6 mice³⁸ and in the colon tissue of IBD patients.³⁹ Thus, differential up-regulation of CCL3 expression in *Sdc1* KO mice as a secondary consequence of increased leukocyte recruitment may have promoted exaggerated inflammation. VCAM-1 was differentially up-regulated in *Sdc1* KO mice during DSS colitis.

Moreover, we could identify increased interaction of Sdc1-deficient neutrophils with ICAM-1 as a molecular mechanism of increased leukocyte influx leading to an overshooting inflammatory reaction as well as to a failure to resolve the leukocyte infiltrate and potentially to increased lethality of Sdc1-deficient mice in the DSS colitis model. Recent results suggest that Sdc1 modulates the direct interaction of ICAM-1 with its integrin counterreceptor, because CD18-blocking antibodies are able to reduce increased binding of Sdc1 KO PMN cells to ICAM-1.¹² In contrast to ICAM-1, no difference in E-selectin and P-selectin binding was observed between Sdc1 KO and wild-type neutrophils. These findings are in accordance with previous intravital microscopy data, suggesting that integrin-mediated tight leukocyte adhesion, rather than selectin-dependent leukocyte rolling, is primarily affected in Sdc1 KO mice.¹⁰ Although heparin and heparan sulfate can modulate endothelial leukocyte recruitment *in vivo* at the level of selectin-mediated rolling,⁴⁰ a later step of leukocyte recruitment appears to be affected in Sdc1 KO mice.

Sdc1 and Intestinal Inflammation

In this report, we were able to show that Sdc1 is essentially involved in controlling intestinal inflammation, because Sdc1-deficient mice exhibit a significantly worsened course of DSS colitis with a high lethality, failure to resolve leukocyte infiltration, and a disturbed, prolonged epithelial healing. Sdc1 is the most important HSPG of the epithelial cell surfaces,^{1,13} serving multiple roles in proliferation and cell repair, and as a coreceptor for cytokine and chemokine signaling.^{2,5,6,10,11} First evidence for the role of Sdc1 in the field of IBD came from Day et al¹⁵ in 1999, who showed immunohistochemically that Sdc1 is less expressed in ulcers of ulcerative colitis patients. *In vitro*, shedding of the extracellular domain of syndecan in intestinal epithelial cells disrupted fibroblast growth factor-dependent proliferation, which could be restored by the adding of the soluble ectodomain containing mainly heparan sulfate side chains as well as by adding heparin, a chemically similar molecule of the heparan sulfate side chains of the syndecans. These observations lead to the hypothesis that this altered biology of syndecans and their heparan sulfate side chains plays a role during the pathogenesis of IBD, opening the view for a new possible mechanism for the intended use of heparin in ulcerative colitis patients. In a recent study, investigating the role of syndecan for the integrity of the epithelial barrier, Bode et al⁴¹ showed that syndecan plays a critical role during protein-losing enteropathy and that the loss of Sdc1 increases basal protein leakage in a dose-dependent fashion. Furthermore, Sdc1 deficiency promoted cytokine associated protein leakage. TNF- α and Sdc1 acted synergistically in this process. Since the epithelial barrier function is essential for the pathogenesis of IBD,⁴² and given these data, it can be speculated that challenging the animals with DSS, triggering TNF- α production (especially in a system already lacking the protective factor of Sdc1 for the gut) promoted lethality in our model.

Sdc1 Modulates Intestinal Epithelial Cell Proliferation

Apart from increased leukocyte recruitment, the lack of Sdc1 lead to decreased cell proliferation, prolonged apoptosis and delayed tissue repair. These findings are in accordance with previous publications on the coreceptor role of Sdc1 in mitogenic growth factor signaling^{1,2,10,11,13} and on delayed skin and corneal wound repair in Sdc1 KO mice, which is partially due to decreased epithelial cell proliferation rates.⁵ Our *in vitro* data in Sdc1 silenced Caco-2 cells confirm and extend these findings, because Sdc1 mRNA knockdown resulted in reduced bFGF-mediated MAPK activation and reduced cell proliferation. Therefore, reduced epithelial cell proliferation due to a lack of the coreceptor function of Sdc1 for tyrosine kinase receptor emerges as an additional possible mechanism of the increased severity of DSS colitis in Sdc1 KO mice, which promotes delayed wound repair and reepithelialization.

Enoxaparin as a Substitute for Sdc1 Function

Unfortunately, the pharmacotherapy of IBD comes along with significant side effects.⁴³ Accordingly, investigation in alternative fields of new anti-inflammatory drugs or mechanisms is mandatory. The worsened outcome of Sdc1 KO mice after DSS challenge could be reversed by treatment with the low molecular weight heparin, enoxaparin. Wild-type animals treated with enoxaparin showed a nonsignificant tendency for increased mortality compared with placebo-treated animals.

Because of its similar ligand binding properties,^{1,3,4} it can be expected that heparin can substitute for a variety of biological functions of HSPG. For example, both heparin and the Sdc1 ectodomain can be a coreceptor of the fibroblast growth factor in epithelial cell lines.^{15,25} It was suggested that heparan sulfate might be secreted by epithelial cells to retain an intact intestinal barrier after shedding of dead epithelial cells.⁴⁴ A lack of this mechanism might contribute to the worse outcome of Sdc1 KO mice, and substitution by heparin might restore this mechanism. Different work groups were able to reproduce the positive effect of heparan sulfate glycosaminoglycans, especially heparins and heparin derivatives in colitis models, eg, in trinitrobenzene sulfonic acid or oxazolone induced colitis.^{14,45} However, since soluble heparin can act as a mimic of both *cis*- (cell surface) and *trans*- (shed) conformations of Sdc1, we cannot fully differentiate between possible differential roles of soluble and membrane-bound forms of Sdc1 in the DSS colitis model. In addition, it has to be considered that heparin may also mimic the effects of other HSPGs. However, very little is known about the role of these HSPGs in the context of colonic inflammation. Because of the less-extended conformation of the glypican core proteins and the closer localization of their HS chains to the plasma membrane,¹ the HS chains of Sdc1 are more likely to form the first line of binding sites for extracellular ligands than glypican HS. Basement membrane HSPG such as perlecan may play a role in sustaining epithelial integrity

and barrier function; however, they should be similarly affected in *Sdc1* KO and wild-type mice during DSS colitis.

Mechanistically, additional aspects of heparin or heparin-derivative effects, such as TNF- α neutralization,⁴⁶ inhibition of P-selectin-mediated leukocyte migration,⁴⁷ or CD14 dependent macrophage activation,⁴⁸ need to be considered.

Finally, the beneficial effect of heparin on TNF- α induced protein-losing enteropathy,¹⁹ may have contributed to the improved outcome in *Sdc1* KO mice, because *Sdc1* deficiency exacerbates protein-losing enteropathy. In our experimental model, enoxaparin treatment not only helped to decrease inflammation in the colon but also effectively abolished increased leukocyte-endothelial interactions and ICAM-mediated leukocyte adhesion *in vitro*. Enoxaparin could inhibit leukocyte-endothelial interactions as efficiently as conventional heparin.⁹ The differential modulation of leukocyte recruitment by heparin in *Sdc1* KO and wild-type mice may therefore represent an important mechanistic aspect of the beneficial effect of heparin in this model.

A few clinical trials with heparin treatment in steroid-resistant ulcerative colitis were performed. In some cases, this therapeutic approach was successful.^{16,17} However, a meta-analysis¹⁸ could not prove efficacy in the treatment of patients with ulcerative colitis.

With respect to the underlying causes of decreased *Sdc1* expression in ulcerative colitis patients,¹³ it is not yet clear if the reduced *Sdc1* expression is a secondary consequence of the active colitis or if a pre-existing reduction in *Sdc1* expression makes a subgroup of patients more susceptible for ulcerative colitis. In the latter case, a differential diagnosis of these patients may be required, which may constitute a potential limitation for a general therapeutic application of heparinoids in IBD. At the same time, the existence of such a subgroup could serve to explain controversial results on the efficacy of heparin therapy in IBD. Therefore, further histopathological studies on these subjects would be desirable.

Conclusions

The observation of a worse course of DSS colitis in *Sdc1*-deficient mice supports the hypothesis that altered expression of the main HSPG, *Sdc1*, plays a substantial role during the process of colon inflammation. Heparin derivatives and formulations with increased potency of targeting heparan-sulfate associated anti-inflammatory mechanisms have to be evaluated, because different mechanisms connected to *Sdc1* function are beneficial in this process. Our findings show that HSPGs such as *Sdc1* contribute to the mucosal integrity by affecting epithelial cell function, cell proliferation, expression of chemokines, and cytokines, as well as by influencing the function of cell adhesion molecules. Mechanistically, the lack of *Sdc1* promoted increased leukocyte recruitment under inflammatory conditions, possibly by removal of a steric hindrance of ICAM-1-counterreceptor interactions. Increased leukocyte recruitment *in vivo* promoted differentially increased

expression of cytokines and chemokines, among which TNF- α is known to be a major player in the pathogenesis of DSS colitis. The lack of *Sdc1* further hampered efficient wound repair, because it is required for tyrosine kinase receptor-mediated mitogenic signaling. Enoxaparin is able to reduce lethality in the course of DSS colitis in *Sdc1* KO mice. In addition, enoxaparin modifies the complex biological dysfunction of missing *Sdc1* during leukocyte recruitment *in vitro*. Therefore, targeting HSPG interactions might provide novel therapeutic approaches for the treatment of IBD, with a potential benefit for patient collectives displaying low colonic *Sdc1* expression.

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