Proteoglycans (PGs) are distributed ubiquitously as a component of extracellular matrix (ECM) at the cell surface and bear glycosaminoglycans (GAGs) such as heparan sulfate (HS) and chondroitin sulfate (CS). HSPGs are critically involved in various physiological and pathological conditions. One important feature of HSPG is the ability to interact with a variety of growth factors, cytokines, chemokines, and ECM proteins, thereby regulating wound healing, tissue remodeling, hemostasis, and inflammation (1). Syndecans are the major cell-surface PGs expressed by virtually all epithelial cells. Syndecan-4 is a member of the syndecan family and is an important constituent of host defense mechanisms by acting as a regulatory receptor that monitors dynamic changes in ECM composition and organization during tissue injury and remodeling (2–6). However, the molecular mechanism by which syndecan-4 regulates complex host defense responses remains to be elucidated. Osteopontin (OPN) is a T helper type 1 immunoregulatory cytokine that plays a critical role in various inflammatory disorders. OPN exerts proinflammatory reactions through interaction with integrin receptors. OPN function can be modulated by protease digestion. However, the molecular mechanisms that regulate OPN function in vivo have not been elucidated. There are two putative heparin-binding domains (HBDs) within the OPN molecule, which may bind both heparin and heparin-like glycosaminoglycans such as syndecan. We show that expression of OPN and syndecan-4 is significantly up-regulated after concanavalin-A (ConA) injection. Syndecan-4 binds to one of the HBDs of OPN, which overlaps with the thrombin cleavage site of OPN. When OPN is associated with syndecan-4, syndecan-4 masks both the thrombin cleavage and the integrin binding sites within OPN. Importantly, syndecan-4-deficient (Syn4KO) mice are more susceptible to hepatic injury, and the thrombin-cleaved form of OPN is significantly elevated in Syn4KO mice as compared with wild-type mice after ConA injection. Finally, we demonstrate that administration of purified syndecan-4 protects mice from ConA-induced hepatic injury. Thus, syndecan-4 is a critical intrinsic regulator of inflammatory reactions via its effects on OPN function and is a potential novel therapeutic tool for treating inflammatory diseases.
RESULTS AND DISCUSSION
Specific binding of heparin and syndecan-4 to the HBD of OPN
We initially examined whether heparin binds to OPN. The binding of biotinylated heparin to the full-length form (OPN full/glutathione S-transferase [GST]), the amino-terminal half of thrombin-cleaved form (OPN N half/GST), and the carboxy-terminal half of thrombin-cleaved form (OPN C half/GST) of human OPN was tested. Heparin bound to Escherichia coli–derived OPN full/GST (Fig. 1 A). In contrast, the same heparin preparation failed to bind to both OPN N half/GST and OPN C half/GST. OPN is heavily glycosylated in vivo (17) and E. coli–derived OPN lacks glycosylation. To exclude that the lack of sugar moiety on OPN affects binding of OPN to heparin, we prepared the glycosylated form of recombinant OPN from Chinese hamster ovary (CHO) cells. Again, heparin bound only to the full-length form of OPN in a dose-dependent manner (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20071324/DC1). Note that the amino acid sequences that correspond to two putative HBDs are (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20071324/DC1) (17). It is unknown whether HBDs within OPN really bind to heparin and HS. Syndecan-4 is known to regulate thrombin activity (18, 19), and OPN function is regulated by thrombin cleavage (12, 14, 17, 20). Therefore, it is of importance to examine whether syndecan-4 can associate with OPN and modulate OPN function. In this report, we show that one of the HSGLPs, syndecan-4, specifically binds to the HBD of OPN via HS moiety. Syndecan-4, when bound to OPN, masks not only thrombin cleavage but also integrin binding sites within OPN molecules, thus regulating various OPN functions. The absence of syndecan-4 results in the exacerbation of concanavalin-A (ConA)–induced hepatic injury and augmentation of the production of the thrombin-cleaved form of OPN, which plays a critical role in hepatic tissue injury (14). Importantly, administration of exogenous syndecan-4 protects mice from ConA-induced hepatic injury. Thus, we show that syndecan-4 is a critical intrinsic regulator of inflammatory reactions through the regulation of OPN functions.

Figure 1. The specific binding of OPN to heparin and HSPG, a syndecan-4. (A) Biotinylated heparin binds to the nonglycosylated form of full-length OPN. Plates were precoated with recombinant OPN, derived from E. coli, as indicated. (B) Biotinylated heparin binds to the synthetic peptide corresponding to one of two putative HBDs of OPN. (C) The binding of 3 μg/ml of recombinant Syn4lg to the plate precoated with either the full-length or thrombin-cleaved form of human OPN (OPN or T-OPN, respectively), or 10 μg/ml of the full-length form of mouse OPN (mOPN). In some experiments, the HS or CS chain of Syn4lg was digested by either HSase or CSase, respectively. Data are presented as means ± SEM. **, P < 0.005. (D) The interaction of the native form of OPN and syndecan-4. The ability of human cell lines to secrete OPN, syndecan-4, and HS were listed. NRC-12 or OPN/Namalwa cells were stimulated with PMA. Culture supernatants were applied to the affinity column coupled with anti-syndecan-4 antibody, anti-OPN antibody, or control antibody. After washing, eluates were applied to SDS-PAGE analysis, transferred to membranes, and immunoblotted (IB) with either anti-OPN or anti-syndecan-4 antibody. ND, not detected.
OPN and syndecan–4 proteins were immunoblotted in the bound fraction from the anti–syndecan–4 antibody–coupled column but not from the control column, indicating the presence of association between OPN and syndecan–4. Culture supernatants were also applied to the affinity column coupled with anti-OPN antibody. The syndecan–4 was immunoblotted in the bound fraction derived from NRC-12 cells but not from OPN/Namalwa cells (Fig. 1 D). In addition, we confirmed the association of OPN and syndecan–4 by using genetically manipulated CHO cells transiently expressing human OPN, syndecan–4, or both OPN and syndecan–4 (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20071324/DC1). We also found that, like heparin, the binding of Syn4Ig was specific to the one of two putative HBDs of OPN that overlaps the thrombin cleavage site (Fig. S5). Thus, the physiological association of HBDs of OPN and HS moiety of syndecan–4 exists, and the biological significance of this association remains to be elucidated.

**Syndecan–4 modulates OPN functions by masking functional domains of OPN**

We thus examined whether the association of OPN and syndecan–4 modulates the availability of OPN to thrombin and/or its receptors. OPN proteins were mixed with either human IgG or Syn4Ig. Addition of thrombin resulted in the significant reduction of full-length OPN levels in OPN preparation when mixed with human IgG. However, when OPN was mixed with Syn4Ig, OPN was resistant to thrombin digestion (Fig. 2 A). Because the binding domain of syndecan–4 to OPN overlaps the thrombin cleavage site, the thrombin resistance may be achieved through the masking of the thrombin-cleaved site by syndecan–4. Syndecan–4 was originally detected in microvascular endothelial cells as an antithrombin binding molecule (19, 26). Antithrombin is a plasma serine protease inhibitor and, thus, inhibits thrombin activity by forming a complex with thrombin.

**Figure 2.** The masking of functional domains of OPN by syndecan–4. (A) OPN becomes less sensitive to thrombin digestion when bound by syndecan–4. Recombinant OPN (50 μl of 3 μg/ml) was mixed with either 50 μl of human IgG1 (hIgG) or 6 μg/ml Syn4Ig. The mixture was subjected to thrombin digestion (2 U/ml) for 37°C for 30 min. The full-length form of OPN was measured using ELISA. Note that this ELISA is unable to detect thrombin-cleaved OPN. (B) OPN recognition by integrin receptors is inhibited by syndecan–4. The binding of CHO cells to OPN is RGD dependent. The binding of CHO cells to either precoated OPN alone or OPN together with Syn4Ig was examined. (C) The binding of CHO cells expressing α4 integrin (α4/CHO) to OPN or OPN bound by syndecan–4 was examined in the presence or absence of 100 μg/ml GRGDS or 10 μg/ml anti-α4 integrin antibody. Data are presented as means ± SEM. *, P < 0.05; **, P < 0.005.
covalent complex with thrombin in a 1:1 ratio. Importantly, after binding to syndecan-4 in vivo, antithrombin exhibits a 500-fold increase of its thrombin inhibitory activity (27). Therefore, it is likely that in vivo syndecan-4 regulates formation of thrombin-cleaved OPN by masking the thrombin cleavage site of OPN and/or inhibiting thrombin activity via association with the antithrombin molecule.

OPN can bind to integrin receptors in at least two ways. First, a classical integrin binding domain, 159RGD161, within the OPN molecule exists close to the consensus thrombin cleavage site, 160R/S163 (17). Therefore, the recognition of OPN by an RGD-recognizing integrin receptor such as αvβ3 can be blocked when OPN is bound by syndecan-4. The binding of CHO cells to OPN was specifically inhibited by GRGDS peptides, confirming that this binding is RGD dependent. The binding of CHO cells to OPN was significantly inhibited when OPN was mixed with syndecan-4 but not with human IgG (Fig. 2 B). Note that OPN binds to the plates comparably well in the presence or absence of Syn4Ig in cell adhesion assays (Fig. S6, available at http://www.jem.org/cgi/content/full/jem.20071324/DC1), indicating that Syn4Ig competes for CHO cell adhesion to plate-coated OPN rather than preventing OPN from binding to the plates. Second, the 162SVVYGLR168 domain, which can be recognized by α4β1 integrin (12, 14), exists also very close to the thrombin cleavage site (17). We therefore tested whether the binding of CHO cells expressing α4β1 integrin (α4/CHO) to OPN can be inhibited by Syn4Ig. Because CHO cells express an RGD-recognizing receptor, a binding assay was performed in the presence of GRGDS peptide, thus allowing the binding of α4/CHO cells to OPN to be α4 integrin dependent. As expected, the binding of α4/CHO cells to OPN was specifically inhibited not only by anti-α4 integrin antibody but also by syndecan-4 (Fig. 2 C). It is known that OPN, αvβ3, and α4β1 (also known as very late antigen 4) integrins are involved in recruitment and cell adhesion of inflammatory cells (7, 10, 12, 14, 15, 17), and it is likely that the association of syndecan-4 and OPN may result in the inhibition of integrin-mediated inflammation. It should be remembered that OPN also putatively binds and mediates cell migration through CD44 (7, 10, 17). Thus, it is of interest in future studies to test whether syndecan-4 regulates the interaction of OPN and CD44.

Both OPN and syndecan-4 expression are up-regulated in a mouse model of ConA-induced hepatic injury

To investigate whether the described scenario operates in vivo, we examined the role of syndecan-4 in ConA-induced hepatic injury in mice. As expected, both plasma OPN and alanine aminotransferase (ALT) levels were significantly elevated after ConA injection (Fig. 3 A). Because antibodies against mouse syndecan-4 and HS are commercially available, we constructed a sandwich ELISA to measure mouse syndecan-4. Plasma syndecan-4 levels were elevated after ConA injection (Fig. 3 B). We also noted that syndecan-4 gene expression in the liver was significantly up-regulated at 2 h and persisted up to 12 h after ConA injection (Fig. 3 C).

Figure 3. The augmented expression of OPN and syndecan-4 in a mouse model of fulminant hepatitis. C57BL/6 mice were intravenously injected with ConA (n = 3). (A) The plasma levels of ALT and OPN after ConA injection. (B) The plasma levels of syndecan-4 after ConA injection. (C) RT-PCR analysis and real-time PCR analysis of syndecan-4 expression in liver after ConA injection. Data are presented as means ± SEM.
Absence of syndecan-4 exacerbates ConA-induced acute hepatic injury and augments production of the thrombin-cleaved form of OPN

If syndecan-4 plays a critical role in the regulation of OPN cleavage by thrombin in vivo, the syndecan-4 deficiency should result in the exacerbation of hepatic injury after ConA injection. Under the condition in which control mice developed mild hepatic injury after ConA injection, syndecan-4-deficient (Syn4KO) mice developed significantly severe hepatic injury, as reflected by the elevation of ALT and aspartate aminotransferase (AST) levels (Fig. 4A). This finding correlated well with the histology in which control mice showed minor hepatic necrosis, whereas there was massive necrosis in Syn4KO mice (Fig. 4B). Consistent to these findings, the survival rate of mice treated with ConA was significantly reduced in Syn4KO mice as compared with control mice. Note that plasma levels of the full-length form of OPN were not different between wild-type and Syn4KO mice after ConA injection, indicating that the absence or presence of syndecan-4 did not influence the induction of OPN expression itself. However, importantly, thrombin-cleaved OPN levels were elevated in Syn4KO mice after ConA injection as compared with those in control mice (Fig. 4C), indicating that levels of the thrombin-cleaved form of OPN correlate well with the degree of inflammation and tissue injury as previously reported (28). The specificity of ELISA used for the detection of the thrombin-cleaved form of mouse OPN is shown in Fig. S7 (available at http://www.jem.org/cgi/content/full/jem.20071324/DC1).

To further test whether OPN is really responsible for the exacerbation of inflammation after ConA injection in Syn4KO mice, mice were treated with a neutralizing antibody raised against the cleaved form of OPN (M5) (12, 14). Syn4KO mice treated with anti-OPN (M5) antibody showed significant amelioration of ConA-induced hepatic injury as judged by the level of plasma ALT and liver histology (Fig. 4D).

Administration of exogenous recombinant syndecan-4 protects mice from ConA-induced hepatic injury

The final issue we addressed was whether the exogenous purified syndecan-4 can protect mice from ConA-induced hepatic injury. Syndecan-4-injected mice were protected from hepatic injury, as reflected by reduced levels of ALT and IFN-γ after ConA injection compared with those in control mice (Fig. 5A). It has been previously shown that OPN is

Figure 4. The high susceptibility of Syn4KO mice to ConA-induced hepatic injury. (A) The plasma levels of ALT and AST in wild-type (Cont) and Syn4KO mice at 12 h after ConA injection (n = 6 per each group). (B) Representative histology of liver sections stained by H-E 12 h after ConA injection, and the survival curve after ConA injection (n = 8 and 13 wild type and Syn4KO, respectively). Dotted lines indicate the area of liver degeneration. The degenerative area per liver section was quantified by using ImageJ (n = 3 per each group). Bar, 200 μm. (C) The plasma levels of full-length and thrombin-cleaved OPN in wild-type (Cont) and Syn4KO mice at 12 h after ConA injection (n = 4 and 6 wild type and Syn4KO, respectively). (D) The attenuation of ConA-induced hepatic injury in Syn4KO mice by anti-OPN (M5) antibody treatment (n = 5 per group). Syn4KO mice were intraperitoneally treated with M5 antibody and control normal rabbit IgG (Cont Ig; 400 μg/head) at 15 h before ConA injection. Plasma ALT levels and representative histology (HE) are shown. Degenerative area per liver section was quantified by using ImageJ (n = 5 per each group). Data are presented as means ± SEM. *, P < 0.05; **, P < 0.005. Bar, 500 μm.
involved in cell adhesion, cell migration, and inflammation via integrin receptors (7, 10, 12, 14, 15, 17). Therefore, we tested whether administration of syndecan-4 would inhibit OPN-mediated inflammatory responses by flow cytometry. The infiltration of inflammatory cells into liver tissues in ConA-injected mice was significantly inhibited by the administration of exogenous syndecan-4 (Fig. 5 B). Infiltrated leukocytes and macrophages/Kupffer cells were defined by the expression of CD45 and F4/80, respectively. Nevertheless, hepatic necrosis in histology was significantly reduced by the administration of syndecan-4 (Fig. 5 C).

Thus, our report reveals a novel role of HSPG in protection against OPN-mediated hepatic injury. One of the major HSPGs, syndecan-4 is a critical intrinsic regulator of inflammatory reactions in vivo by regulating various OPN functions. We identified the primary amino acid sequence within a putative HBD within OPN, which interacts with the HS moiety of syndecan-4. The putative in vivo role of syndecan-4 in OPN-mediated inflammatory reactions is schematically shown in Fig. 5 D. Initial inflammation insult induces the up-regulation of OPN and syndecan-4 expression, and activation of thrombin at the site of insult. OPN induced the migration and cell adhesion of inflammatory cells through interaction with integrins. Thrombin cleaves OPN specifically and augments the production of the thrombin-cleaved form of OPN, which favors the exacerbation of inflammatory reaction and tissue injury. Meanwhile, syndecan-4 acts as a negative regulator of inflammatory responses by binding to HBDs of OPN, thus interfering with integrin-mediated cell adhesion and migration and/or masking the thrombin cleavage site of OPN, leading to the inhibited formation of the thrombin-cleaved form of OPN. Further research is needed to determine whether OPN function is also regulated by other HSPGs such as syndecan-1, -2, and -3 and glypican. HSPG deserves further investigation as a novel therapeutic tool for various inflammatory diseases.

MATERIALS AND METHODS

OPN and syndecan-4 preparations. The three human OPN complementary DNAs (cDNAs) were inserted into pGEX-4T vector (GE Healthcare) in the same reading frame as the carrier gene GST and were transformed in E. coli JM109 cells. Thus, three unglycosylated human OPN/GST fusion proteins were produced: OPN full/GST (M1-N314), OPN N half/GST (M1-R168), and OPN C half/GST (K170-N314). These proteins were purified as described previously (29). To prepare the glycosylated forms of OPN (OPN/CHO and OPN N half/CHO), the full-length human and mouse OPN and OPN N half cDNAs were inserted into pcDNA3 (Invitrogen) and transfected to CHO-K1 cells with Lipofectamine 2000 (Invitrogen). The glycosylated form of human OPN was purified with a formyl-cellulofine column (Seikagaku Kogyo) coupled with anti-OPN (O-17) antibody (Immuno-Biological Laboratories)

Figure 5. Exogenous syndecan-4 protects mice from ConA-induced hepatic injury. (A) Exogenous syndecan-4 protects mice from ConA-induced hepatic injury, as judged by plasma ALT levels and plasma IFN-γ levels at 12 h after ConA injection. Normal human IgG (Cont Ig) was used as a control IgG (n = 10 per each group). (B) Liver-infiltrating leukocytes were prepared from Syn4Ig- or control normal human IgG (Cont Ig)-treated mice at 12 h after ConA injection (n = 5 per group). The number of CD45-positive leukocytes and F4/80-positive macrophages was determined by flow cytometry. (C) Representative histology of liver sections stained by H-E at 24 h after ConA injection. The degenerative area per liver section was quantified by using ImageJ (n = 4 per each group). Data are presented as means ± SEM. *, P < 0.05; **, P < 0.005. Bar, 500 μm. (D) Schematic illustration of the in vivo role of syndecan-4 in OPN-mediated inflammatory reactions.
to syndecan-4, NRC-12 cells and OPN/Namalwa cells, stimulated with PMA for 30 min in serum-free medium, were cultured for an additional 48 h without PMA. The supernatant of NRC-12 cells was applied to an anti-syndecan-4 antibody–coupled formyl-cellulose column and washed extensively. A rabbit IgG–coupled formyl-cellulose column was used as a control column. Elute fraction with 0.2 M glycine–HCl, pH 2.5, was immediately neutralized and electrophoresed through 12% SDS-PAGE gel and probed with anti-OPN (1B20) or anti-syndecan-4 antibody. The supernatants of NRC-12 and OPN/Namalwa cells were also applied to an anti-OPN antibody (O-17; B1)–coupled formyl-cellulose column and Western blotted. The supernatants obtained from CHO cells, transiently transfected with OPN alone, syndecan-4 alone, or both OPN and syndecan-4 cDNA, were also tested for the presence of association between OPN and syndecan-4.

**Thrombin treatment of OPN.** 50 µl of 3 µg/ml hOPN/CHO protein was mixed with either 50 µl of human IgG1 (hIgG) or 6 µg/ml Syn4Ig and incubated for 1 h at 37°C, then digested with 2 U of thrombin for 30 min.

**Cell adhesion test.** The 96-well plates were precoated with 10 µg/ml OPN/CHO protein in the presence or absence of 20 µg/ml Syn4Ig overnight at 4°C, followed by treatment with 0.5% BSA in TBS for 1 h at room temperature. Cells were suspended in DMEM containing 0.25% BSA, and 200 µl of cell suspension (at a cell density of 5 × 10^4 cells per well) was applied to 96-well plates and incubated for 1 h at 37°C. The medium was removed from the plates, and all wells were washed twice. The adherent cells were fixed and stained by 0.5% crystal violet in 20% methanol for 30 min. All wells were rinsed three times with water, and adherent cells were then lysed with 20% acetic acid. The resulting supernatants from each well were analyzed by an immunoreader (Immuno Mini NJ-2300; Nolge Nunc International), and the absorbance at 590 nm was measured to determine the relative number of cells adhered to wells. The binding of cells to OPN was expressed as 100%.

**ELISA.** OPN, human syndecan-4 (JBL), and IFN-γ (BD Biosciences) concentrations were measured by using ELISA kits as specified by the manufacturers. The plasma level of mouse syndecan-4 was measured by using an ELISA system, which was established using 10 µg/ml of rabbit anti–mouse syndecan-4 antibody (B1) for capture antibody and 3 µg/ml of biotinylated anti-HS antibody (Seikagaku Kogyo) for detection antibody. Purified mouse syndecan-4 Ig was used for standard. The thrombin-cleaved form of mouse OPN was detected by ELISA obtained from JBL. The detailed information on this ELISA for the thrombin-cleaved form of OPN is shown in Fig. S7.

**Induction of ConA-induced liver injury in mice.** C57BL/6 mice were injected intravenously with 10 mg ConA (Sigma–Aldrich) per kilogram of body weight, dissolved in pyrogen-free PBS. In some experiments, 150 µg Syn4Ig or human IgG was administered to mice intraperitoneally 15 h before ConA challenge. Liver damage was evaluated by measuring the serum activity of ALT and AST by using a standard clinical autoanalyzer (DRI-Chem 5500V; Fujifilm).

**Histology.** Livers were harvested at various times after ConA injection. All specimens were fixed in 10% buffered formalin and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin (H&E). Light microscopy was performed to assess liver injury. Necrotic areas were measured in each section by using ImageJ (version 1.37; National Institutes of Health), followed by calculation of the necrotic area per section. For each tissue, data were obtained using at least three high power fields (×100).

**Flow cytometry.** Liver-infiltrating leukocytes were isolated as previously described (14). In brief, livers were minced after a few minutes of perfusion, pressed through a stainless steel mesh, and suspended in PBS. After washing, cells were resuspended in 33% Percoll solution and centrifuged at 2,000 rpm for 15 min to remove liver parenchymal cells. The pellet was treated with red blood cell lysis solution, washed with PBS, and resuspended in 10% FCS–DMEM. The numbers of leukocytes and macrophages were determined.
Analysis of messenger RNA (mRNA) expression. Total RNA from livers was extracted by TRIzol (Invitrogen). The following primers were by flow cytometry using monoclonal antibodies reacting to CD45 (BD Biosciences). Differences were considered to be significant when \( P < 0.05 \) (**).

Statistical analysis. Data are presented as means ± SEM and are representative of at least three independent experiments. Significant differences between experimental groups in the adhesion test, thrombin-resistance test, and ConA-induced hepatitis model were analyzed using the Student’s t-test. Significant differences between groups were considered to be significant when \( P < 0.05 \) (**).

Online supplemental material. Fig. S1 shows the structure of OPN. Fig. S2 shows the binding of heparin to the full-length form of OPN. Fig. S3 shows the structure of syndecan-4 and Syn4Hg. Fig. S4 shows the association between OPN and syndecan-4 in the supernatant of CHO transfectant cells. Fig. S5 shows the binding of Syn4Hg to the HBID of OPN. Fig. S6 shows the binding of OPN to plates in the presence or absence of SynHg. Fig. S7 shows the specificity of the ELISA system for detection of the thrombin-cleaved form of mouse OPN. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20071324/DC1.

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