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Fibrinogen protects against barrier dysfunction through maintaining cell surface syndecan-1 *in-vitro*

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Abstract

Background—We have shown that fresh frozen plasma's (FFP) protection of pulmonary endothelial barrier integrity following hemorrhage shock is due in part to restoration of endothelial syndecan-1. In the present study, we investigated the role of fibrinogen, a major component of FFP, as an endothelial protector and hypothesize that fibrinogen stabilizes cell surface syndecan-1 to restore endothelial barrier integrity.

Methods—Pulmonary endothelial cells were incubated in FFP, fibrinogen, or lactated Ringers (LR) then immunostained with anti-syndecan-1 or fibrinogen and barrier integrity assessed. In some experiments, cells were exposed to fibrinogen depleted plasma.

Results—Cell surface syndecan-1 was increased by FFP and fibrinogen compared with LR-treated cells while barrier integrity was augmented by FFP and fibrinogen compared to LR. The physiological concentration of 2.5 mg/ml fibrinogen was sufficient to increase cell surface syndecan-1. Co-localization and co-immunoprecipitation experiments demonstrated that fibrinogen associates with syndecan-1. Fibrinogen deficient plasma was unable to augment syndecan-1 immunostaining and lost its endothelial protective effect on barrier integrity.

Conclusion—These data suggest that in-vitro, fibrinogen associated with cell surface syndecan-1 and enhanced endothelial barrier integrity.

Keywords

fresh frozen plasma; pulmonary endothelial cells; endothelial permeability; actin stress fibers

INTRODUCTION

The endothelial glycocalyx is a complex network that projects from the cell surface of the endothelium into the vessel lumen. Proteoglycans provide structural support for the glycocalyx, which in endothelial cells, consists of the transmembrane core protein syndecan-1. Cell surface syndecan-1 with attached heparan sulfate glycosaminoglycan side chains anchor the glycocalyx to the endothelial cell membrane to protect vessel integrity (1,

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2). We have been interested in the role of syndecan-1 in endothelial dysfunction following hemorrhagic shock (2 - 4). Hemorrhagic shock causes shedding of the syndecan-1 ectodomain with loss of the glycocalyx. Ectodomain shedding contributes to endothelial injury (2, 3) with subsequent hyperpermeability and tissue edema, both which contribute to organ injury and dysfunction (3, 5). We have also shown that hemorrhagic shock downregulates lung endothelial syndecan-1 expression and enhances lung injury (2 - 4).

Both civilian and military studies have documented improved survival with the early administration of plasma following hemorrhagic shock (6, 7). Plasma's protection is due in part to restoration of endothelial syndecan-1 (2, 8). We will show in the current study that fibrinogen, as a major protein found in plasma, contributes to plasma's protection. Our investigations into fibrinogen were based on the simple observation that plasma, but not serum, lessened pulmonary endothelial cell permeability (unpublished data). As human serum lacks fibrinogen (and other clotting factors) found in plasma, we further investigated this finding. Low systemic concentrations of fibrinogen are associated with increased severity of injury and coagulopathy and are independently predictive of mortality after hemorrhage shock (9, 10). Fibrinogen concentrates have been suggested as an alternative to plasma in some instances (11 – 13). In the present study, we investigate fibrinogen as an endothelial protector and hypothesize that fibrinogen stabilizes cell surface syndecan-1 to restore endothelial barrier integrity.

MATERIALS AND METHODS

Primary cell culture

Human lung microvascular endothelial cells (HLMVEC; PromoCell) were grown to confluence in endothelial basic medium-2 (EBM-2; Lonza) supplemented with 10% fetal bovine serum (FBS), human recombinant epidermal growth factor, human recombinant insulin-like growth factor-1, human basic fibroblast growth factor, vascular endothelial growth factor, hydrocortisone, ascorbic acid, heparin, gentamicin, and amphotericin B. Endothelial cells (passages 6–8) were incubated with EBM-2 containing 2% FBS prior to experiments. Lactated Ringer's solution (LR), fresh frozen plasma (FFP), and fibrinogen depleted plasma (FDP; Sekisui Diagnostics, Germany), were diluted in media to 10% as we have previously demonstrated this to be optimal for endothelial cell function (4, 14). Fibrinogen-deficient plasma was purified using human thrombin but still contains a small amount of fibrinogen (0.08 mg/ml). Fibrinogen (F3879, Sigma-Aldrich) contains 50-70% protein (80% of protein is clottable) was added to medium at 2.5, 5.0 and 10.0 mg/ml.

Endothelial barrier integrity

To determine endothelial monolayer permeability, HLMECs were plated on gelatin-coated culture inserts (0.4- μ m pore size) in 12-well companion plates and grown to confluence in the growth medium. Subsequently, FITC-labeled dextran (40 kDa, Sigma) was added to the upper chamber (100 μ g/ml), and PBS to the lower chamber (to prevent the formation of an oncotic pressure gradient) for 1 hour. Medium was collected from the lower chamber, and the fluorescence was measured using a fluorimeter (485 nm excitation, 530 nm emission). The fold change in FITC-dextran fluorescence intensity over controls (LR) was used as a

measure of monolayer permeability. Actin stress fibers were also stained in these cells, as stress fiber formation causes cell contraction and disrupts the endothelial barrier (15, 16). Cells were grown on gelatin-coated 8-well chamber slides and fixed in 4% paraformaldehyde then permeabilized with 0.2% Triton X-100. After washing and blocking with 1% bovine serum albumin (BSA), cells were incubated with 5 U/ml of Texas Red-X phalloidin (Molecular Probes) which is specific for F-actin. The immunofluorescence intensity was quantified using Quantity One software (Bio-Rad, Hercules, Ca).

Immunofluorescence staining

HLMECs monolayers were grown directly on 8-well chambers (4×10^4 ; Corning). After being treated with the test fluids for 6 hours, cells were fixed in 4% paraformaldehyde for 15 minutes and blocked with 1% bovine serum albumin (BSA) in PBS for 1 hour at room temperature. Cells were then incubated with anti-syndecan-1 antibody or anti-fibrinogen antibody (1:200; Cell Signaling) in 1% BSA at 4°C overnight and followed by incubating with Alexa fluor 488 conjugated anti-rabbit IgG (1:400; Invitrogen) in 1% BSA for one hour at room temperature. The fluorescence intensity was quantified using Quantity One and reported as relative fluorescence units (RFU).

Co-immunoprecipitation

Endothelial cells grown in 10-cm plates were washed with ice-cold PBS and incubated on ice for 15 minutes with 500 μ l lysis buffer (20 mM Tris-HCl, pH 7.5, 0.5% NP-40, 137 mM NaCl, 1 mM PMSF, 1 μ M pepstatin, and 1 μ M aprotinin). The cells were scrape-harvested, and nuclear and cellular debris removed by centrifugation for 10 minutes at 12,000 g at 4°C. Cell lysates were incubated with anti-syndecan-1 antibody for 1 hour at 4°C, followed by addition of 40 μ l protein A/G agarose beads (50% slurry) for 2 hours at 4°C. The immunoprecipitates were washed three times with ice-cold lysis buffer, separated by centrifugation for 30 seconds at 12,000 g , and then heated with Novex LDS sample buffer for electrophoresis and Western blot analysis.

Statistics

All data were obtained from four independent experiments, each performed in triplicate. Results are presented as mean $\pm$ SEM. Statistical significance was determined by ANOVA with Bonferroni correction, $p < 0.05$ for significance.

RESULTS

Fibrinogen increases cell surface syndecan-1

Syndecan-1 protein (55 kDa) was detectable in cells treated with FFP and fibrinogen but not LR (Fig. 1A). Similarly, cells treated with FFP and fibrinogen demonstrated comparable syndecan-1 staining (82.7 ± 12 RFU for FFP vs 73.1 ± 1.6 RFU for fibrinogen; $p > 0.05$) which was significantly higher than LR (18.9 ± 6.5 RFU, $p < 0.01$) for both FFP and fibrinogen (Fig. 1A). Notably, fibrinogen at doses of 2.5, 5.0 and 10.0 mg/ml equally increased cell surface syndecan-1 (Fig. 1B). Human plasma fibrinogen concentration is approximately 2.5 mg/ml, suggesting that physiologic fibrinogen concentration is sufficient to maintain endothelial syndecan-1 under normal conditions. When cell surface staining of

fibrinogen was assessed, cells treated with LR displayed low levels of fibrinogen on the cell surface (Fig. 1C). There was a significant increase in cell surface fibrinogen staining when cells were treated either FFP (51.9 ± 7.1 RFU) or FIB (62.7 ± 2.8 RFU) compared to LR (22.5 ± 1.1 RFU, $p < 0.01$).

Fibrinogen associates with cell surface syndecan-1 and enhances endothelial barrier integrity

To further examine the potential of fibrinogen to bind to endothelial cell syndecan-1, we performed co-localization and co-immunoprecipitation experiments. When cells were co-immunostained with anti-syndecan-1 and anti-fibrinogen antibodies (Fig. 2A), syndecan-1 and fibrinogen co-localized on the cell membrane while only weak co-localization occurred in LR treated cells (Fig. 2A). Further, fibrinogen protein bands were detected on fibrinogen-treated cells but only weak fibrinogen bands on LR-treated cells (Fig. 2B). We did not detect either syndecan-1 or fibrinogen bands on the isotope control immunoprecipitates (Fig. 2B).

We next sought to determine the effect of fibrinogen on endothelial cell barrier integrity by assessing stress fiber formation and permeability. Actin stress fiber formation generates contractile tension, leading to endothelial barrier disruption (15, 16). In LR-treated endothelial cells, we observed increased F-actin stress fibers compared to both FFP- and fibrinogen-treated cells (Fig. 2C). Consistently, treatment with either FFP or fibrinogen significantly decreased the monolayer permeability to FITC-dextran compared with LR treatment (0.23 ± 0.10 fold change for FFP and 0.30 ± 0.10 fold change for FIB vs 1.00 ± 0.20 control for LR, both $p < 0.01$) (Fig. 2C). Together, these data indicate that fibrinogen associates with cell surface syndecan-1 and correlates with enhanced endothelial barrier integrity.

Fibrinogen is responsible for FFP's endothelial cell protection

The data presented suggest fibrinogen plays a pivotal role in protecting against endothelial barrier dysfunction. As a first step in determining if fibrinogen was necessary and sufficient for FFP's protection to the endothelium, syndecan-1 immunostaining was compared after incubation of endothelial cells with FFP or fibrinogen-depleted plasma (FDP). As shown in Fig. 3A, compared to LR, FFP significantly increased cell surface syndecan-1 (115.2 ± 7.5 RFU for FFP vs 38.3 ± 13.9 RFU for LR, $p < 0.05$), whereas fibrinogen-depleted plasma was comparable to LR (37.7 ± 12.3 RFU for FDP vs 38.3 ± 13.9 RFU for LR, $p > 0.05$). Large amounts of stress fibers were observed in LR- and FDP-treated cells but not in FFP-treated cells (Fig. 3B). Lastly, fibrinogen depleted plasma-treated cells demonstrated comparable levels of permeability to LR-treated cells (0.89 ± 0.1 fold change for FDP vs 1.0 ± 0.1 control for LR, $p > 0.05$), but FFP-treated cells had significantly lower permeability (0.58 ± 0.05 fold change, $p < 0.05$ vs both LR and FDP) (Fig. 3B). These data together indicate that fibrinogen plays a pivotal role in the protection by plasma of the endothelial barrier.

DISCUSSION

We have shown for the first time that fibrinogen, as an isolated protein or as a component of plasma, associates with cell surface syndecan-1 and contributes to pulmonary endothelial

barrier integrity. Our dose response study revealed that only a low concentration of fibrinogen is needed for this protective effect. Additionally, as shown by fibrinogen depletion experiments, fibrinogen is responsible for FFP's endothelial barrier protection, as shown by increased permeability and stress fiber formation.

We have previously demonstrated that loss of syndecan-1 was associated with enhanced pulmonary permeability and inflammation in our mouse model of hemorrhage shock (4, 17). Further, we have shown that resuscitation with FFP restored cell surface syndecan-1 expression and mitigated pulmonary hyperpermeability, inflammation and injury in vitro and in vivo (2, 3, 17, 18). Once on the cell surface, our current data suggests that the fibrinogen component of plasma binds to and stabilizes cell surface syndecan-1.

Cell surface syndecan-1 can function as a receptor for heparan sulfate-binding molecules (19, 20), capturing distant ligands found in plasma by binding to their heparin binding domains (21). As fibrinogen possesses heparin binding domains (22), we hypothesize that the mechanism by which fibrinogen engages with cell surface syndecan-1 is binding of the heparan sulfate moieties attached to syndecan-1 to the heparin binding sites on fibrinogen. Fibrinogen is a 340-kDa homodimeric glycoprotein comprised of two alpha, two beta, and two gamma polypeptide chains linked by disulfide bridges. Binding to syndecan-1 most likely occurs with the beta chain of fibrinogen as this chain possesses the heparan sulfate-binding sites (23, 24). Specifically, the 15–42 sequence of the beta chain of fibrinogen has been shown to mediate platelet and endothelial cell spreading, fibroblast proliferation, and capillary tube formation. Interactions between this sequence and the endothelial cell receptor, vascular endothelial (VE)-cadherin has also been shown to promote capillary tube formation and angiogenesis (25). It is possible, though less likely, that syndecan-1 binds to the alpha chain of fibrinogen.

A second potential mechanism by which fibrinogen could stabilize cell surface syndecan-1 is prevention of syndecan-1 cleavage by sheddases. Cell surface expression of syndecan-1 is influenced either by sheddases or regulated by gene expression (2). The sheddases, such as activated matrix metalloproteinases ADAM-17 and tumor necrosis factor alpha, cause syndecan-1 ectodomain release from the cell surface (26). Shed ectodomains are associated with enhanced shock, inflammation, and endothelial damage (3) and independently predict mortality in injured patients (27, 28). Binding of fibrinogen to syndecan-1 may shield its cleavage site, thus stabilizing syndecan-1 on the cell surface.

Fibrinogen is a major plasma protein that plays an important role in hemostasis. It is used in bleeding patients either as an isolated component or as cryoprecipitate in the United States. Our results demonstrate an endothelial barrier protective effect for fibrinogen, which may support the early use of fibrinogen as a therapeutic for hemorrhagic shock. Fibrinogen has also been shown to reduce neutrophil adhesion to the activated endothelium suggesting an additional benefit to supplementation (29).

The current study has a number of limitations. Because plasma contains hundreds of proteins, proteins other than fibrinogen may bind to syndecan-1. However, we would not anticipate other proteins to significantly participate in barrier integrity and/or not be present

in human plasma in sufficient quantities to provide protection. The exception to this could be albumin, as it has been reported to provide protection to the endothelium in a rodent model of hemorrhage (30). As this study represents our initial findings into the key role of fibrinogen as an endothelial protector, future studies are needed to define the precise mechanisms for this protection. We have not definitively demonstrated binding between fibrinogen and syndecan-1. However, our co-localization and co-immunoprecipitation experiments are highly suggestive. Also, the specific fibrinogen chain responsible for binding to fibrinogen has not been elucidated. Finally, we have not tested recombinant fibrinogen to ensure it is fibrinogen alone responsible for protection to the endothelium. Studies to confirm our *in vitro* studies *in vivo* are in progress. As the present study was performed *in vitro* only it therefore has limitations as to its translatability to *in vivo* situations.

In summary, the results from present study demonstrate that fibrinogen associates with endothelial cell surface syndecan-1 to maintain its expression. Association of fibrinogen to syndecan-1 results in less stress fiber formation and decreased permeability. Our data further indicate that fibrinogen recapitulates plasma's protection of endothelial barrier integrity and in fact may be responsible for its protection. These data support further investigations into the precise mechanisms by which fibrinogen interacts with cell surface syndecan-1 and suggests that fibrinogen as an endothelial therapeutic should be further explored.

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Fig. 1. Fibrinogen increases cell surface syndecan-1

A. Human lung microvascular endothelial cells (HLMCEs) were cultured in EBM-2 containing 2% FBS for 2 hours and then incubated with the media containing 10% LR, 10% FFP, and 10 mg/ml fibrinogen for 6 hours. Cell lysates were analyzed with Western blot for syndecan-1 protein levels (top panel). Cells were also immunostained with anti-syndecan-1 antibody and images were captured with Nikon E800 fluorescence microscope with an original magnification of 600 (middle panel, same below). The fluorescence intensity was quantified using Quantity One and reported as relative fluorescence units (RFU). Results are presented as means $\pm$ SEM, n=4/group (bottom panel). **B.** Fibrinogen increases cell surface syndecan-1 at a dose comparable to FFP. HLMCEs were cultured in EBM-2 containing 2% FBS for 2 h and then incubated with the media containing 10% LR, 2.5, 5.0 and 10.0 mg/ml fibrinogen for 6 h. Cells were immunostained with anti-syndecan-1 antibody and images were captured (top panel). The fluorescence intensity results are presented as means $\pm$ SEM, n=4/group (bottom panel). **C.** Fibrinogen increases cell surface fibrinogen. HLMCEs were treated as described in A. Cells were immunostained with anti-fibrinogen antibody and the images were captured (top panel). Fibrinogen fluorescence intensity was reported as relative fluorescence units (RFU). Results are presented as means $\pm$ SEM, n=4/group (bottom panel).

Fig. 2.

Engagement of syndecan-1 and fibrinogen protects endothelial barrier. HLMECs were treated as described in Fig. 1A. Cells were co-immunostained with anti-syndecan-1 and anti-fibrinogen antibodies. The merged images show co-localization of syndecan-1 and fibrinogen in the perinuclear regions and on the membrane. **B.** Western blot analysis of syndecan-1 immunoprecipitates confirmed that fibrinogen and syndecan-1 bind together. Shown are representative results of 3 independent experiments. **C.** Fibrinogen maintains endothelial barrier function, an effect similar to FFP. HLMECs were treated with 10% LR, 10% FFP, and 10 mg/ml fibrinogen for 6 hours before following assays. Top panel: the stress fiber images were captured with Nikon E800 fluorescence microscope with an original magnification of 600. Bottom panel: the monolayer permeability was assessed by FITC-labeled dextran. Results are presented as means $\pm$ SEM, n=4/group.

Fig. 3. Fibrinogen-depleted plasma loses protection on cell surface syndecan-1 and endothelial barrier function. HLMECs were treated with 10% LR, 10% FFP and 10% FDP for 6 hours before following assays. Top panel: the stress fiber images were captured with Nikon E800 fluorescence microscope with an original magnification of 600. Bottom panel: the monolayer permeability was assessed by FITC-labeled dextran. Results are presented as means $\pm$ SEM, n=4/group.