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Role of heparan sulfate in mediating CXCL8-induced endothelial cell migration

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Role of heparan sulfate in mediating CXCL8-induced endothelial cell migration

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Running title: HS in mediating EC migration

Abstract

Several positively charged epitopes on the surface of CXCL8 involved in the binding of the major components of endothelial glycocalyx, sulfated glycosaminoglycans (GAGs). In the present study, we aimed to test the hypothesis that the surface GAGs—heparan sulfate (HS) is a crucial prerequisite for enhancement of endothelial cell migration by CXCL8, and to explore its underlying mechanism by detecting the changes in expression of Rho-GTPases and in the organization of actin cytoskeleton after enzymatic removal of HS on human umbilical vein endothelial cells (HUVECs) by using heparinase III. Our results revealed that the reduction of wound area by CXCL8 was greatly attenuated by removal of HS. The upregulations of Rho-GTPases, including Cdc42, Rac1, and RhoA by CXCL8 were suppressed by removal of HS. The polymerization and polarization of actin cytoskeleton, and the increasing of stress fibers by CXCL8 were also abolished by heparinase III. Taken together, our results demonstrated an essential role of HS in mediating CXCL8-induced endothelial cell migration, and highlighted the biological relevance of the CXCL8 and GAGs interactions in endothelial cell migration.

Keywords: Heparan sulfate; CXCL8; HUVEC; Rho GTPases; Cell migration

Introduction

Inflammatory cytokines are always promoted by vascular injury. In turn, CXCL8 are among the inflammatory cytokines known to upregulate endothelial cell (EC) adhesion molecules, to recruit leukocytes and to induce EC and smooth muscle cell migration, contributing to the wound healing in vascular homeostasis and overall health. CXCR1 and CXCR2 are two kinds of G protein-coupled receptors for CXCL8 (Zeng et al. 2011). Activation of those receptors by CXCL8 causes phosphorylation of protein kinase B, calcium influx, formation of F-actin, and cytoskeleton rearrangement. Those events are very important for directed chemotactic movement of leukocytes and ECs (Baggiolini 2015; Gales et al. 2013). Recently, it was demonstrated that several positively charged epitopes on the surface of CXCL8 involved in the binding of the major components of endothelial glycocalyx, sulfated glycosaminoglycans (GAGs) (Pichert et al. 2012a). Those binding sites for GAGs are well separated with the binding sites for receptors, allowing CXCL8 to interact closely with both components simultaneously (Pichert et al. 2012b; Tarbell et al. 2014). GAGs binding to CXCL8 promotes its oligomerization and its retention on cell surfaces (Proudfoot 2006), which can build up a chemotactic gradient at inflammatory loci and thereby causes a priming of cells and modulates the cell migration spatiotemporally. The immobilization of CXCL8 on the glycocalyx at the abluminal surface of the endothelium modulates the leukocyte recruitment (Carveth et al. 1989). Once the C-terminal GAG-binding domain on CXCL8 was deleted, it was failed to attract leukocyte with the same extent as native CXCL8 *in vitro* and *in vivo* (Middleton et al. 1997), indicating the binding of CXCL8 to surface endothelial GAGs is a crucial prerequisite for cell migration.

GAGs are heterogeneous unbranched polysaccharides with high charge densities (Fu & Tarbell 2013; Tarbell & Ebong 2008; Tarbell & Pahakis 2006; Weinbaum et al. 2007). The most prominent GAG in the vasculature is heparan sulfate (HS), accounts for >50% of the total GAG, usually is

present in the endothelial glycocalyx layer as proteoglycan attachments (Tarbell et al. 2014). Recently, it was demonstrated that the involvement of HS in the cell motility of EC under fluid shear stress (Moon et al. 2005; Thi et al. 2004; Yao et al. 2007). However, the role and underlying mechanism of HS in chemotactic movement of EC by CXCL8 are largely unclear.

In mechanism, cell migration was spatiotemporally regulated by multiple intertwined signaling networks (Vicente-Manzanares et al. 2005). Rho-family GTPases (Rho GTPases), including Cdc42, Rac1, and RhoA, play a central role in cell migration (Nobes & Hall 1995). Cdc42 controls the formation of protrusion and filopodia. Rac1 induces the formation of protrusion and lamellipodia. RhoA mediates the stress fibers formation. A previous work in our laboratory demonstrated that CXCL8 induces the endothelial cell (EA.hy926 cell line) migration via Rac1/RhoA pathway (Lai et al. 2011).

In the present study, we aimed to test the hypothesis that the surface GAGs is a crucial prerequisite for enhancement of endothelial cell migration by CXCL8, and to explore its underlying mechanism by detecting the changes in expression of Rho-GTPases and in the organization of actin cytoskeleton after enzymatic removal of HS on human umbilical vein endothelial cells (HUVECs) by heparinase III. Our results demonstrated an essential role of HS in mediating CXCL8-induced endothelial cell migration, and highlighted the biological relevance of the CXCL8 and endothelial GAGs interactions in endothelial cell migration.

Material and Methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Allcells, USA. HUVECs

were grown in complete culture media for HUVECs (Allcells, USA) in a humidified 5%/95% CO_2/air incubator at 37°C. Fluorescence-activated cell sorting (FACS) analysis showed that almost all of the cells (>96%) take up high amount of acetylated LDL and are positive for the presence of CD31, CD45, CD62, and von Willebrand factor (vWF). Cells (passages 3 to 5) were plated on to glass slides or 6-well plates at a density of 1×10^5 cells/cm^2 and cultured for 3~5 days until they attained confluence.

Heparinase III and/or CXCL8 treatments

F. heparinum heparinase III (Aglyco, China) selectively cleaves HS of the glycocalyx (Zeng et al. 2012a). It was demonstrated that HS was dramatically degraded by 15 mU/ml heparinase III in ECs, including HUVECs (Giantsos-Adams et al. 2013). Cells were treated with 15 mU/ml heparinase III and/or 100 ng/ml CXCL8 (Pepro-Tech, NJ, USA) in basic HUVEC culture medium (Allcells, USA) with 1% bovine serum albumin (BSA) for indicated times, respectively.

Scratch wound assay

For scratch wound assay (Zeng et al. 2011), prior to the heparinase III/CXCL8 treatments, HUVEC monolayers were scratched by a yellow tip after confluence in 6-well plates, followed by twice-washed to remove the cell debris. After treatment for indicated times, photographs were taken with an invert contrast microscopy (Olympus CKX41, Jap) and digitized using a digital camera (Cannon Powershot G11), and the wound areas were calculated to evaluate the cell migration capacity by using ImageJ 1.50b (National Institutes of Health, USA).

Western Blot

After treatments, expressions of Rho GTPases in HUVECs were performed by Western Blot. Cells were washed and then lysed on ice for 10 min in RIPA Lysis Buffer (Beyotime, China) with an addition of protease inhibitor cocktail (1:100, BestBio Science, China), phosphatase inhibitor

cocktail (1:100, BestBio Science, China) and 10 mM phenylmethylsulfonyl fluoride (PMSF). Protein concentration was measured by a Protein Determination Kit (Cayman). Proteins were size fractionated using SDS-PAGE and electrotransferred onto PVDF membrane (Bio-Rad) and hybridized with monoclonal antibodies (Santa Cruze, USA), including mouse anti-RhoA antibody (1:500), Rabbit anti-Rac1 antibody (1:200), mouse anti-Cdc42 antibody (1:200). Detection was carried out using peroxidase-conjugated secondary antibodies (goat anti-mouse or goat anti-rabbit, 1:5000) and enhanced chemiluminescence reagents (BeyoECL Plus, Beyotime, China). Blots were imaged by Molecular Image® ChemiDoc™ XRS+ with Image Lab™ Software (Bio-Rad, USA). Quantitative data were obtained by using ImageJ 1.50b Gel Analyzer.

Visualization of actin cytoskeleton and confocal microscopy

Immediately after treatments, HUVECs were fixed in 2% paraformaldehyde, permeabilized with 1% Triton X-100, and stained with BODIPY® FL phallacidin (Invitrogen, USA) to visualize the actin cytoskeleton. Cell nuclei were stained by DAPI. All samples were imaged with a Leica TCS SP5 laser scanning confocal microscopy (Sichuan University). The max-intensity Z-projection images were shown as described previously (Zeng et al. 2014; Zeng et al. 2012a; Zeng & Tarbell 2014; Zeng et al. 2013).

Statistical analysis

Data are presented as means ± SD. Statistical analysis was performed by one-way ANOVO with either the least significant difference (LSD) test or Tamhane's T2 test (depending on Levene's statistic for homogeneity of variance), using the SPSS 21 software package. Differences in means were considered significant if $P<0.05$.

Results

An essential role of heparan sulfate in CXCL8-induced HUVEC migration

The migration capacity of HUVEC was evaluated by wound assay (Fig. 1). The results revealed that the wound area in CXCL8 treatment at 8 h significantly lower than that in control at 8 h. And the wound area in CXCL8 treatment was further decreased at 24 h. Those changes in wound area by administering CXCL8 were greatly attenuated by heparinase III while the wound area had not further reduced by heparinase III compared with that in control at the same time, suggesting CXCL8 induces cell migration and plays an essential role of HS in the CXCL8-induced HUVEC migration.

Heparan sulfate mediates the regulation of Cdc42 by CXCL8 in HUVECs

The expression of Cdc42 induced by CXCL8 was time-dependent (Fig. 2A and B). Compared with control, CXCL8 significantly induced the expression of RhoA at 12 h, but did not at 4h. The changes at 12 h in Cdc42 expression induced by CXCL8 was significantly abolished by heparinase III. At 4 h, heparinase III with or without CXCL8 enhanced the expression of Cdc42, and CXCL8 slightly reduced Cdc42 (Fig. 2A), with no significant differences as compared with that without treatment (Fig. 2B).

Heparan sulfate mediates the regulation of Rac1 by CXCL8 in HUVECs

The expression of Rac1 in HUVECs in the presence of CXCL8 was time-dependent (Fig.2C and D). At 4 h, it was not observed significant changes in expression of Rac1 in HUVEC that were treated with heparinase III and/or CXCL8. At 12 h, CXCL8 significantly increased the Rac1 level, and this was abolished by the presence of heparinase III.

Heparan sulfate mediates the regulation of RhoA by CXCL8 in HUVECs

The expression of RhoA induced by CXCL8 was also time-dependent (Fig. 2E and F). Compared with control, CXCL8 significantly induced the expression of RhoA at 12 h, but did not at 4h. The

changes at 12 h in RhoA expression induced by CXCL8 was significantly abolished by heparinase III. At 4 h, heparinase III with or without CXCL8 enhanced the expression of RhoA (Fig. 2E), with no significant differences as compared with that without treatment (Fig. 2F).

Remodeling of actin cytoskeleton by CXCL8 is also heparan sulfate mediated

The changes in actin cytoskeleton in the presence of CXCL8 and/or heparinase III were investigated (Fig. 3). As shown in static conditions (control), the dense peripheral actin bands were present at the cell periphery of HUVECs, in which, the disordered actin filaments were organized into a loose network. In the presence of heparinase III, the actin network did depolymerized and the dense peripheral bands were greatly diminished. CXCL8 induced an obvious polymerization and polarization of actin cytoskeleton, and increased stress fibers with duration, indicating an increased tension with the time of CXCL8 exposure. Co-treatment of heparinase III with CXCL8 almost completely suppressed the remodeling of actin cytoskeleton by CXCL8 alone, showing an important role of heparan sulfate in cytoskeleton reorganization.

Discussion

We presently validated the crucial prerequisite of the most prominent GAG, heparan sulfate for induction of wound healing and cell migration of HUVECs by chemokines, and mechanistically demonstrated critical roles of heparan sulfate in the regulation of Rho-GTPases, including Cdc42, Rac1, and RhoA by CXCL8, and in the reorganization of actin cytoskeleton by CXCL8.

It was demonstrated that GAGs interact selectively with chemokines (Kuschert et al. 1999). The residues, including H18, K20, R60, K64, K67 and R68 in CXCL8 that participated in its interaction with heparin and heparan sulfate were identified, based on site-directed mutagenesis, molecular

182 modeling and NMR spectroscopy (Pichert et al. 2012b). GAGs modulate the cellular responses
183 and leukocyte recruitment (Carveth et al. 1989). Once the C-terminal GAG-binding domain on
184 CXCL8 was deleted, it was failed to attract leukocyte with the same extent as native CXCL8 in
185 vitro and in vivo (Middleton et al. 1997). On the other hand, soluble GAGs binding with CXCL8
186 to form complexes that are unable to bind the G-protein-couple chemokine receptor CXCR1 and
187 CXCR2, inhibit the CXCL8-induced neutrophil calcium flux (Kuschert et al. 1999). Those
188 findings indicate the binding of CXCL8 to surface endothelial GAGs is a crucial prerequisite for
189 cell migration. In the present study, by using heparinase III to selectively cleave the HS of the
190 glycocalyx on HUVECs, the reduction of wound area by CXCL8 was greatly attenuated. This
191 highlighted the biological relevance of the CXCL8 and endothelial GAGs interactions in EC
192 migration.

193 Cell migration is a complex process, which integrated the extracellular signals that initiated by
194 chemokine or mechanical stimuli into cellular migration machinery via triggering the activation or
195 aggregation of receptors at the cell surface and transcriptional regulation of motogenic gene
196 products, such as Rho GTPases (Zeng et al. 2012b). Rho-family GTPases (Rho GTPases),
197 including Cdc42, Rac1, and RhoA, play a central role in cell migration (Nobes & Hall 1995).
198 Cdc42 controls the formation of protrusion and filopodia. Rac1 induces the formation of protrusion
199 and lamellipodia. RhoA mediates the stress fibers formation. CXCL8 activated both the receptors
200 CXCR1 and CXCR2 in human lung microvascular endothelial cells (HLMECs) and immortalized
201 dermal human microvascular endothelial cells (HMECs) (Schraufstatter et al. 2001). Role and
202 underlying mechanism of CXCR1/2 in CXCL8-induced EC migration have been well-
203 investigated. It was suggested that CXCL8 initially activates RhoA and actin stress fiber formation
204 in ECs due to activation of the CXCR1 (1-2 min), and that Rac mediated the responses of cell

retraction and gap formation between neighboring cells is a function of activation of CXCR2 (15 min) in HMECs (Schraufstatter et al. 2001). We previously also demonstrated a quick upregulation of Rac1 expression by CXCL8 within 5 min to induce membrane ruffles via phosphoinositide 3-kinase (PI_3K), and a upregulation of Rac1 and RhoA expression for longer time (1 h to 6 h) to induce the formation of stress fibers in EA.hy926 cells (Lai et al. 2011). As endothelial glycocalyx covered over the cell surface, the receptors might be concealed by the GAGs. After removal the HS GAGs, we found the significant reduction of migration during 4-12 h, and the dramatically suppressed expression of Cdc42, Rac1, and RhoA at 12 h, respectively, compared with that in the presence of CXCL8 alone. Interestingly, the wound healing/migration, and the reorganization of actin cytoskeleton were significantly attenuated by removal of heparan sulfate, but we have not detected significant changes in Cdc42, Rac1, and RhoA expression at 4h. It seems that the roles of HS in CXCL8-induced EC migration during the first 4 h was Rho-GTPases-independent, and the details mechanism during the first 4h remains a mystery and is worth to investigate in the future. Nevertheless, we confirmed the role of HS in CXCL8-induced EC migration at longer time (12 h) is associated with Rho-GTPases.

In conclusion, there is an important biological relevance between the CXCL8 and endothelial GAGs in EC migration. As the chemokines induced cell migration of endothelial cells might contribute to the wound healing after vascular injury, and to the cell recruitment in inflammation and metastatic tumor to promote angiogenesis, targeting to GAGs provides a promise strategy for therapeutic angiogenesis.

Conflict of interest

None declared.

Acknowledgement

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Figure Legends

Figure 1. Heparinase III attenuated the CXCL8-induced wound closure. HUVEC monolayers were scratched by a yellow tip after confluence in 6-well plates, followed by twice-washed to remove the cell debris, and then were treated with 15 mU/ml heparinase III (HepIII), 100 ng/ml CXCL8, or both for indicated times (4, 8, and 12 h), respectively. Normal cell without treatment was set as control (CT). At each time point, photographs were taken with an invert contrast microscopy (Olympus CKX41, Jap) and digitized using a digital camera (Cannon Powershot G11) (A), and the wound areas were calculated to evaluate the cell migration capacity (B). Compared with CT, faster wound closure was only observed in CXCL8 treatment cells during 4~8 h and 8~12 h, suggesting CXCL8 induced cell migration and this could be attenuated by heparinase III. Scale bar: 400 μm; n=4; $*P< 0.05$, $**P < 0.01$ vs. control at each time point.

Figure 2. Effect of heparinase III on the CXCL8-modulated expression of Rho GTPases. HUVECs were treated with with 15 mU/ml heparinase III (HepIII), 100 ng/ml CXCL8, or both for indicated times (4 and 12 h), respectively. Normal cell without treatment was set as control (CT). After treatment, the expression of Rho-GTPases was detected by western blot, and quantitative data were obtained using ImageJ 1.50b Gel Analyzer (National Institutes of Health, USA). (A and B) Cdc42; (C and D) Rac1; (E and F) RhoA. $*P< 0.05$ vs. CT; $\#P< 0.05$.

Figure 3. Heparinase III inhibited the CXCL8-induced actin cytoskeleton reorganization. HUVECs were treated with with 15 mU/ml heparinase III (HepIII), 100 ng/ml CXCL8, or both for indicated times (4 and 12 h), respectively. Normal cell without treatment was set as control (CT). Then, cells were fixed in 2% paraformaldehyde, permeabilized with 1% Triton X-100, and stained with BODIPY® FL phallacidin to visualize the actin cytoskeleton (Green) by confocal microscopy. Blue indicates cell nuclei stained by DAPI. Red arrow heads indicate stress fibers.

Scale bar: 50 μm.

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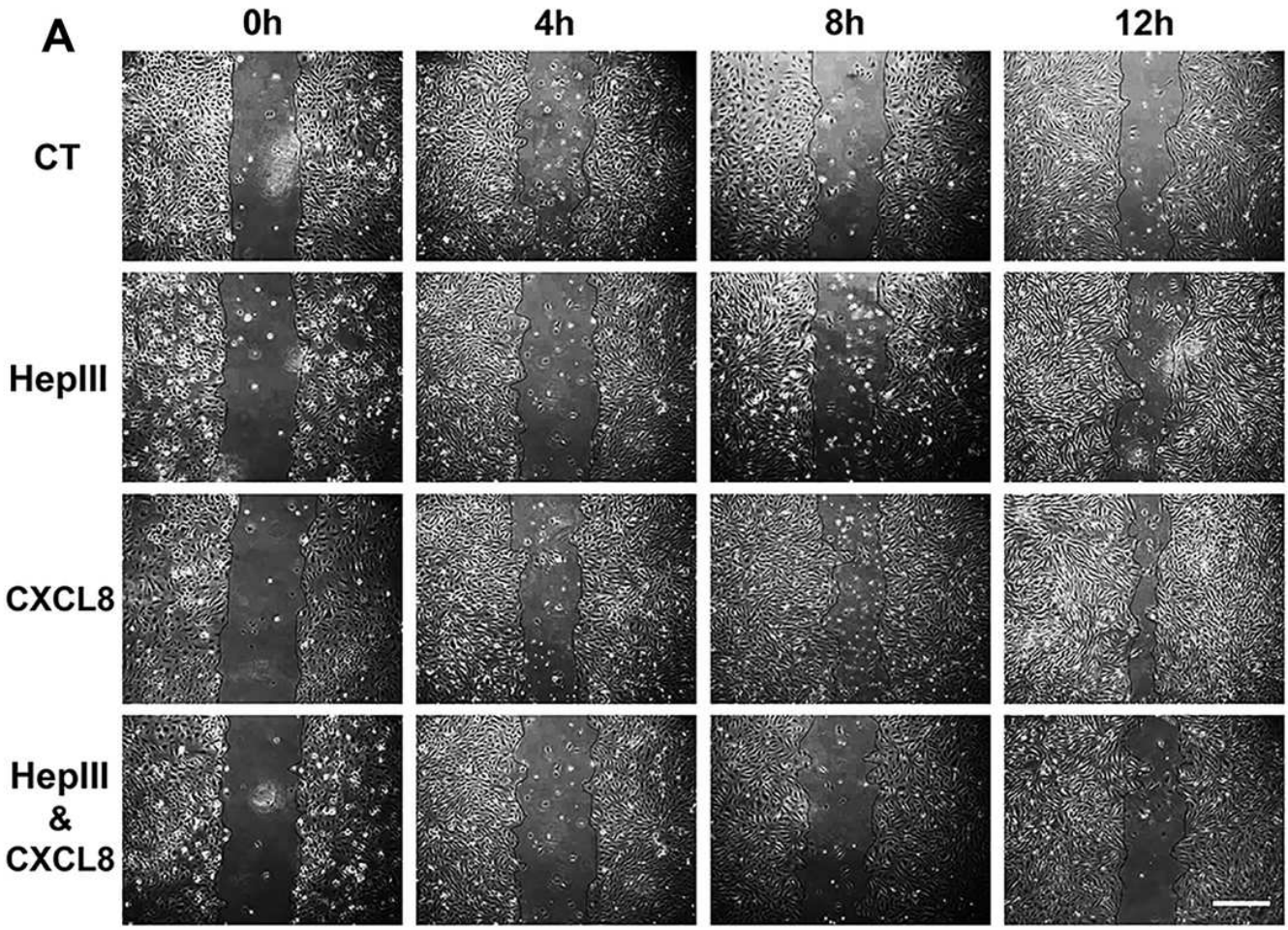
Figure 1

Figure 1. Heparinase III attenuated the CXCL8-induced wound closure. HUVEC monolayers were scratched by a yellow tip after confluence in 6-well plates, followed by twice-washed to remove the cell debris, and then were treated with 15 mU/ml heparinase III (HepIII), 100 ng/ml CXCL8, or both for indicated times (4, 8, and 12 h), respectively. Normal cell without treatment was set as control (CT). At each time point, photographs were taken with an invert contrast microscopy (Olympus CKX41, Jap) and digitized using a digital camera (Cannon Powershot G11) (A), and the wound areas were calculated to evaluate the cell migration capacity (B). Compared with CT, faster wound closure was only observed in CXCL8 treatment cells during 4~8 h and 8~12 h, suggesting CXCL8 induced cell migration and this could be attenuated by heparinase III. Scale bar: 400 μm ; n=4; *P< 0.05, **P < 0.01 vs. control at each time point. "


A
0h
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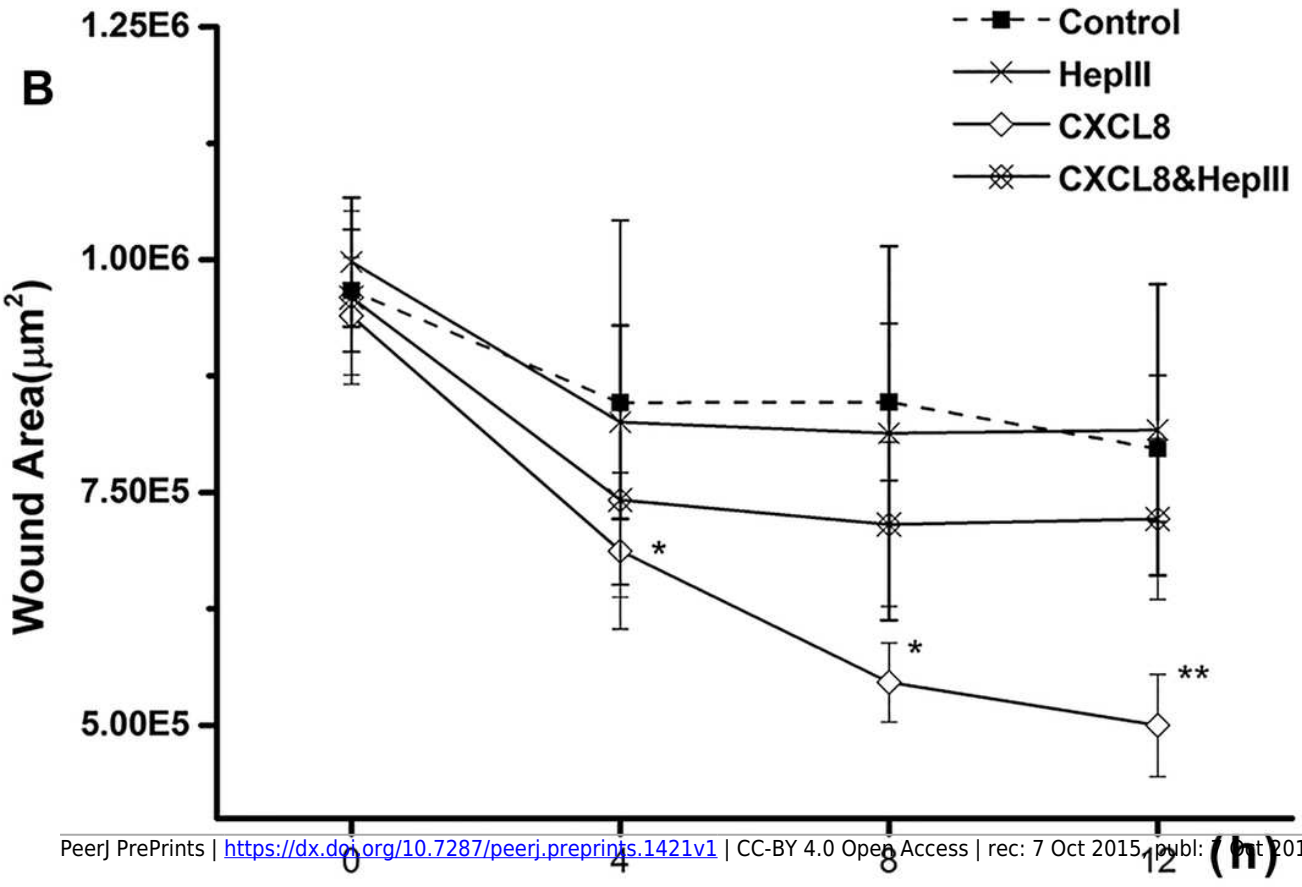
HepIII & CXCL8


B
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CXCL8&HepIII
Wound Area(μm²)
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1.00E6
7.50E5
5.00E5
0
4
8
12
(h)
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Figure 2

Figure 2. Effect of heparinase III on the CXCL8-modulated expression of Rho GTPases. HUVECs were treated with with 15 mU/ml heparinase III (HepIII), 100 ng/ml CXCL8, or both for indicated times (4 and 12 h), respectively. Normal cell without treatment was set as control (CT). After treatment, the expression of Rho-GTPases was detected by western blot, and quantitative data were obtained using ImageJ 1.50b Gel Analyzer (National Institutes of Health, USA). (A and B) Cdc42; (C and D) Rac1; (E and F) RhoA. *$P < 0.05$ vs. CT; #$P < 0.05$.

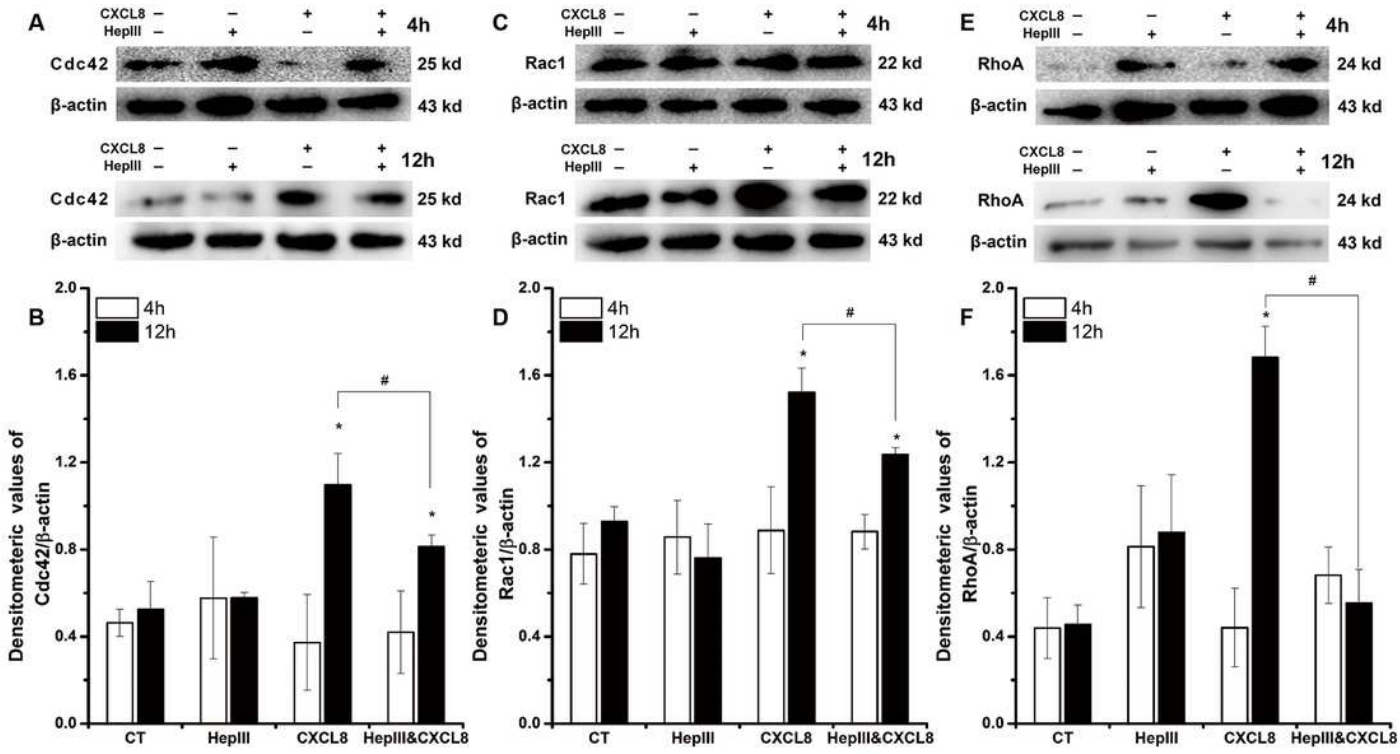

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Figure 3

Figure 3. Heparinase III inhibited the CXCL8-induced actin cytoskeleton reorganization. HUVECs were treated with with 15 mU/ml heparinase III (HepIII), 100 ng/ml CXCL8, or both for indicated times (4 and 12 h), respectively. Normal cell without treatment was set as control (CT). Then, cells were fixed in 2% paraformaldehyde, permeabilized with 1% Triton X-100, and stained with BODIPY® FL phallacidin to visualize the actin cytoskeleton (Green) by confocal microscopy . Blue indicates cell nuclei stained by DAPI. Red arrow heads indicate stress fibers. Scale bar: 50 μm.