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

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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.

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

2

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18 **Running title: HS in mediating EC migration**

19

20

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

44 Inflammatory cytokines are always promoted by vascular injury. In turn, CXCL8 are among the
45 inflammatory cytokines known to upregulate endothelial cell (EC) adhesion molecules, to recruit
46 leukocytes and to induce EC and smooth muscle cell migration, contributing to the wound healing
47 in vascular homeostasis and overall health. CXCR1 and CXCR2 are two kinds of G protein-
48 coupled receptors for CXCL8 (Zeng et al. 2011). Activation of those receptors by CXCL8 causes
49 phosphorylation of protein kinase B, calcium influx, formation of F-actin, and cytoskeleton
50 rearrangement. Those events are very important for directed chemotactic movement of leukocytes
51 and ECs (Baggiolini 2015; Gales et al. 2013). Recently, it was demonstrated that several positively
52 charged epitopes on the surface of CXCL8 involved in the binding of the major components of
53 endothelial glycocalyx, sulfated glycosaminoglycans (GAGs) (Pichert et al. 2012a). Those binding
54 sites for GAGs are well separated with the binding sites for receptors, allowing CXCL8 to interact
55 closely with both components simultaneously (Pichert et al. 2012b; Tarbell et al. 2014). GAGs
56 binding to CXCL8 promotes its oligomerization and its retention on cell surfaces (Proudfoot
57 2006), which can build up a chemotactic gradient at inflammatory loci and thereby causes a
58 priming of cells and modulates the cell migration spatiotemporally. The immobilization of CXCL8
59 on the glycocalyx at the abluminal surface of the endothelium modulates the leukocyte recruitment
60 (Carveth et al. 1989). Once the C-terminal GAG-binding domain on CXCL8 was deleted, it was
61 failed to attract leukocyte with the same extent as native CXCL8 *in vitro* and *in vivo* (Middleton
62 et al. 1997), indicating the binding of CXCL8 to surface endothelial GAGs is a crucial prerequisite
63 for cell migration.

64 GAGs are heterogeneous unbranched polysaccharides with high charge densities (Fu & Tarbell
65 2013; Tarbell & Ebong 2008; Tarbell & Pahakis 2006; Weinbaum et al. 2007). The most prominent
66 GAG in the vasculature is heparan sulfate (HS), accounts for >50% of the total GAG, usually is

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

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

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

85

86

87 **Material and Methods**

88 **Cell culture**

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

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

96 **Heparinase III and/or CXCL8 treatments**

97 F. heparinum heparinase III (Aglyco, China) selectively cleaves HS of the glycocalyx (Zeng et al.
98 2012a). It was demonstrated that HS was dramatically degraded by 15 mU/ml heparinase III in
99 ECs, including HUVECs (Giantsos-Adams et al. 2013). Cells were treated with 15 mU/ml
100 heparinase III and/or 100 ng/ml CXCL8 (Pepro-Tech, NJ, USA) in basic HUVEC culture medium
101 (Allcells, USA) with 1% bovine serum albumin (BSA) for indicated times, respectively.

102 **Scratch wound assay**

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

109 **Western Blot**

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

113 cocktail (1:100, BestBio Science, China) and 10 mM phenylmethylsulfonyl fluoride (PMSF).
114 Protein concentration was measured by a Protein Determination Kit (Cayman). Proteins were size
115 fractionated using SDS-PAGE and electrotransferred onto PVDF membrane (Bio-Rad) and
116 hybridized with monoclonal antibodies (Santa Cruze, USA), including mouse anti-RhoA antibody
117 (1:500), Rabbit anti-Rac1 antibody (1:200), mouse anti-Cdc42 antibody (1:200). Detection was
118 carried out using peroxidase-conjugated secondary antibodies (goat anti-mouse or goat anti-rabbit,
119 1:5000) and enhanced chemiluminescence reagents (BeyoECL Plus, Beyotime, China). Blots were
120 imaged by Molecular Image® ChemiDoc™ XRS+ with Image Lab™ Software (Bio-Rad, USA).
121 Quantitative data were obtained by using ImageJ 1.50b Gel Analyzer.

122 **Visualization of actin cytoskeleton and confocal microscopy**

123 Immediately after treatments, HUVECs were fixed in 2% paraformaldehyde, permeabilized with
124 1% Triton X-100, and stained with BODIPY® FL phallacidin (Invitrogen, USA) to visualize the
125 actin cytoskeleton. Cell nuclei were stained by DAPI. All samples were imaged with a Leica TCS
126 SP5 laser scanning confocal microscopy (Sichuan University). The max-intensity Z-projection
127 images were shown as described previously (Zeng et al. 2014; Zeng et al. 2012a; Zeng & Tarbell
128 2014; Zeng et al. 2013).

129 **Statistical analysis**

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

134

135 **Results**

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

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

144 **Heparan sulfate mediates the regulation of Cdc42 by CXCL8 in HUVECs**

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

151 **Heparan sulfate mediates the regulation of Rac1 by CXCL8 in HUVECs**

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

156 **Heparan sulfate mediates the regulation of RhoA by CXCL8 in HUVECs**

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

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

162 **Remodeling of actin cytoskeleton by CXCL8 is also heparan sulfate mediated**

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

172

173

174 **Discussion**

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

179 It was demonstrated that GAGs interact selectively with chemokines (Kuschert et al. 1999). The
180 residues, including H18, K20, R60, K64, K67 and R68 in CXCL8 that participated in its interaction
181 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

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

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

225

226 **Conflict of interest**

227 None declared.

228

229 **Acknowledgement**

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233 Sichuan University of China.

234

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298

299

300 **Figure Legends**

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

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

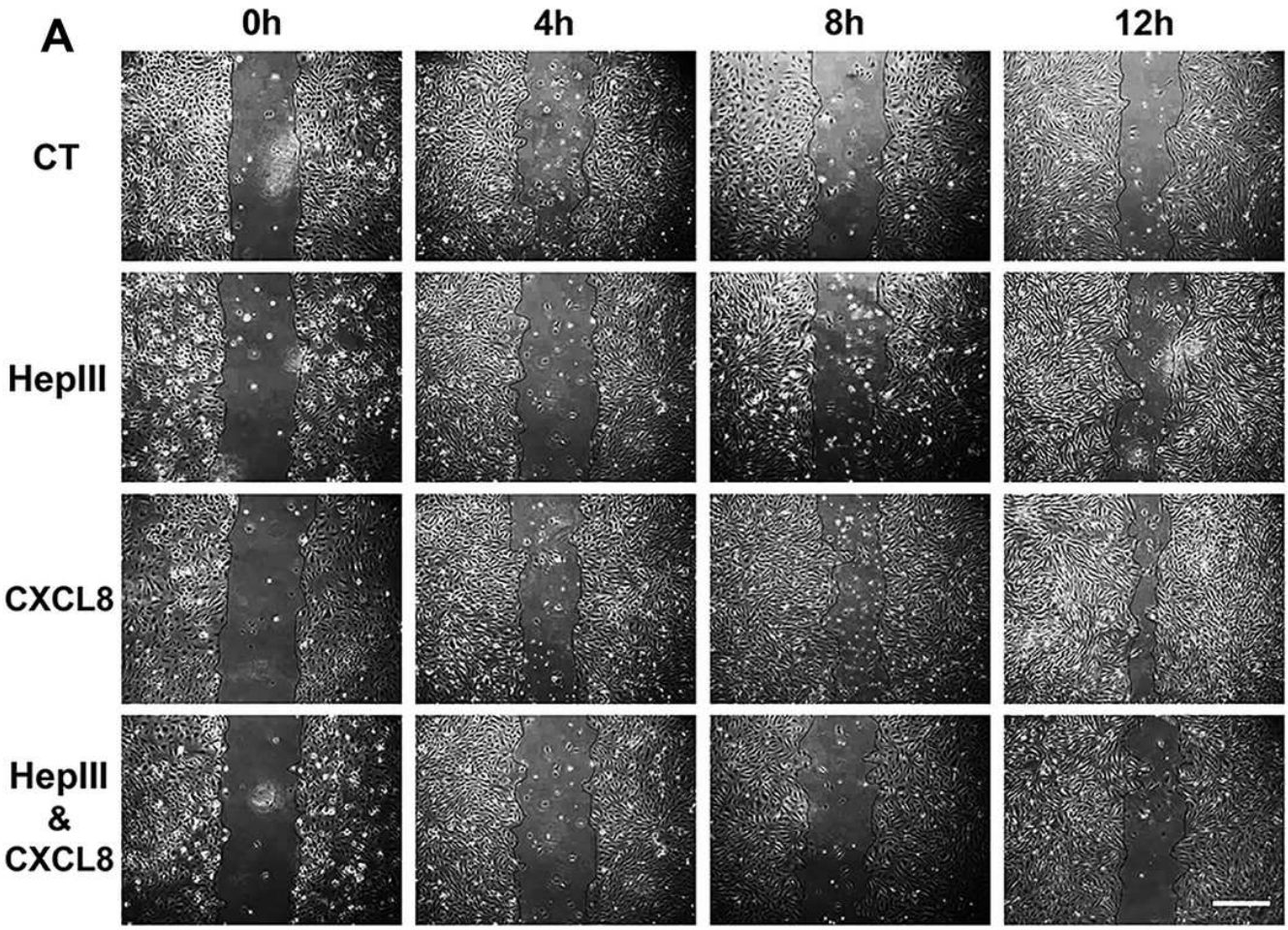
317 **Figure 3. Heparinase III inhibited the CXCL8-induced actin cytoskeleton reorganization.**
318 HUVECs were treated with with 15 mU/ml heparinase III (HepIII), 100 ng/ml CXCL8, or both
319 for indicated times (4 and 12 h), respectively. Normal cell without treatment was set as control
320 (CT). Then,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

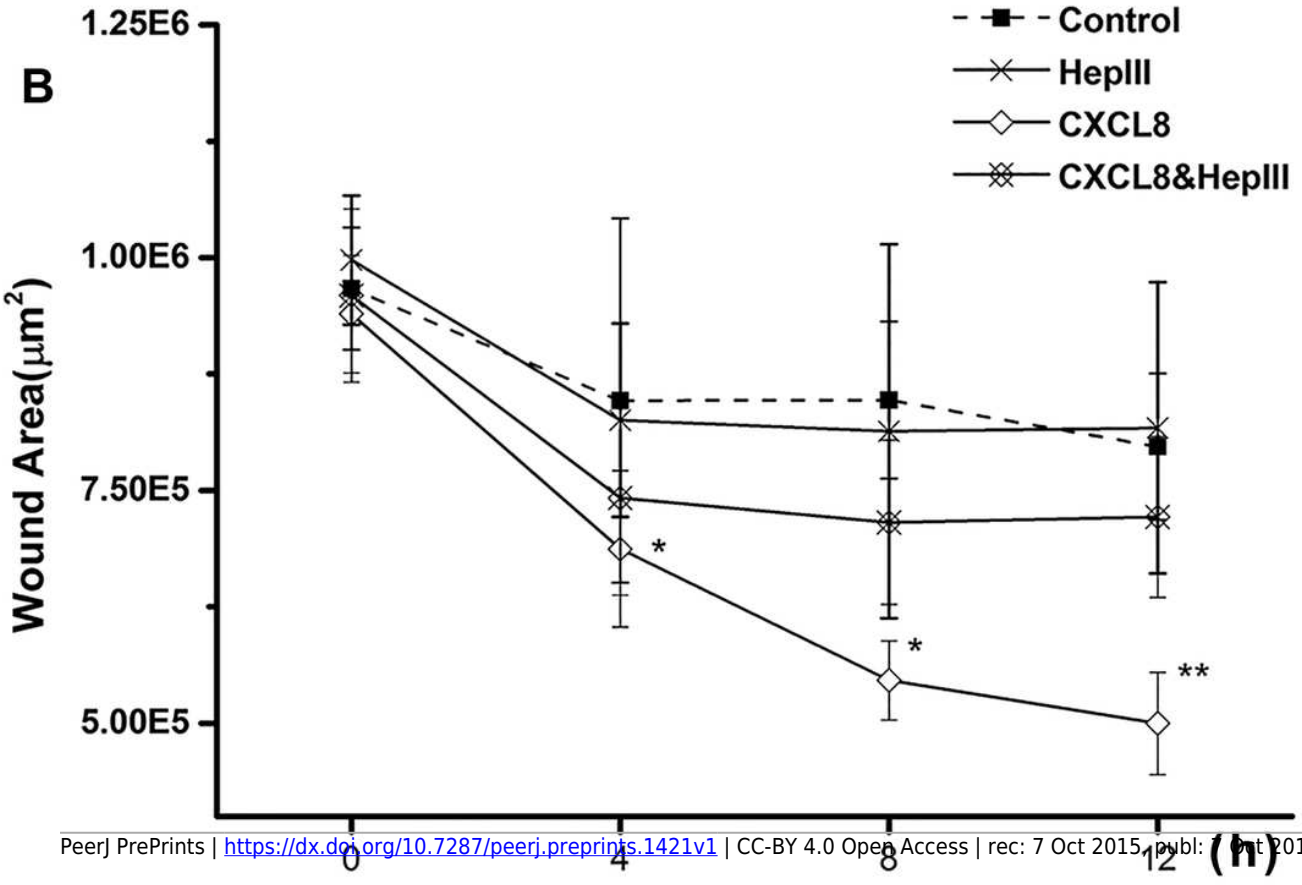
Scale bar: 50 μm.

1

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. "





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2

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; #$P < 0.05$.

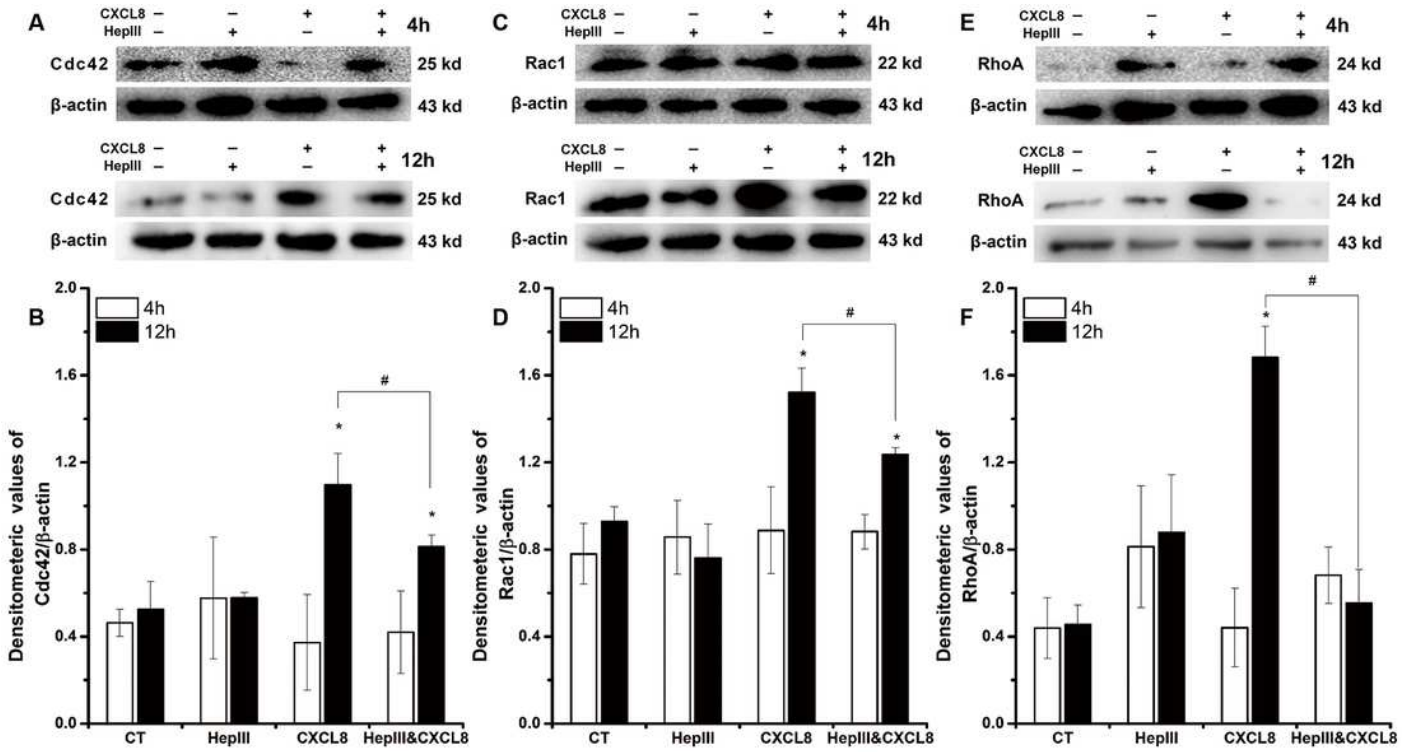

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Figure 3

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.