YKL-40 Enhanced the Permeability of HDMECs with Histamine Through Activating Akt and p38 Pathways

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Research

Keywords: YKL-40, CSU, HDMECs, permeability, VE-cadherin

DOI: https://doi.org/10.21203/rs.3.rs-674027/v1

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Abstract

Background, YKL-40 is currently considered as an important marker of endothelial dysfunction. Chronic spontaneous urticaria (CSU) is a common vascular skin disease. The increased vascular permeability play an important role in the occurrence and pathogenesis of CSU.

Objective, the aim of this study is to explore the role of YKL-40 on the permeability of HDMECs.

Methods, in this study, the mRNA level of YKL-40 in human mast cell line (HMC-1) were detected by RT-PCR. The effects of YKL-40 on vascular permeability, VE-cadherin release, VE-cadherin disruption in human dermal microvascular endothelial cells (HDMECs) were investigated by transwell, ELISA or immunofluorescence. The phosphorylation of VE-cadherin, p38 and Akt, in histamine plus YKL-40 treated HDMECs were detected by Western Blot.

Results, we found that YKL-40 signicantly promoted the permeability changes and leaded to the released, disruption of VE-cadherin in HDMECs induced by histamine. Furthermore, YKL-40 also enhanced the Akt and p38 pathways.

Conclusion, we suggest that YKL-40 may serve as pro-permeability cytokines, and play a role in the pathogenesis of CSU. This study will help to further elucidate the pathogenesis of CSU and provide a new target for the development of anti-histamine resistance drugs for CSU.

Introduction

Chronic spontaneous urticaria (CSU), a common vascular skin disease with high incidence and recurrent attacks, is one of the skin diseases that seriously affect the quality of patients’ life(1). Some CSU patients have resistance to treatment. Although the pathogenesis of CSU is complex and not yet fully clear, lots of studies had demonstrated that histamine is the major mediator responsible for CSU. Histamine and other vasoactive substances, released by mast cell degranulation, can ultimately lead to increased vascular permeability, which is considered to be the central link in the pathogenesis of CSU. Bossi et al had reported that the supernatant of human mast cell line (HMC-1), which treated with serum from CSU patients, could increase the permeability of human dermal microvascular endothelial cells (HDMECs)(2).

The stability of vascular permeability depends on the normal function of vascular endothelial barrier. As an important intercellular adhesion molecule, vascular endothelial cadherin (VE-cadherin) plays an important role in the changes of vascular permeability induced by various vasoactive substances (including histamine)(3). The function of endothelial barrier depends on the correct location and function of VE-cadherin. VE-cadherin at the endothelial junction stabilizes the function of endothelial barrier under the action of integrin β1 of endothelial cells(4). During vascular injury, VE-cadherin of endothelial cells can be degraded, part of which enters the cytoplasm through endocytosis, and part of which will dissociate into the blood and form soluble VE-cadherin through hydrolysis.
YKL-40, also described with human cartilage glycoprotein-39 and chitinase-3-like-1, can be secreted and expressed by a series of inflammatory-related cells, including tumor cells, vascular smooth muscle cells, mature neutrophils, articular synovial cells, chondrocytes, hepatic stellate cells, colon epithelial cells, airway epithelial cells and tubular epithelial cells. YKL-40 plays an important role in cell proliferation, microangiogenesis, acute or chronic inflammation. Clinical studies showed that YKL-40 is closely related to many diseases, such as tumors(5), rheumatoid arthritis(6), coronary artery disease(7), pneumonia(8), liver fibrosis(9), chronic pancreatitis(10) and other diseases. The serum level of YKL-40 was increased in many allergic diseases, such as atopic dermatitis(11), asthma(12), and was positively related to the severity of these disease. The expression of YKL-40 in peripheral blood of patients with psoriasis(13) and diabetes(10) were also significantly increased. Therefore, YKL-40 is currently considered to be an important marker of endothelial dysfunction. Our previous study examined the serum levels of YKL-40 in patients with CSU and healthy controls. We found that the serum YKL-40 levels were significantly increased in patients with CSU compared with healthy controls, which indicated that YKL-40 may play an important role in the pathogenesis of CSU(14).

To explore the role of YKL-40 in pathogenic mechanism of CSU, this study investigated the effects of YKL-40 on histamine-stimulated HDMECs in this study.

**Materials And Methods**

**Cell Culture**

HMC-1 cells were cultured in IMDM with 100 U/ml of penicillin and streptomycin, and 10% fetal bovine serum (FBS) at 37°C in 5% CO₂. The cells were stimulated with 50 nM of phorbol 12-myristate 13-acetate (PMA) plus 1 μM of A23187 (calcium ionophore) and incubated at 37°C for 8 h.

The HDMECs were cultured in DMEM medium supplemented with 10% PBS, 100 U/mL of penicillin, and 100 U/mL of streptomycin. In this study, HDMECs were seeded in plates at 1 x 10⁵ cells/cm². After a 48-hour incubation, HDMECs were treated with recombinant human YKL-40 (10-500 ng/ml) or histamine plus YKL-40 (10-500 ng/ml) for different times.

**Real-time quantitative PCR**

HMC-1 cells were seeded in a 6-wells plate and then stimulated with 50 nM of PMA plus 1 μM of A23187 and incubated at 37°C for 8 h. PBS were as controls. The mRNA levels of YKL-40 were determined by real-time quantitative PCR. Total RNA was extracted by Trizol reagent and the cDNA was synthesized from the total RNA by using RT reagent Kit with gDNA Eraser (Takara, Dalian, China). The following primers were used: YKL-40 (5′-AATTCGGCCTTCTTCTTTCC-3′, and 5′-GATAGCCTCAACACCCAGA-3′), GAPDH (5′-CGGAGTCAACCGGATTTGTCG-3′ and 5′-CGGTGCCAATGGATTTGCCA-3′). The mRNA levels of YKL-40 were expressed as relative mRNA levels compared with control and determined by the 2^ΔΔCt method.

**Measurement of vascular permeability**
As described methods(15), HDMECs were cultured on the upper chamber of transwell (Transwell membrane, 0.4 μM pore size; Corning Costar). After 48 h, the integrity and homogeneity of the endothelial monolayer were examined by microscope. The confluent monolayers were incubated with histamine (100 μmol/L), YKL-40 (500 ng/ml), histamine (100 μmol/L) plus YKL-40 (10-500 ng/ml) for 2 h. Antihistamine (loratadine or ranitidine, 10 μmol/L) were pretreated for 20 minutes. After treatment, FITC- dextran (1mg/ml) was added into the upper chambers, and fluorescence in the lower chamber was measured one hour later with fluorescence reader.

**Assay for soluble VE-cadherin levels**

Levels of soluble VE-cadherin in cultured supernatants of HDMECs were detected with Human VE-Cadherin ELISA Kit (Boster Biological Technology, Wuhan, China. Catalog #EK1317) according to the manufacturer’s instruction.

**Immunofluorescence**

The expression of VE-cadherin in HDMECs was detected by immunofluorescence. HDMECs, grown on glass coverslips, were treated with histamine (100 μmol/L), YKL-40 (500 ng/ml), histamine (100 μmol/L) plus YKL-40 (10-500 ng/ml) for 2 h. Antihistamine (loratadine or ranitidine, 10 μmol/L) were pretreated for 20 minutes. Cells were fixed by 4% paraformaldehyde for 1h, and then incubated with VE-cadherin antibody at 4°C overnight (1:50 dilution; Cell Signaling Technology). 4’-6-diamidino-2-phenylindole (DAPI) was used to counterstain the nuclei for 5 minutes. Fluorescence images were captured by laser scanning confocal microscopy (Olympus, Tokyo, Japan).

**Western Blot Analysis**

The expression of VE-cadherin, akt and mitogen-activated protein kinases (MAPKs) in HDMECs was measured by western blot. HDMECs were treated with histamine (100 μmol/L), YKL-40 (500 ng/ml), histamine (100 μmol/L) plus YKL-40 (10-500 ng/ml) for 15 min. Antihistamine (loratadine or ranitidine, 10 μmol/L) were pretreated for 20 minutes. After treatment, total protein was extracted. Protein samples of 40 mg were electrophoresed on 12% or 6% Tris-glycine gels, subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride membranes. Subsequently, membranes were incubated with primary antibody at 4°C overnight and with the appropriate HRP-conjugated secondary antibody for 1 hour. The expression of VE-cadherin, akt and MAPKs (ERK, JNK and p38) was determined with enhanced chemiluminescence reagents. The results were normalized to the expression of β-actin.

**Statistical analysis**

The data showed in Table 1 and Figure1 were expressed as median ± interquartile range with Mann-Whitney test and Wilcoxon sign-rank test. Other datas were expressed as mean ± SD. One-way analysis of variance was used to compare statistical differences between groups. P < 0.05 was set as the statistically significant.
Results

Increased YKL-40 levels in HMC-1 stimulated by PMA and A23187

To explore the origin of YKL-40 in pathogenesis of CSU, we assayed mRNA expression of YKL-40 in HMC-1 stimulated by PMA and A23187. As presented in Figure 1, the mRNA levels of YKL-40 in HMC-1 stimulated by PMA and A23187 were markedly increased when compared with PBS controls (p<0.01).

Effect of YKL-40 on the permeability of HDMECs

As described in the method, the amount of FITC-dextran in the lower chamber leaked from the HDMECs layer was detected to scale the permeability of HDMECs. The permeability of HDMECs with different treatments was quantified by the percentage of OD490 change. As shown in Figure 2A, YKL-40 treated alone had no effect on the permeability of HDMECs. Figure 2B showed that YKL-40 treated with histamine could significant enhanced the permeability of HDMECs when compared with histamine alone group. This study also investigated the effects of different antihistamines on the permeability of histamine-YKL-40-treated HDMECs. Our data suggested that H1 antihistamine (loratadine) or H2 antihistamine (ranitidine) cannot suppress the hyperpermeability of HDMECs induced by histamine plus YKL-40 when compared with the histamine alone group.

YKL-40 induces sVE-cadherin release from HDMECs

The previous study had indicated histamine could induce sVE-cadherin release in HMEC-1 cells and sVE-cadherin levels was reached their peak value after 20 minutes to 4 hours. As presented in Figure 3, sVE-cadherin levels of histamine treated alone group were significantly increased when compared with PBS control group. Moreover, sVE-cadherin levels of YKL-40 treated with histamine together group were significantly increased when compared with histamine group. This study also investigated the effects of different antihistamines on sVE-cadherin release in histamine-YKL-40-treated HDMECs. HDMECs were pretreated with different antihistamines (10 μmol/L for 20 minutes) and then incubated with 100 μmol/L of histamine and 500 ng/ml of YKL-40 for 2 h. As shown in Figure 3, H1 antihistamine (loratadine) or H2 antihistamine (ranitidine) cannot block the release of sVE-cadherin in histamine-YKL-40-treated HDMECs when compared with the histamine alone group.

YKL-40 leads to VE-Cadherin Disruption

The disruption of VE-cadherin in HDMECs treated by histamine plus YKL-40 was observed by double staining for FITC-VE-cadherin and DAPI. As presented in Figure 4A, abundant expression of VE-cadherin was observed in control HDMECs. Obvious VE-cadherin cleavage was found in histamine-treated HDMECs. And the more disruption of VE-cadherin was observed in histamine plus YKL-40-treated cells. In contrast, H1 antihistamine (loratadine) and H2 antihistamine (ranitidine) cannot attenuated the change.

YKL-40 induces VE-Cadherin phosphorylation
The expression of phosphorylated VE-cadherin in HDMECs was detected by western blot. As presented in Figure 4B, histamine upregulated expression levels of phosphorylated VE-cadherin in HDMECs. The expression levels of phosphorylated VE-cadherin in histamine-YKL-40-treated HDMECs were obviously enhanced when compared to the HDMECs induced by histamine alone. Meanwhile, H1 antihistamine (loratadine) and H2 antihistamine (ranitidine) cannot block the phosphorylation of VE-cadherin in histamine-YKL-40-induced HDMECs.

**Effects of YKL-40 on activation of Akt and p38**

To evaluate the mechanisms underlying the effects of YKL-40, we examined the potential effects of IL-35 on activation of Akt and MAPKs (ERK, JNK and p38). As shown in Figure 4C, the treatment of HDMECs with histamine plus YKL-40 resulted in an increased phosphorylation of Akt and p38 after 15 min. H1 antihistamine (loratadine) and H2 antihistamine (ranitidine) cannot attenuate histamine plus YKL-40-induced phosphorylation of Akt and p38.

**Discussion**

YKL-40 plays an important role in cell proliferation, microangiogenesis, acute or chronic inflammation and it is currently considered to be an important marker of endothelial dysfunction. Salomon et al had reported that YKL-40 serum level is increased in patients with atopic dermatitis and reflects the severity of symptoms(11). The enhanced levels of YKL-40 had also been found in many diseases such as asthmatic, psoriasis, secondary diabetes and peripheral artery disease. Our previous study examined the serum levels of YKL-40 in patients with CSU and healthy controls. We found that the serum YKL-40 levels were significantly increased in patients with CSU compared with healthy controls, which indicated that YKL-40 may play an important role in the pathogenesis of CSU(14).

YKL-40 can be secreted by a series of inflammatory-related cells, including tumor cells, vascular smooth muscle cells, mature neutrophils, articular synovial cells. Mast cells, as one of the major effector cells in CSU, play an integral role in the inflammatory response by accumulating at sites of inflammation and mediating the production of inflammatory cytokines(16). In this study, we found YKL-40 can be secreted by activated HMC-1 cells which stimulated by PMA and A23187.

CSU is a common vascular skin disease. Abundant evidence had indicated that increased vascular permeability play an important role in the occurrence and pathogenesis of CSU. Recent studies have found that YKL-40 can up-regulate the expression of vascular endothelial growth factor (VEGF) in vascular endothelial cells by inducing the coordination of membrane-bound receptor syndecan-1 and integrin αvβ3, thus promoting the formation of microvessels in vascular endothelial cells(17). It is suggested that YKL-40-related receptors could be expressed in vascular endothelial cells. Muszyski et al suggested that YKL-40 can decrease the stability of blood-brain barrier and increase vascular permeability in patients with Alzheimer's disease(18). However, the effect of YKL-40 on skin vascular permeability in CSU patients is still not reported. In this study, the effect of YKL-40 on the permeability of
HDMECs was detected. Our results suggested that YKL-40 alone cannot affect the permeability of HDMECs. However, YKL-40 could promote the permeability of HDMECs induced by histamine.

VE-cadherin is an important intercellular adhesion molecule, which plays an important role in maintaining and restoring the function of endothelial barrier. The destruction or decomposition of VE-cadherin can further lead to hemorrhage and inflammatory cell infiltration. Studies have shown that VE-cadherin can release a soluble fragment into the blood under some pathological conditions involving vascular injury. Chen et al had indicated that the serum soluble VE-cadherin in patients with CSU was significantly higher than those in patients with atopic dermatitis and healthy controls, and it is positively correlated with the disease score(19). They also found histamine could induce the release of soluble VE-cadherin in endothelial cells. However, the effect of YKL-40 synergistic with histamine to increase the permeability of HDMECs on the normal expression of VE-cadherin and the release of soluble VE-cadherin has not been reported. In this study, our data indicated that YKL-40 treated with histamine together could significantly increased the released sVE-cadherin levels when compared with histamine group. And we also found that histamine plus YKL-40 could lead to the more disruption of VE-cadherin in HDMECs observed by double staining for FITC-VE-cadherin and DAPI. The expression levels of phosphorylated VE-cadherin in histamine-YKL-40-treated HDMECs were obviously enhanced when compared the HDMECs induced by histamine alone.

Because endothelial barrier dysfunction and VE-cadherin expression are often associated with the activation of MAPKs and phosphoinositide 3-kinase (PI3-K)/AKT pathway(20; 21). To evaluate the mechanisms underlying the effects of YKL-40, we examined the potential effects of IL-35 on activation of Akt and MAPKs (ERK, JNK and p38). As shown in Fig. 5C, the treatment of HDMECs with histamine plus YKL-40 resulted in an increased phosphorylation of Akt and p38 after 15 min.

Although the second generation antihistamines are used as the first-line treatment for CSU in domestic and foreign guidelines, there are still some intractable cases that are ineffective for various antihistamines. In our study, we also study the treatment effect of H1 antihistamine (loratadine) and H2 antihistamine (ranitidine) on the HDMECs treated with histamine plus YKL-40. However, H1 antihistamine (loratadine) and H2 antihistamine (ranitidine) cannot attenuate the effect including the permeability changes and the expression of VE-cadherin. It is suggested that YKL-40 may be an inflammatory molecule that is not inhibited by antihistamines, which mediates the increase of vascular permeability and thus induces the occurrence and development of CSU.

Taken together, this study provides first observations on the association of YKL-40 and CSU, and showed the increased YKL-40 serum levels in CSU patients. In vitro, YKL-40 significantly promoted the permeability changes and leaded to the released, disruption of VE-cadherin in HDMECs induced by histamine. Furthermore, YKL-40 also enhanced the Ark and p38 pathways. Therefore, we suggest that YKL-40 may serve as pro-permeability cytokines, and play a role in the pathogenesis of CSU. This study will help to further elucidate the pathogenesis of CSU and provide a new target for the development of anti-histamine resistance drugs for CSU.
Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and materials: All data generated or analysed during this study are included in this published article

Competing interests: The authors declare no conflict of interest.

Funding: The study was supported by Health and Family Planning Commission of Sichuan Provincial (130409).

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manuscript review: Lin Wang

Acknowledgements: Not applicable

Conflict of interest statement

The authors declare no conflict of interest.

Clinical Trial Registration:

Trial registration: Not applicable

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**Tables**

Table 1 is not available with this version

**Figures**

![Bar Chart: YKL40 Expression](chart.png)
Figure 1

Enhanced mRNA expression of YKL-40 in HMC-1 cells stimulated by PMA and A23187. HMC-1 cells were stimulated with PMA (50 nM) plus A23187 (1 μM) for 8 h. After that, mRNA expression of YKL-40 in HMC-1 cells were measured by Real-time quantitativePCR. The mRNA expression of YKL-40 in simulated HMC-1 were significantly increased when compared with control cells. All data are expressed as Mean ± SD. P values are based on the t test. n = 10. **P <0.01.

Figure 2

YKL-40 enhanced the permeability of HDMECs induced by histamine. As described in the Methods, the OD490 in the lower chamber were calculated for displaying the permeability of HDMECs. A: Effect of YKL-40 at different concentrations on the permeability of HDMECs. B: Effect of YKL-40 and Histamine on the permeability of HDMECs. #P<0.05 compared to the control group. *P<0.05 compared to the group only treated with histamine.
Figure 3

YKL-40 increased the sVE-cadherin release from HDMECs cells induced by histamine. HDMECs were treated with histamine (100 μmol/L), YKL-40 (500 ng/ml), histamine (100 μmol/L) plus YKL-40 (10-500 ng/ml) for 15 min. Antihistamine (loratadine or ranitidine, 10 μmol/L) were pretreated for 20 minutes. H1 antihistamine (loratadine) or H2 antihistamine (ranitidine) cannot block the release of sVE-cadherin in histamine-YKL-40-treated HDMECs when compared with the histamine alone group. Values were expressed as mean ± SD. One-way analysis of variance was used to compare statistical differences between groups. ###P<0.0001 compared to the control group. **P<0.01 compared to the group only treated with histamine.
YKL-40 promoted the VE-cadherin phosphorylation and VE-cadherin disruption and increased phosphorylation of Akt and p38 in HDMECs induced by histamine. HDMECs were treated with histamine (100 μmol/L), YKL-40 (500 ng/ml), histamine (100 μmol/L) plus YKL-40 (10-500 ng/ml) for 15 min. Antihistamine (loratadine or ranitidine, 10 μmol/L) were pretreated for 20 minutes. (A) After treatment, the expression of VE-cadherin in HDMECs was observed by immunofluorescence double staining for FITC-VE-cadherin and DAPI. (B) Afterward, protein samples were obtained to measure expression levels of phosphorylated VE-cadherin by western blots. (C) Phosphorylation of Akt and p38 MAPKs was analyzed by western blotting.
Figure 5

Figure not available in this version