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Article type : Letter to the Editor

Title;

Increase of tissue factor expression on the surface of peripheral monocytes of patients with chronic spontaneous urticaria

To the Editor,

The involvement of the coagulation cascade has been demonstrated in the pathogenesis of chronic spontaneous urticaria (CSU) by a number of observations, including successful treatment of CSU with anticoagulants[1,2]. We previously reported that blood coagulation potential is elevated in CSU, and that histamine and agonists for toll-like receptors (TLRs) may synergistically induce tissue factor (TF) expression on vascular endothelial cells[3,4]. Immunohistochemical staining revealed TF expression by eosinophils in the lesion of CSU[5]. However, monocytes are considered to be the main source of TF in the blood, causing clot formation[6]. We here investigated the level of TF expression on monocytes in the blood of patients with CSU, and the potential of TF

on monocytes to trigger the extrinsic coagulation cascade and consequential inter-cellular gap formation of vascular endothelial cells.

Peripheral Blood Mononuclear Cells (PBMC) were obtained from 79 patients and 10 healthy donors. No statistical differences were detected between the groups in terms of age and sex by the Mann Whitney test and the Chi-square test, respectively (Table S1). Clinical features of patients with CSU are summarized in Table S2. Based on the cytometric analysis, TF expression on monocytes in CSU patients was significantly higher than that in healthy donors (Fig 1a). We then investigated TF expression on monocytes in response to TLR agonists and/or histamine *in vitro*. Monocytes were stimulated with various TLR agonists and/or histamine. As shown in Fig 1b, c, monocytes from normal donors stimulated by agonists for TLR 1,2,4,5 enhanced both TF mRNA and TF protein expressions, whereas those by agonists for TLR 3,6,7,8,9 and histamine did not.

Considering the association of *Helicobacter pylori* infection and CSU in many reported cases, we initially studied the effect of Lipopolysaccharide (LPS), which derives from Gram-negative bacteria and activates TLR4, on the TF expression of monocytes. The expression of TF mRNA and cell surface TF protein by the monocytes from normal donors increased in response to LPS stimulation in a dose-dependent manner (Fig S1a, b). To test if the LPS-induced TF of monocytes have procoagulant activity, monocytes isolated from normal donors were stimulated with LPS and measured for their TF activity. TF activity assay showed an enhanced activity of TF in an LPS-dose-dependent manner from the range of 0.1 pg/mL to 100 pg/mL (Fig 1e).

To evaluate the effect of TF of monocytes on vascular permeability, we performed impedance analysis, which reflects the area of cellular adhesion and morphology the electrodes as Cell Index (CI)[4]. In Fig. 2, the black line shows the decrease of CI, which reflects inter-cellular gap formation of histamine- and LPS-treated human umbilical vein epithelial cells (HUVECs) cultured on the electrodes in the presence of factor VIII-deficient plasma (positive control). A decrease of CI was also observed in the presence of sufficient numbers of TF-expressing monocytes from normal donors (10^4 cells / well). However, CI did not decrease in the presence of

a lower number of monocytes (10^3 cells /well) treated with LPS and factor VIII-deficient plasma, or in the presence of a large numbers of unstimulated monocytes (10^3 cells or 10^4 cells /well) and factor VIII-deficient plasma. Moreover, a CI decrease was not observed in the presence of LPS-activated monocytes and factor X-deficient plasma.

These results suggest that LPS increased TF expression on monocytes resulting in the activation of factor X-dependent, but factor VIII-independent, coagulation cascade followed by the gap formation of vascular endothelial cells. Because of the increase of the intrinsic coagulation potential in CSU[3)], the activation of factor X and II in the common pathway of coagulation, and the induction of vascular endothelial cells gap formation by these factors may be further enhanced in CSU.

Autoantibodies against IgE or its receptor (FcεRI) on mast cells are known to be involved in the pathogenesis of CSU. Moreover, the presence of histamine-releasing factors has also been reported in the plasm of patients with CSU [7,8)]. We presume that plasma leakage caused by the activation of the coagulation cascade triggered by TF expressed on either endothelial cells or monocytes allows these factors to move out to the extravascular space and encounter dermal mast cells, which then degranulate and cause massive vascular hyperpermeability and wheal formation. It is still unclear if these factors activate dermal mast cells only in the skin. Skin specific molecules, such as mas-related G-protein coupled receptor member X2 (MRGPRX2) expressed by mast cells only in the skin, may also be necessary for wheal formation in urticaria. In any case, it is noteworthy that TF expression on endothelial cells, but not monocytes may be inhibited by H_1-antihistamines, and thus, may account for the pathomechanism of CSU refractory to H_1-antihistamines.

An association of chronic infection and CSU has been described for a long time, and the eradication of *Helicobacter pylori* may bring about resolution of urticaria [9)]. In this study, LPS and FLA-ST, more efficiently enhanced TF expression than Pam3CSK4 and HKLM. Since LPS and FLA-ST are components of gram-negative bacteria, and HKLM is from gram-positive bacteria, the effect of chronic infection of gram-negative bacteria,

such as *Helicobacter pylori,* may be more dominant than that of gram-negative bacteria in the pathogenesis of antihistamine-resistant CSU via the induction of TF expression on monocytes.

Development of acute urticaria after bacterial or viral infection is commonly experienced in clinical practices [10]. However, in most cases, it disappears within a few weeks. Therefore, there are likely to be other causal mechanisms underlying the pathogenesis of the chronic stage of spontaneous urticaria. Since the activation of TLRs transduces various cascades of the cells, other stimuli that share intracellular signal transductions with TLRs that induce monocyte TF expression may also contribute to the histamine-resistant or partially resistant CSU.

In conclusion, monocyte TF expression is enhanced in CSU patients compared to healthy donors. It may be induced by agonists for TRL-1,2,4,5 so as to trigger the exogenous coagulation pathway and vascular hyperpermeability in a histamine-independent manner. Consequently, TF expression on monocytes may be utilized as a marker for CSU pathological conditions, and as a therapeutic target for severe and refractory CSU.

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Conflict of interest; none declared.

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Fig 1. TF expression and activity on monocytes

The relative fluorescence index (rFIs) of monocytes of healthy controls and patients were plotted, respectively. Relative fluorescence index = (median fluorescence intensity (MFI) anti-TF antibody - MFI of isotype control) /MFI isotype control. Statistical significance between two groups was determined by Mann-Whitney U test. (a)

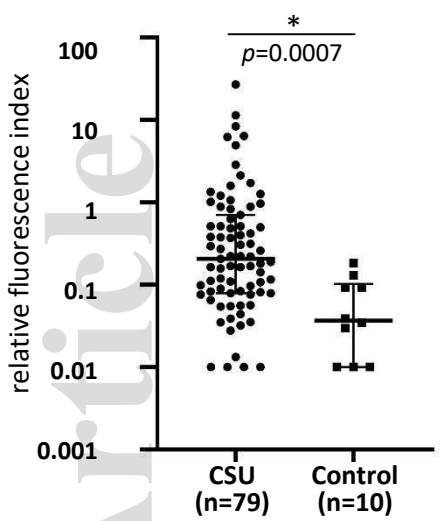
The expression of mRNA (b,d) and surface protein of TF (c) on monocytes isolated from healthy donors. Monocytes were stimulated with TLR1-9 agonists and/or histamine. TF expression on monocytes was enhanced by stimulation with TLR 1, 2, 4, 5 agonists.

TF procoagulant activity increased in a dose-dependent manner and reached a peak at 100 pg/mL of LPS stimulation. (e)

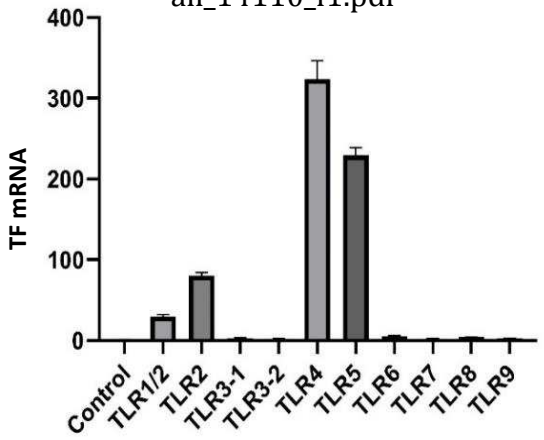
Fig 2. Enhanced TF expression on monocytes increases inter-cellular gap formation of HUVECs in the presence of plasma

CI declined in the presence of TF-expressing monocytes treated with LPS and factor VIII deficient-, but not factor X deficient-plasma.

(a)

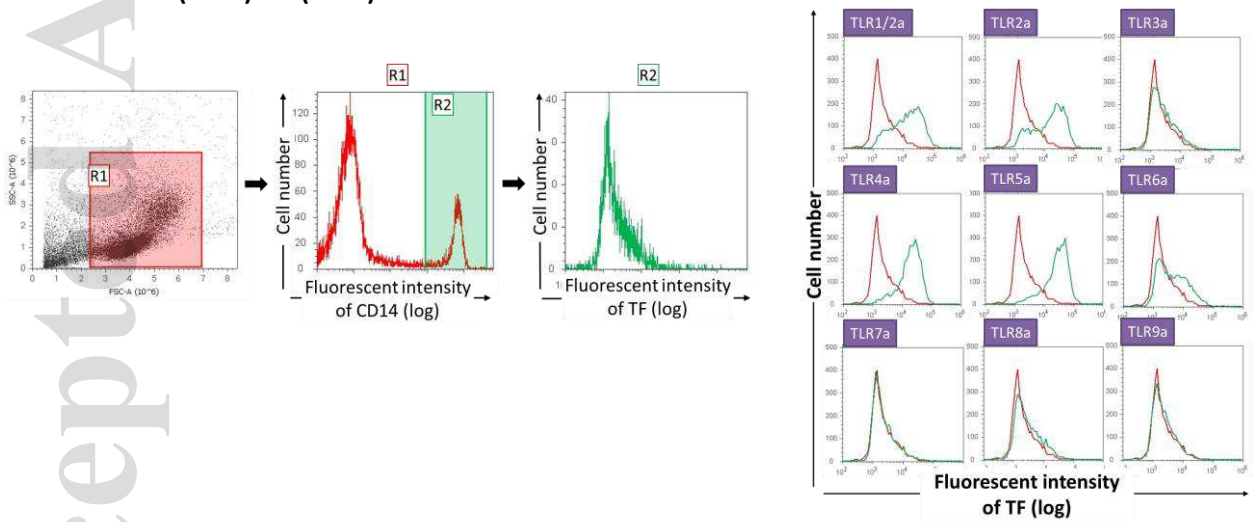


(b)

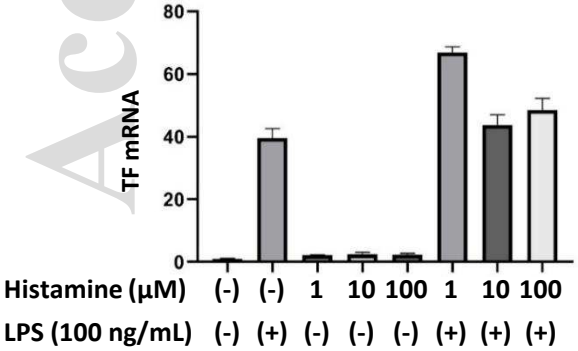


TLR1/2 agonist : Pam3CSK4 (1 μg/mL)
TLR2 agonist : HKLM (10^8 cells)
TLR3 agonist-1 : Poly(I:C) HMW (10 μg/mL)
TLR3 agonist-2 : Poly(I:C) LMW (10 μg/mL)
TLR4 agonist : LPS-EK standard (10 μg/mL)
TLR5 agonist : FLA-ST standard (1 μg/mL)
TLR6/2 agonist : FSL-1 (1 μg/mL)
TLR7 agonist : Imiquimod (1 μg/mL)
TLR8 agonist : ssRNA40 (1 μg/mL)
TLR9 agonist : ODN2006 (5 μM)

(c)



(d)



(e)