Elevated Levels of Soluble and Neutrophil CD146 in Active Systemic Vasculitis

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Abstract

Background: Cell adhesion molecules have been implicated to be up-regulated in systemic vasculitis and could be potentially used as clinical diagnostic biomarkers. As a newly identified adhesion molecule, the expression level of CD146 in vasculitis has never been determined.

Methods: Clinical samples were collected and levels of soluble CD146 were measured in sera using sandwich ELISA and neutrophil CD146 using FACS analysis in 59 patients with active systemic vasculitis and 34 healthy controls.

Results: Expression of CD146 was significantly elevated in circulating neutrophils of patients with active small-vessel vasculitis (WG and MPA), but not in patients with large-vessel vasculitis (TA), compared with samples from healthy controls. Interestingly, elevated levels of neutrophil CD146 were dramatically decreased after treatment with immunosuppressive drugs. Moreover, a significantly increased level of soluble CD146 was found in sera from patients with MPA and vasculitis-induced renal failure.

Conclusion: The clinical study indicated that CD146 expression was elevated in active vasculitis and correlated with its pathological progression and post-treatment remission.

Vasculitis is a common autoimmune disease involving the inflammation of blood vessels characterized as severe vascular damage caused by inflammatory mediators, such as NO, ROS, and cytokines, which are mainly released by circulating leukocytes pathologically recruited to the inflammation site.[1,2] Inflammatory cytokines, such as IL-6 and TNF, could stimulate the production of C-reactive protein (CRP),[3] which in turn up-regulates expression of cell adhesion molecules (CAMs) in both leukocytes and endothelial cells[4] and then promotes the adhesion of circulating leukocytes to endothelium and followed transendothelial migration.[5] Thus, accumulation of CAMs, including PECAM-1, ICAM-1, VCAM-1, and E-selectins on leukocytes (especially neutrophils) is a very important hallmark for early vasculitic lesion. However, the molecular mechanism for active systemic vasculitis is still complicated and some novel players, particularly the ectopically expressed membrane protein mediating neutrophil-endothelium contact, need to be identified.

CD146, a cell adhesion molecule belonging to the Ig superfamily, was originally identified as a melanoma progression marker[6,7] and later recognized as an endothelial biomarker.[8-10] Our previous studies found that endothelial CD146 is involved in neovascular formation, especially tumor angiogenesis,[11-13] suggesting a role of CD146 in vascular biology. Moreover, significantly elevated levels of soluble CD146 (sCD146) have been found in synovial fluid of patients with rheumatoid arthritis[14] and in patients with chronic renal failure.[15] These observations indicated that CD146 is probably involved in chronic inflammatory disease. More importantly, CD146 has been recently found to be involved in the initial steps of lymphocyte-endothelium interaction,[16] which proposed the possible mechanism of how CD146 takes part in the inflammation process. However, the expression level of CD146 in vasculitis and whether CD146 participates in the inflammatory reaction of vasculitis are still undetermined.

Materials and Methods

Patients and Samples

In the current study, 59 patients at Peking Union Medical College Hospital with systemic vasculitis were classified as Wegener's granulomatosis (WG, n=24, 36 ± 13 years, 7 males), microscopic polyangiitis (MPA, n=21, 43 ± 14 years, 9 males), and Takayasu's arteritis (TA, n=14, 35 ± 14 years, 7 males) according to the American College of Rheumatology (ACR) 1990 criteria. In addition, 34 healthy volunteers from this hospital (H, 40 ± 16 years, 18 males) served as controls. Disease activity of systemic vasculitis was evaluated using the Birmingham vasculitis activity score (BVAS).[17] Patients with a BVAS above 5 were considered active, while those with scores lower than 1 were defined as remission. The serum CRP concentration and erythrocyte sedimentation rate (ESR) were measured as biochemical proof. The histological diagnosis showed that among the 59 patients, 14 MPA patients and 5 WG patients had renal failure. All patients who participated in the study provided informed consents.

Peripheral blood from each patient was sampled twice, before and after 2 to 4 weeks of treatment with immunosuppressive drugs including corticosteroids and cyclophosphamide. Serum was prepared by centrifugation of blood at 2,000 rpm for 5 min and then stored at –70°C until testing.

Detection of Serum Soluble CD146 (sCD146) by ELISA

The concentration of sCD146 in serum was measured using a CyQuant ELISA assay kit (Bioxytex, Marseille, France) according to the manufacturer's instructions. Briefly, 20 µL of sera were first diluted to 200 µL with a dilution buffer from the kit and then incubated for 30 min in a plate precoated with anti-CD146 antibody at room temperature. After washing, the plates were incubated for a further 30

min with another HRP-conjugated anti-CD146 antibody at room temperature. The color development was initiated by adding substrate and was measured using a microplate reader (Model550, Bio-Rad, Hercules, CA) at 495 nm.

Flow Cytometry Analysis of CD146 in Circulating Neutrophils

In order to avoid any cell stimulation during isolation from blood, we used whole blood for FACS analysis. One hundred µL of blood with anticoagulant from an individual patient was diluted with 200 µL PBS and incubated with anti-CD146 monoclonal antibody AA98[11] or isotype-matched murine IgG (Sigma, St. Louis, MO) for 40 min, followed by incubation with FITC-conjugated anti-mouse IgG (Jackson Laboratory, West Grove, PA) for another 30 min. Red cells in the samples were then removed using BD lysis solution (BD/Pharmingen, San Diego, CA). After centrifugation at 1,000 rpm for 5 min and washing with PBS/BSA, the cells were analyzed on a BD FACSCalibur. Leukocytes from each sample were divided into 3 groups: neutrophils designated as R1, monocytes designated as R2, and lymphocytes designated as R3 (supplemental data, **Figure S1**) on the basis of forward and sidelight scatter properties according to previous reports.[18] Neutrophils were gated as R1 and distinguished from other cells by the distinct scatter characteristics and further confirmed by staining with anti-CD15 antibody (Jingmei Biotech, Beijing, China). Within the group of neutrophils, mIgG or AA98 staining was analyzed. Two thresholds of fluorescent intensity, marker 1 (M1) and marker 2 (M2), were defined using mIgG staining histogram (supplemental data, **Figure S2**). Cells stained with AA98 and having a fluorescent intensity within the M2 were considered as CD146-positive neutrophils. The ratio of CD146-positive cells in the total neutrophils was calculated and presented as the neutropil CD146 level for every clinical sample.

RT-PCR and Western-blotting

Neutrophils were isolated from the blood of patients using a Dextran sedimentation, followed by the Hypaque-Ficoll sedimentation procedure.[19] More than 98% of isolated cells were determined as neutrophils using a FITC-conjugated anti-CD15 antibody (Jingmei Biotech). Total RNA was isolated from neutrophils. CDNA, synthesized using random primers, served as a template for PCR with the following pair of CD146 primers: forward: 5′-CATC-CAGCTCCGCGTCTACA-3′; and reverse: 5′-ACCAGCT-GTGTGCGGTTCAG-3′. After 35 PCR cycles (94°C for 30 s, 52°C for 30 s, 72°C for 1.5 min, and 72°C for 7 min), the PCR products were separated using a 1% agarose-gel.

Neutrophil lysates were first subjected to a 10% SDS-PAGE and then transferred to a Hybond membrane (Amersham, Piscataway, NJ). The membrane was blocked with 5% dry nonfat milk in PBS with 0.1% Tween-20 for 1 h followed by incubation for 2 h with AA98 antibody, then probed for 1 h with secondary HRP-conjugated anti-mouse IgG (Pierce Biotechnology, Rockford, IL). After extensive washing, the immunoreactive proteins were detected on the membrane by enhanced chemiluminescence (Pierce Biotechnology).

Induction of Neutrophil CD146 Expression by Sera from Patients With Vasculitis

Neutrophils were isolated as described above. The isolated neutrophils were then cultured in 6-well plates (10^6 cells/well) with 2 mL of RPMI 1640 medium (Gibco BRL Life Technologies, Rockville, MD) in the presence of 200 µL of serum (from MPA patients or healthy donors) with or without prednisolone (PSL) (Sigma, Deisenhofen, Germany) at 37°C with 5% CO_2. After culture for 12 h, the neutrophil CD146 was detected by FACS as described above.

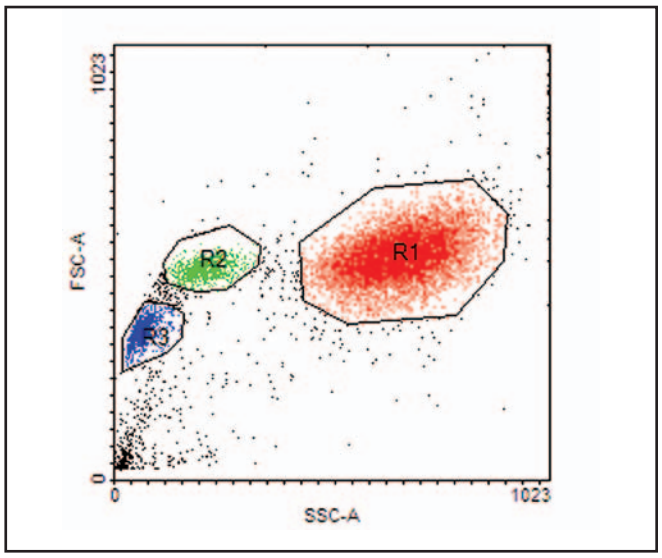

Figure S1_Flow cytometry scatter plot demonstrating the 3 leucocyte populations: group 1, designated as R1, is neutrophils; group 2, designated as R2, is monocytes; and group 3, designated as R3, is lymphocytes on the basis of forward and sidelight scatter properties.

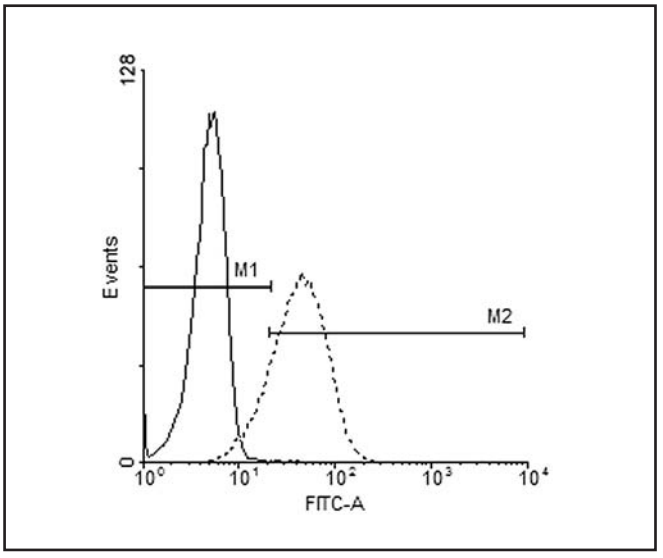

Figure S2_Overlapped histograms of neutrophils—the left is a histogram of gated neutrophils staining with mIgG isotype control; the right is a histogram of gated neutropphils CD146 staining (in this sample, 88.3% of gated cells express CD146).

Statistical Analysis

Statistical analysis was conducted using SPSS 11.0. The Kruskal-Wallis test was used to detect differences between more than 2 groups (nonparametric one-way ANOVA). Unpaired Student's *t*-tests were used to compare results from patients with active disease, and paired Student's *t*-tests were used to compare results from patients before and after treatment. Results were expressed as mean ± SD. A *P* value lower than 0.05 was considered significant.

Results

Significantly Increased Levels of Soluble and Neutrophil CD146 Found in Patients With Active Vasculitis

For the purpose of investigating the expression level of CD146 in active systemic vasculitis, we collected clinical samples, both sera and leukocytes, from the blood of 59 patients with several kinds of systemic vasculitis and 34 healthy donors. The patients were further grouped as TA (n=14), WG (n=24), and MPA (n=21) according to the ACR 1990 criteria. First, we tested the concentrations of soluble CD146 (sCD146) in the sera from patients and healthy volunteers using a sandwich ELISA kit with anti-CD146 antibodies. The results showed a significant increase of sCD146 levels in MPA sera (445 ± 124 ng/mL versus 309 ± 82, *P*=0.009), but not in sera of patients with TA (259 ± 100 ng/mL, *P*=0.08) or WG (299 ± 114 ng/mL, *P*=0.71), when compared with the samples from healthy donors (309 ± 82 ng/mL) in the statistic analysis (**Figure 1A**). Subsequently, we determined the expression level of membrane CD146 in neutrophils, which is the major population (70%) of leukocytes, by flow cytometry assay labeling neutrophils with anti-CD15 antibody and testing them with anti-CD146 staining. The results, shown in **Figure 1B**, presented that the percentages of CD146-positive neutrophils were dramatically increased in the patients with WG (28.6 ± 18.8, *P*=0.001) or MPA (20.5 ± 25.0, *P*=0.025), but not in the samples from TA patients (8.2 ± 4.1, *P*=0.11), compared with neutrophils taken from healthy people (3.2 ± 1.8).

Since renal failure is a common complication of severe systemic vasculitis in clinic, patients (14 MPA patients and 5 WG patients) with renal failure were cross-checked. It was found that the increased sCD146 levels were correlated with the disrupted kidney functions because the significantly higher levels of sCD146 presented in the sera from patients with renal failure (476 ± 96 ng/mL) compared with samples from patients without renal failure (278 ± 104 ng/mL, *P*=0.0000) or healthy donors (309 ± 82 ng/mL, *P*=0.0000) (**Figure 1C**). Together, clinical data displaying the elevated levels of neutrophil CD146 in small-vessel vasculitis, specifically WG and MPA, and increased sCD146 levels in MPA and vasculitis-induced renal failure, indicated the pathologically up-regulated CD146 expression in the inflammatory reactions of active systemic vasculitis.

Ectopically Increased Neutrophil CD146 in Vasculitis Were Diminished by Immunosuppressive Treatments

The levels of neutrophil CD146 were further monitored in patients following the treatment of immunosuppressive agents, including corticosteroids and cyclophosphamide. Interestingly, elevated neutrophil CD146 in WG and MPA patients remarkably decreased after the immunosuppressive treatment and significant expressions of neutrophil CD146 were no longer seen in patients in remission (WG 3.4 ± 2.3 and MPA 2.6 ± 2.0) compared with the levels of healthy donors (**Figure 2A**). These observations were then confirmed by biochemical methods, RT-PCR, and Western blotting (**Figure 2B**), using neutrophils from 2 typical MPA patients. Results showed that both mRNA and protein levels of CD146 in these cells were diminished after immunosuppressive treatments, indicating the correlation of CD146 protein expressed

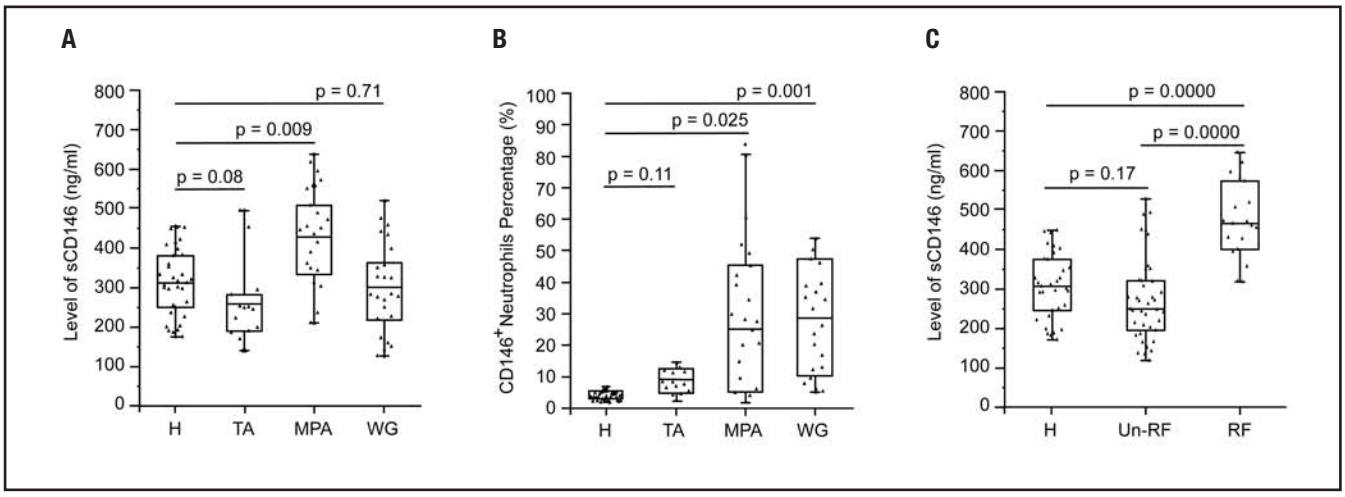

Figure 1_Both soluble and neutrophil CD146 levels were raised in systemic vasculitis. (**A**) Concentrations of soluble CD146 in the samples from the indicated groups were measured by sandwich ELISA methods. (**B**) Cells in the blood samples from indicated groups were stained by anti-CD146 antibodies in flow cytometry analysis. Neutrophils were distinguished from other cells by scatter properties and anti-CD15 labeling and ratios of positive cells in the group of neutrophils were calculated. (**C**) Patients were further grouped as renal failure and non-renal failure according to their kidney functions. Soluble CD146 levels were then determined. Statistic analysis was performed to determine the *P* values between different groups.

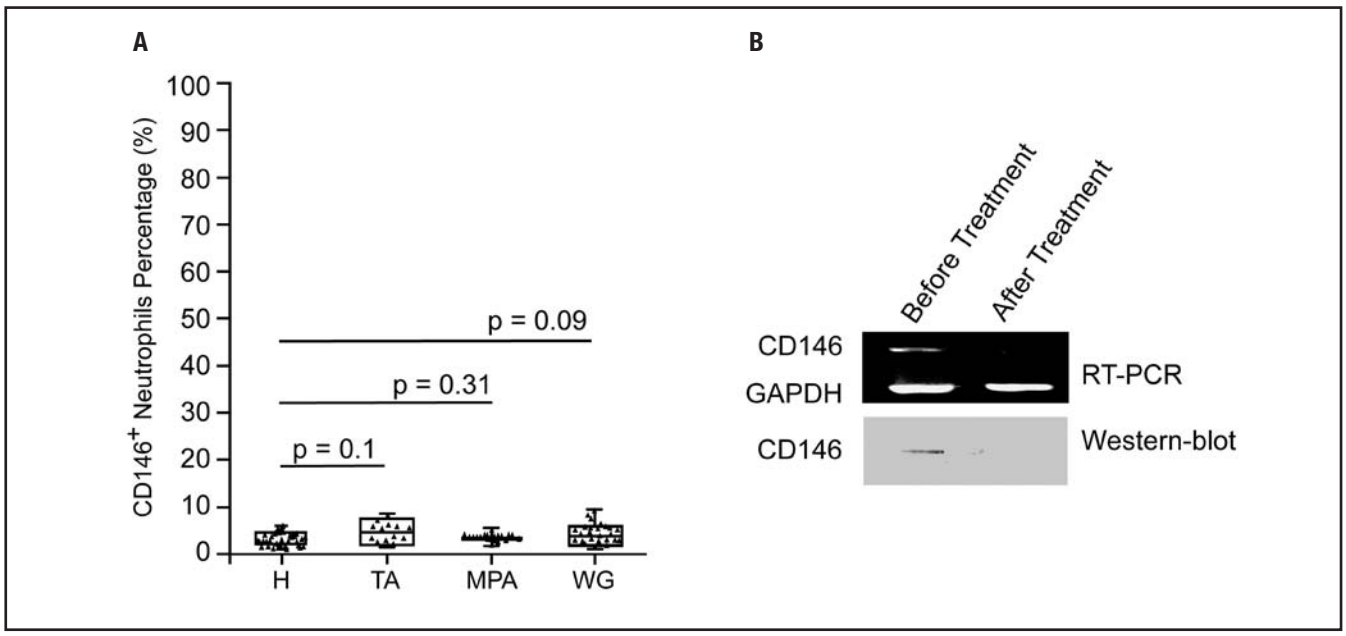

Figure 2_Increased neutrophil CD146 expression vanished after immunosuppressive treatment. (**A**) Percentages of CD146-positive neutrophils in the blood of the patients from the indicated groups were tested after treatment by the methods described in Figure 1B. Statistical analysis was performed to determine the *P* values between different groups. (**B**) Neutrophils from the blood of 2 typical MPA patients were isolated before and after the treatment. RT-PCR and Western blotting were performed using these neutrophils to test the mRNA and protein levels of CD146.

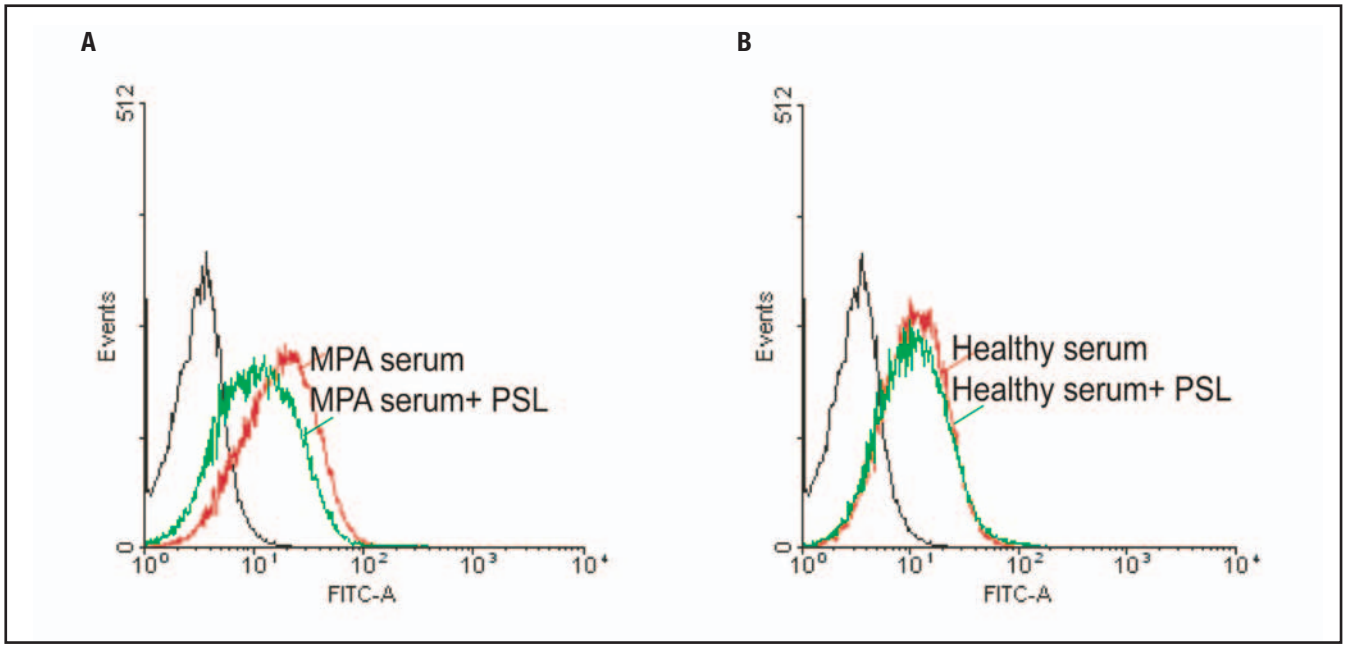

Figure 3_Immunosuppressive agents inhibited neutrophil CD146 expression induced by MPA patient sera. (**A**) Normal neutrophils were isolated from the blood of healthy volunteers and cultured in vitro with or without the serum taken from MPA patients. Prednisolone was added to the cells with serum. After being cultured for 12 hours, cells were subjected to flow cytometry analysis with anti-CD146 antibodies. (**B**) The same assays were also performed under the stimulation of sera from healthy donors.

by circulating neutrophils with the progression and remission of active systemic vasculitis.

MPA Patient Sera-Induced CD146 Expression on Neutrophils was Suppressed by Immunosuppressive Drugs in Vitro

To further confirm the pathologically inducible neutrophil CD146 expression, an in vitro model was established to investigate the regulation of CD146 expression during the progression and remission of vasculitis using sera from MPA patients with or without treatment to stimulate normal neutrophils taken from healthy donors. Results showed that neutrophil CD146 expression was induced by MPA serum to a greater extent than by healthy serum (percentages of CD146 positive cells: 31.5 ± 4.9 versus 13.9 ± 1.0, P=0.0017, see **Figure 3**). As observed in patients who received immunosuppressive treatment, the neutrophil CD146 induced by MPA serum was also significantly inhibited by PSL, the active form of immunosuppressive drug prednisone (31.5 ± 4.9 without PSL versus 15.5 ± 3.2 with PSL, P=0.0024), whereas healthy serum-induced neutrophil CD146 was almost unaffected by PSL (13.9 ± 1.0 without PSL versus 14.4 ± 1.9 with PSL, P= 0.69). In contrast to neutrophils, lymphocyte CD146 expression was not induced by the MPA serum (data not shown), indicating a distinct mechanism for its expression regulation in lymphocytes. Together, our in vitro studies verified the effect of immunosuppressive treatment in reducing abnormally up-regulated neutrophil CD146 expression and implied the possible involvement of CD146 in inflammatory progression of vasculitis.

Discussion

Pathologically increased recruitment of leukocytes to the endothelium and followed transendothelial migration, which are mainly mediated by CAMs, are the most common and important characteristics in inflammatory reactions such as systemic vasculitis. Indeed, some adhesion molecules, notably PECAM-1, ICAM-1, VCAM-1, and E-selectin, were reported to be up-regulated on leukocytes during inflammation. As a newly identified cell adhesion molecule, CD146 was found to be widely expressed on endothelial cells and activated T cells. Moreover, the homophilic interaction between membrane CD146 proteins was involved in the initial step of lymphocyte endothelium interaction,[16] indicating a role of CD146 in inflammatory processes. However, the expression level of CD146 on leukocytes in typical chronic inflammation diseases, such as vasculitis, had never been studied.

In the present clinical test with statistic analysis, we first discovered a significant elevation in CD146 expression levels on neutrophils in active small-vessel vasculitis, WG, and MPA, but not large-vessel vasculitis, TA, in comparison with samples from healthy donors. Owing to the distinct clinical symptoms, angiographic presentations and pathological causes between different kinds of vasculitis, it is possible that neutrophil CD146 is only involved in ANCA-related small-vessel vasculitis, which is implicated in different pathogenesis from large-vessel vasculitis.[20] For example, small-vessel vasculitis usually affects the small-vessel-size system such as capillaries, venules, arterioles, and arteries, and causes severe systemic vessel damage and fatal implications such as renal failure,[21] whereas large-vessel vasculitis characteristically affects the branches of the carotid arteries and is not associated with the histopathologic finding of fibrinoid necrosis.[22] Therefore, neutrophil CD146 might potentially be used in clinical diagnosis of systemic small-vessel vasculitis.

Interestingly, ectopic expression of neutrophil CD146 vanished after the treatment of immunosuppressive therapy in our clinical observations and was further confirmed by biochemical studies. Our in vitro model, showing that immunosuppressive treatment could suppress MPA patients' sera-induced neutrophil CD146 expression, implied that the immunosuppressive drugs might directly affect neutrophil function. Indeed, it was reported that PSL interferes with neutrophil-endothelial adhesion and neutrophil mediated endothelial injury in vitro.[23] Thus, decreasing the expression of CAMs, such as CD146, and then suppressing neutrophil adhesion and motility might be one of the protective effects of corticosteroid therapy in the treatment of vasculitis. Furthermore, the correlation between the decrease of neutrophil CD146 from pathological up-regulation and post-treatment remission implied the utility of CD146 levels in monitoring response to treatment in vasculitis.

Previous studies had evidenced increased levels of sCD146 in the synovial fluid of patients with rheumatoid arthritis[14] and sera of patients with polymyositis,[24] indicating the involvement of sCD146 in chronic local inflammation. Here, we observed a significant up-regulation of sCD146 level in the sera from MPA patients suffering from systemic vessel inflammation. Moreover, sCD146 was also found to increase in the sera of patients with chronic renal failure[15] and diabetic nephropathy,[25] suggesting a correlation between its levels and compromised kidney function. It is consistent with present studies showing that raised sCD146 levels were related with vasculitis-induced renal failure in WG and MPA patients. In fact, many pathological disorders are associated with renal failure, including vasculitis. About 90% of MPA patients have renal diseases in the form of pauci-immune necrotizing and crescentic glomerulonephritis as was seen in WG.[26] Moreover, elevated inflammatory cytokines, such as TNF-α and IL-1β, play a pivotal role in chronic renal failure.[27] Since these cytokines could stimulate the expression of endothelial CD146,[28] it is possible that increased soluble CD146 in vasculitis is caused by up-regulated endothelial CD146. As a newly identified endothelial marker, CD146 was found to be located in the junction of endothelial cells,[29] and fluctuation of its expression level reflected the alterations in endothelial junction, followed by changes of endothelial cells layer. Attributed to the utility of sCD146 level in monitoring the endothelial monolayer integrity and the enrichment of blood vessels in kidney, sCD146 might be a novel indicator for vasculitis-caused renal failure. LM

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