Increased Expression of PAD4 in Neutrophils of MPO-ANCA Associated Vasculitis and its Correlation with the Vasculitis Activity

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Abstract

Myeloperoxidase (MPO)-specific anti-neutrophil cytoplasmic autoantibody (ANCA) associated vasculitis (MPO-AAV) is the most common ANCA associated vasculitis (AAV) in china and other East Asian countries. Its pathogenesis remained elusive. We evaluated the expression of peptidylarginine deiminase (PAD4) in neutrophils from MPO-AAV and explored the relationship between the expression and the vasculitis activity, and also investigated the pathogenic mechanism. 40 patients initially diagnosed with MPO-AAV in the active state and 40 comparable healthy controls (HC) were enrolled in this study. The patient disease activity were evaluated with Birmingham Vasculitis Activity Index version 3 (BVAS-V3). The expression of PAD4 in their neutrophils, which was described as percentage of PAD4+ neutrophils in all neutrophils and mean fluorescence intensity (MFI) of PAD4+ neutrophils, was measured by flow cytometry (FCM). Their serum level of NETs, C5a and MPO-ANCA were detected using enzyme linked immunosorbent assay (ELISA). The percentage of PAD4+ neutrophils, MFI of PAD4+ neutrophils, the levels of serum NETs and C5a were markedly higher in patient group than that in HC group. Meanwhile, in patient group, the percentage of PAD4+ neutrophils, MFI of PAD4+ neutrophils and MPO-ANCA presented significantly correlation with NETs. Although univariable analysis showed the percentage of PAD4+ neutrophils, MFI of PAD4+ neutrophils, NETs, C5a and MPO-ANCA had respective correlation with BVAS, multivariable analysis indicated that only the percentage of PAD4+ neutrophils as well as the level of serious MPO-ANCA owned independent correlations with BVAS.PAD4 might be a new potential target for MPO-AAV treatment in the future.

Introduction

Anti-neutrophil cytoplasmic antibody-associated vasculitis (AAV) is a group of necrotizing vasculitis that primarily affect small blood vessels with pauci-immune complex deposits. It was mainly divided into microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA) and eosinophilic granulomatosis with polyangiitis (EGPA)[1]. The positive antineutrophilic cytoplasmic antibodies (ANCA) is the hallmark of AAV, and ANCA with specificity for proteinase-3 (PR3-ANCA) is predominantly detected in GPA while ANCA against myeloperoxidase (MPO-ANCA) is the most frequent autoantibody in MPA and EGPA[2]. In China as well as in other East Asian countries, MPA with MPO-ANCA is the most common AAV whereas in western countries, GPA with PR3-ANCA positivity is the most prevalent AAV instead[3, 4]. Also, compare to GPA, MPA usually occurred in more elderly population and had higher mortality due to more severe multisystem damage [4, 5]. Of marked interest was the result from a genomewide association study, which revealed that GPA and MPA were genetically distinct and their strongest genetic associations were with the antigenic specificity of ANCA, but not with the clinical syndrome[6]. So, it would be more validate that researches on the pathogenesis of AAV should be based on MPO-AAV mainly consisted of MPA or on PR3-AAV largely composed of GPA.

Although the precise cause of AAV remained elusive, studies had revealed some of factors might involve in the pathogenesis of MPO-AAV [7-9]. MPO-ANCA could activate neutrophils by combining with MPO expressed on neutrophils under the stimulation of inflammatory factors to promote inflammatory reaction[8, 10]. In addition, the complement alternative pathway (cAP) was found to be involved in the pathogenesis of MPO-AAV. The combination of fragment a of fifth complement C5 (C5a) issued from cAP with C5a receptor (C5aR) on neutrophil could improve neutrophil survival, activate its respiratory burst and phagocytosis, which would further aggravate the inflammation and clinic damages[11-13].

Neutrophil extracellular traps (NETs), which was first described in 2004, is a unique innate immune defense mechanism. It is composed of DNA, histones, and granular proteins mainly including MPO, PR3, neutrophil elastase (NE), peptidylarginine deiminase 4 (PAD4) and etc[14]. Studies had revealed NETs was involved in pathogenesis of the vasculitis[15-19]. The formation of NETs was called NETosis, and PAD4 expressed in neutrophil is a critical enzyme to generate NETs by mediating histone citrullination to promote the NETosis[20]. Actually, PAD4 was found to be involved in the pathogenesis of varieties of diseases, including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and tumors[21-23]. However, to our knowledge, there is no research by now to investigated PAD4 expression in neutrophil and its possible pathogenic effect in MPO-AAV. In the present study on patients with active MPO-AAV, we explored the PAD4 expression in neutrophil, its influence on vasculitis activity and the possible mechanism via detecting the PAD4 in neutrophils, checking the NETs, C5a and MPO-ANCA in peripheral blood, and analyzing their relationships.

Patients and methods

Patients

A total of 40 patients with MPO-AAV enrolled in this study were all selected from the First Affiliated Hospital of Anhui Medical University from March 2017 to May 2020. Their AAV were diagnosed according to the classification criteria for GPA and EGPA published by American College of Rheumatology (ACR) in 1990 and the definition for MPA from 2012 Chapel Hill Consensus Conferences (CHCC) nomenclature of vasculitis[1, 24, 25]. All patients were test positive for p-ANCA and MPO-ANCA by indirect immunofluorescence (IIF) and enzyme-linked immunosorbent assay (ELISA), respectively, and also with negative detection for c-ANCA and PR3-ANCA as well as anti-nuclear antibody (ANA). They were all diagnosed with MPO-AAV in active state for the first time. Their blood samples and clinical documents including disease activity assessment were taken before initiation of any immunosuppressive treatment. The Version 3 of Birmingham Vasculitis Activity Score (BVAS-V3) was employed to evaluate the vasculitis activity. In addition, 40 healthy volunteers were included in this study as controls. This study had been approved by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University, and all subjects have signed informed written consent before enrolled.

PAD4 detected by Flow Cytometry

50μL of peripheral blood was lysed with 1mL of FACS Lysing Solution (555899, BD, USA) in dark for 10 minutes. CD16 (563830, BD, USA) and CD62L (555543, BD, USA) were used for neutrophil staining. Then, after fixing and permeabilizing, cells were incubated with mouse anti-human PAD4 as primary antibody (ab128086, Abcam, UK) for 1 hour, and stained with PE-rat anti-mouse IgG2a as secondary antibody (12-4210-82, Thermo Fisher, USA) for 30 minutes. Data were detected by a flow cytometer (FACS Calibur, BD), and analyzed with FlowJo7.6 software.

NETs assayed by ELISA

The serum level of NETs was measured using the capture ELISA protocol as described previously[16, 26]. Briefly, microtiter plates were first coated with the monoclonal anti-MPO antibody (ab25989, Abcam, UK) and incubated overnight at 4°C. After blocking with 3% skimmed milk, the blood sample diluted at 1:50 was added and incubated for 90 minutes. Then the secondary anti-dsDNA antibody (ab27156, Abcam, UK, 1:2000 dilution) was added into the wells as second antibody. After incubation for 2 hours and washed 4

times, horseradish peroxidase-conjugated rabbit anti-mouse IgG H&L antibody (ab6728, Abcam, UK) was put in. After incubation again, the substrate chromogenic agent was added in turn. 10 minutes later, the reaction suspension solution was mixed and the results was calculated with optical density (OD) at the wavelength of 450nm on the ELIASA (PRA ELX800, Bio Tek, Vermont, USA).

C5a determined by EIASA

C5a level in peripheral blood from the 2 groups was detected using Human C5a ELISA kit (BMS2088, Invitrogen, USA) and according to the producer's introduction.

MPO-ANCA tested by ELISA

MPO-ANCA titer in serum samples was test by routine ELISA. In brief, plates were coated with MPO (M6908, Sigma-Aldrich, USA) 2ug/well at 4 overnight, then blocked with 3% skim milk and the serum samples (1:100 dilution) were added. Horseradish-peroxidase–conjugated anti-human Ig (IgG, IgM and IgA) (A18847, Invitrogen, USA) was used as secondary antibody (1:5,000 dilution) and reacted with substrate chromogenic agent for ten minutes. OD of 450nm wavelength was obtained by the same steps as ELISA above. OD value, which was more than mean plus 2 times standard deviation(SD) obtained in healthy control group, were taken as lower threshold for MPO-ANCA positivity in both groups.

Statistical analysis

GraphPad Prism8.0 software (San Diego, CA, USA) was used to analyzed data. Continuous variables were expressed as standard deviation (SD) or median and interquartile range (IQR) according normal or non-normal distribution, respectively. Comparison between two normal distribution groups was performed by two-tailed unpaired t test with Welch's correction. The correlation of two groups with normal distribution was computed by Pearson correlation analysis, while non-normal distribution was calculated by Spearman correlation analysis. In order to perform the multiple factors correlation analysis, the variables in the univariate analysis with p -value less than 0.10 and no multicollinearity were included. Multicollinearity was judged according to the condition indexes and the variance ratio in the fitted equation. p -values less than 0.05 were considered statistically significant.

Results

Demographic and clinical data analysis

The demographic characteristics of involved patients and healthy controls (HC) were described in Table I. A total of 40 eligible patients with MPO-AAV were enrolled in patient group, among whom 39 were diagnosed as MPA and 1 as GPA. The male patients (60.00%) were more than the females (40.00%). Laboratory tests for p-ANCA and MPO-ANCA in patient group were both positive. Their disease duration was 6.75 +- 6.72 months and their mean BVAS was 21.03+-5.97. The HC group also consisted of 40 volunteers with negative test results for both p-ANCA and MPO-ANCA. There was no difference between the 2 group in terms of age (67.65 +- 12.52 *vs.* 65.30 +- 10.13 years old) and gender ratio (60.00% *vs.* 47.51% were male), indicating that the 2 groups were comparable demographically.

The difference of PAD4 expression between patient and HC group

The expression of PAD4 in neutrophils detected by FCM was described by the percentage of neutrophils expressed $PAD4^+$ in all neutrophils and the mean fluorescence intensity (MFI) of $PAD4^+$neutrophils. Statistical analysis showed that the percentage of $PAD4^+$ neutrophils in the patient group was significantly higher than that in HC group (70.48 +- 10.47% *vs* . 25.91 +- 6.33%, p <0.001; Fig.1A). Also, the MFI of $PAD4^+$neutrophils was increased significantly in the patient group compared with the HC group (32.89 +- 4.43 *vs.* 14.41 +- 4.24,p <0.001; Fig.1B).

Comparison of NETs and C5a between the patient and HC group

The NETs and C5a levels in peripheral blood from all patients and controls were compared by the two-tailed unpaired t test with Welch's correction. It was found to that both the variables in patient group were

significantly higher than that in HC group (0.62 +- 0.22 *vs.* 0.26 +- 0.15,p <0.001 and 4.64 +- 1.04ng/ml *vs.* 2.88 +- 0.96ng/ml, p <0.001, respectively) (Fig. 2A and Fig. 2B).

Analysis of the relationships in laboratoryvariables in patient group

It was showed by Spearman correlation analysis that PAD4 expressions in neutrophil, which included the percentage of neutrophils expressed PAD4 in all neutrophils and MFI of PAD4$^+$neutrophils, were correlated with NETs (r =0.432, p =0.0054 and$r$ =0.395, p =0.0117, respectively) (Fig.3A and Fig. 3B). In addition, NETs displayed a statistically significant correlation with MPO-ANCA but not with C5a (r =0.415, p =0.0078 and$r$ =0.270, p =0.0920, respectively) (Fig. 3C, Fig. 3D). Furthermore, MPO-ANCA exhibited significant correlation with C5a (r =0.558, p =0.0002) (Fig. 3E).

Investigation on the relationship between BVAS and variables tested

In patient group, Spearman correlation analysis shown that MPO-AAV disease activity measured with BVAS statistically significantly correlated with the level of percentage of PAD4$^+$ neutrophil in all neutrophils, MFI of PAD4$^+$neutrophils, NETs, C5a and MPO-ANCA (r =0.913, p <0.0001; r =0.881,p <0.0001; r =0.473, p =0.0021;r =0.666, p <0.0001 and r =0.869,p <0.0001, respectively) (Table II). There was neither significant correlation between BVAS and the patient age, and nor between BVAS and disease duration (r =0.011, p =0.9482 and$r$ =0.085, p =0.5865, respectively) (Table II). Nevertheless, multiple linear regression analysis found only percentage of PAD4$^+$ neutrophil and MPO-ANCA had independent correlations with BVAS (p =0.0053 and p =0.0007, respectively) (Table II).

Discussion

In order to avoid the influence of glucocorticoid and any other immunosuppressant on the main outcome measures in this study, all the 40 selected patients with active MPO-AAV were initially diagnosed without any treatment with these agents. Among them, 39 patients (97.5%) had MPA, which was consistent with the fact that MPA with MPO-ANCA was most common AAV in China and other East Asian countries[4]. Also, Age and gender distribution in our study was respectively comparable between the patient and HC group as statistical analysis showed there was no significant difference for them between the 2 groups (Table I).

PAD4 is one of the 5 isoenzymes of PAD (PAD1-4 and PAD6) in human, a family of Ca^{2+}-dependent deiminase enzymes. It is mainly expressed in the nucleus of neutrophil [27, 28]. Li et al. constructed PAD4 knockout mice and stimulated them with chemokines and bacteria. They found chromatin decondensation was inhibited in neutrophils, NETs could not form and the antimicrobial ability of mice was significantly decreased, which indicated PAD4 played an important role in formation of NETs by mediating histone citrullination[29]. Studies shown that PAD4 was also involved in the pathogenesis of both tumor and autoimmune diseases, such as SLE and rheumatoid arthritis (RA) [21, 30-32]. Knight et al. published that MRL/lpr SLE model mice treated by PAD4 inhibitors presented lighter clinical manifestations and disease activity compared to the controls[21]. Moreover, PAD4 in synovial fluid of RA was detected at significantly higher levels than that of osteoarthritis or psoriatic arthritis, which might be account for the generation of autoantigens in RA[33]. In this study, we detected the expression level of PAD4 in neutrophils by FCM, and found both the percentage of PAD4$^+$ neutrophils and the MFI of PAD4$^+$ neutrophils were significantly higher in patient group than in HC group, respectively (Fig.1). This might account for the NETs in patient group was markedly higher than that in HC group (Fig. 2A). Further mover, the percentage of PAD4$^+$ neutrophils and the MFI of PAD4$^+$ neutrophils respectively manifested positive correlation with NETs in patient group (Fig.3A and 3B).

As for NETs, in addition to its basic function of innate immunity against microbial infection[14], it was found to play important roles in pathogenesis of various autoimmune diseases, especially of AAV, by promoting both innate and adaptive immunity pathway. In a vitro experiment, Wang et al. demonstrated NETs could activate the cAP, an innate immune pathway to aggravate inflammation by producing C5a [18]. In AAV, the most common autoantigens (MPO and PR3) were the component of NETs[14], Sangaletti et al. reported

that autoantigens in NETs could be processed and present to adaptive immune T cell more effectively by myeloid dendritic cells to induce the production of autoantibodies including MPO-ANCA[34]. MPO-ANCA combined with MPO on neutrophils and promote the neutrophils to discharge granules, to produce reactive oxygen radicals (ROS), to form NETs and finally to damage the tissues and organs by the inflammatorily destroyed vessels[15, 16]. The discharged granules from the neutrophils provoked by MPO-ANCA contained factors, such as properdin, that could boost cAP to present more C5a[35, 36]. In this study, we found serum C5a of patient group was significantly higher than that in HC group, which supported the activated cAP in active MPO-AAV (Fig. 2B). Although Wang et al. reported NETs could activate the cAP[18], our study indicated that, compare to NETs, MPO-ANCA might play more important roles to activate cAP by releasing granules because C5a was significantly associated with MPO-ANCA but not with NETs (Fig. 3C and Fig. 3D). Our result also showed MPO-ANCA had significant positive correlation with NETs (Fig.3E), which might be attributed to that MPO in NETs could be more effectively processed and present as autoantigen by myeloid dendritic cells to activate adaptive immunity[34]. Kusunoki et al. found in their mice model of MPO-AAV that histone citrullination could decrease by inhibiting PAD4 with Cl-amidine, and then serious MPO-ANCA could also be reduced [37].

As discussed above, PAD4 played a key role in NETs formation, which might promote adaptive immunity to facilitate the generation of MPO-ANCA and also might activate cAP to increase C5a. Both MPO-ANCA and C5a might trigger neutrophils to form NETs, to accelerate cAP, to produce ROS and etc. This then could create a self-fueling inflammatory amplification loop, a "vicious cycle", to aggravate clinical inflammatory damage of MPO-AAV. Actually, Univariable correlation analysis in this study showed that the percentage of $PAD4^{+}$neutrophils, MFI of $PAD4^{+}$neutrophils, NETs, C5a and MPO-ANCA was positively correlated with BVAS, respectively (Tab. II). However, multivariable correlation analysis revealed that only the percentage of $PAD4^{+}$neutrophils and MPO-ANCA was independently correlated with BVAS, respectively (Tab. II). In fact, MPO-ANCA was the hallmark for MPO-AAV as an autoimmune disease. Previous studies had demonstrated MPO-ANCA as a pathogenic autoantibody by experimental research and clinical report[8, 38]. Though PAD4, NETs, C5a and the "vicious cycle" among them might contribute to the production of MPO-ANCA, MPO-ANCA present as an independent factor affecting the vasculitis activity in this clinical study. Meanwhile, PAD4 could have independent influence on BVAS, which should be attributed to its enzymatic function in NETs generation and the NETs pathologic efficacy in MPO-AAV.

Conclusion

Although pathophysiological mechanisms of MPO-AAV involved innate immunity (e.g., role of PAD4, effect of NETs, activation of cAP, and etc.) and adaptive immunity (e.g., generation of MPO-ANCA) and the complex relationships between them, our research result showed that PAD4 as well as MPO-ANCA was independent factor affecting the clinical damage of MPO-AAV. Since PAD4 inhibitor could decrease NETs and MPO-ANCA generation in animal model of MPO-AAV[37], It is tempting to imagine that PAD4 might be a potential new therapeutic target for MPO-AAV.

Conflict of interest

The authors declare no conflict of interest.

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Ethical approval

The grant supporting this review were approved by the Ethics Committee of the Anhui Medical University.

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Figure legends

Figuer 1. Comparison of the expression of PAD4 in neutrophils between patient and HC group.

It showed that the percentage of neutrophils expressed $PAD4^+$ in all neutrophils (Fig. 1A) and MFI of $PAD4^+$neutrophils (Fig. 1B) in patient group was significantly higher than that in healthy control group, respectively.

Graphs represent arithmetic mean +- standard deviation. The two-tailed unpaired t test with Welch's correction was employed as statistical analysis.

HC: healthy control; MFI: mean fluorescence intensity; OD: optical density; PAD4: peptidylarginine deiminase 4.

Figure 2. Comparison of NETs and C5a between the patient and HC group.

Statistical analysis showed that the serum level of NETs (Fig. 2A) and C5a (Fig. 2B) in patient group was significantly higher than that in HC group, respectively.

Graphs represent arithmetic mean +- standard deviation. Statistical analysis was performed by two-tailed unpaired t test with Welch's correction.

C5a: fragment a of the fifth complement; HC: healthy control; NETs: neutrophil extracellular traps; OD: optical density.

Figure 3. Correlation analysis result of laboratory variables in patient group.

Spearman correlation analysis revealed that the percentage of PAD4$^+$ neutrophil in all neutrophils and MFI of PAD4$^+$neutrophils had statistically significant correlations with NETs in peripheral blood, respectively (Fig. 3A and Fig. 3B). The level of NETs statistically significant correlated with MPO-ANCA but not with C5a (Fig. 3C and Fig. 3D). Also, MPO-ANCA showed a positive correlation with C5a. $^*p < 0.05$.

C5a: fragment a of the fifth complement; MFI: mean fluorescence intensity; MPO-ANCA: myeloperoxidase-specific anti-neutrophil cytoplasmic autoantibody; NETs: neutrophil extracellular traps; OD: optical density; PAD4: peptidylarginine deiminase 4.

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