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Identification of target antigens of anti-endothelial cell antibodies in patients with anti-neutrophil cytoplasmic antibody-associated vasculitides: A proteomic approach

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Abstract Anti-endothelial cell antibodies (AECAs) have been reported to cause endothelial cell dysfunction, but their specific targets have never been identified in anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitides (AAVs). Proteins from human umbilical vein endothelial cells (HUVECs) were separated by 2-dimensional electrophoresis (2-DE). 2-D immunoblots were used to compare serum IgG reactivities from 30 patients with AAV and 12 healthy controls (HCs). Proteins identified as target antigens by MALDI- TOF-TOF mass spectrometry included lamin A/C, vimentin, α-enolase, far upstream binding protein 2 (FUBP2) and protein disulfide-isomerase A3 precursor (PDIA3). Antibodies targeting lamin A, vimentin, α-enolase, FUBP2 and PDIA3 were identified in 57.1%, 64.3%, 35.7%, 50% and 0% of patients with microscopic polyangiitis and 8%, 3.3%, 7.2%, 0% and 6.7% of HCs respectively. IgG from patients with microscopic polyangiitis had stronger reactivity against HUVEC than other groups and HCs and induced stronger Erk phosphorylation in HUVECs than IgG from HCs.
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1. Introduction

Granulomatosis with polyangiitis (GPA) (formerly Wegener's granulomatosis), eosinophilic granulomatosis with polyangiitis (EGPA) (formerly Churg-Strauss syndrome) and microscopic polyangiitis (MPA) are pauci-immune necrotizing vasculitides of small-sized vessels associated with anti-neutrophil cytoplasmic antibodies (ANCAs). The immunofluorescence pattern distinguishes cytoplasmic ANCA, which usually targets proteinase 3 (PR3), and perinuclear ANCA, targeting myeloperoxydase (MPO). The spectrum of ANCA-associated vasculitides (AAVs) is wide, and the main clinical manifestations involve the eye, nose, throat, lung, kidney, skin, muscle, joints and peripheral nervous system [1].

In vitro, ANCAs trigger the activation, degranulation and apoptosis of neutrophils, which then cause endothelial cell (EC) damage [2]. Thus, evidence has been obtained for the pathogenic role of ANCAs in vitro and in vivo, mostly involving anti-MPO antibodies, although other mechanisms including B-cell and T-cell activations and granuloma formation occur during AAVs [3]. Recent advances have been made in the treatment of AAVs with the demonstration in 2 prospective randomized studies that rituximab, a chimeric anti-CD20 monoclonal antibody targeting B cells, is as effective as oral and/or intravenous cyclophosphamide [4,5], which emphasizes the pathogenic role of B cells and auto-antibodies.

Interestingly, not all patients with AAVs have detectable ANCAs, and other autoantibodies are probably involved in these conditions. Thus, anti-EC antibodies (AECAs), described in other conditions such as systemic lupus erythematosus [6], systemic sclerosis and pulmonary arterial hypertension [7], have been detected in patients with AAVs. Although the pathogenic role of AECAs remains controversial [8], AECAs have been reported to induce the expression of vascular adhesion protein 1 and major histocompatibility complex (MHC) class I-related antigen A [9] and mediate antibody-dependent cellular cytotoxicity in AAVs [10]. A few target antigens of AECAs have been identified in patients with AAVs and include PR3 and MPO, which may be adsorbed at the surface of ECs. To uncover the target antigens of these AECAs in patients with AAVs, we used 2-D electrophoresis (2-DE), then immunoblotting with proteins of HUVECs and identified proteins involved in cytoskeleton, cell energy metabolism, and other key cellular pathways. Importantly, these target antigens are linked to transforming growth factor β (TGF-β) and the mitogen associated protein kinase (MAPK) family. We confirmed by ELISA tests that α-enolase, vimentin, lamin A and far upstream binding protein 2 (FUBP2) were target antigens of AECAS. Finally, we demonstrated that purified serum IgG from patients with MPA induced stronger Erk phosphorylation than IgG from healthy controls (HCs).

2. Methods

2.1. Patient sera

Sera were obtained from 46 patients with AAVs, including 15 with GPA, 14 with MPA and 17 with EGPA fulfilling the classification criteria of the American College of Rheumatology and/or the Chapel Hill nomenclature [11]. Disease activity was evaluated by the Birmingham vasculitis activity score (BVAS). Vasculitides was considered active with BVAS $\geq$ 3 [12]. Sera from 30 patients including 12 with GPA, 9 with EGPA and 9 with PMA were used for proteomic experiments. Twenty five patients had active vasculitides, as assessed by BVAS $\geq$ 3 and mean disease duration 65 months (range 0–240 months), and 18 patients had detectable ANCAs targeting PR3 or MPO at the time of blood sampling (Additional File 1).

For proteomic experiments, sera were pooled by groups of 3 according to disease type and/or disease activity. Thus, we tested 10 pools of sera from patients: 4 pools from patients with GPA, 3 from patients with MPA, and 3 from patients with EGPA. A pool of sera from 12 HCs was used as a control. All patients and healthy controls gave a written informed consent. Sera were collected with approval of the ethics committee of the "groupe hospitalier Pitié-Salpêtrière" and the study conforms to the principles outlined in the Declaration of Helsinki.

2.2. Cell culture

HUVECs were isolated by digestion of freshly obtained umbilical cords and cultured with microvascular EC growth medium (Promocell, Heidelberg, Germany) as previously described [7,13,14]. Cells at passage 2 were harvested for protein extraction. Immunofluorescence assays were performed at the 3rd passage in multiple chamber slides (Millipore, Billerica, MA, USA).

2.3. Indirect immunofluorescence

Endohelial cells were fixed with 4% paraformaldehyde, washed with phosphate buffer saline (PBS), incubated with sera from patients or healthy controls at a 1:100 dilution for 1 h 30 min at room temperature and finally washed twice with PBS. A goat anti-human IgG secondary antibody conjugated with FITC (Invitrogen, Carlsbad, CA, USA) was then added for 1 h at room temperature. Slides were mounted with a mounting medium containing 4′,6′-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA) and cells visualized under a Zeiss Axio Observer.Z1 microscope equipped for fluorescence (Carl Zeiss, Oberkochen, Germany). All slides from each single experiment were visualized at the same time of exposure. Corrected total cell fluorescence (CTCF) was obtained with Image J and corresponds to cell fluorescence corrected by background noise.

2.4. 2-D immunoblotting

2.4.1. Protein extracts

HUVECs were stored at −80 °C in 1 mM phenylmethanesulfonylfluoride (PMSF) and protease inhibitors (Complete Mini, Roche Diagnostics, Meylan, France). Protein extraction was performed as described [15] and detailed in the Additional File 2. Briefly, cells were suspended at 10^6/ml in a sample solution extraction kit (Kit 3; Bio-Rad, Hercules, CA, USA). Cells were sonicated and the supernatant was collected after ultracentrifugation (Ultracentrifuge Optima L90K; Beckman Coulter, Fullerton, CA, USA) at 150,000 g for 25 min at 4 °C.

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