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Identification of anti-moesin antibodies in the serums of patients with antiphospholipid syndrome☆

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ABSTRACT

Introduction: The antiphospholipid syndrome (APS) is an acquired autoimmune disease characterized by recurrent vascular thrombosis and obstetric complications. However, the precise mechanisms by which the autoantibodies mediate disease remain to be elucidated. Moesin is an intracellular protein that links the cell membrane and cytoskeleton, mediating the formation of microtubules and cell adhesion sites as well as ruffling of the cell membrane, which is crucial for platelet activation.

Materials and methods: We screened the serums from patients with APS for the presence of anti-moesin antibodies (anti-moesin Abs) recognizing antigens derived from prokaryotic expression system, and investigated the effect of murine monoclonal anti-moesin Abs (anti-moesin mAbs) on platelet activation and aggregation by flow cytometry and platelet aggregation assay in vitro to study their potential pathogenic role in APS.

Results: The presence of anti-amino (N)-terminal portion of moesin antibodies (anti-moesin-N Abs) was observed in 73% (73/100) patients with APS, which was significantly higher than anti-cardiolipin antibodies (aCL, 49%) and anti-β_2 glycoprotein I antibodies (anti-β_2GPI, 43%). Moreover, the elevated anti-moesin-N Abs levels significantly correlated with plasma levels of anti-β_2GPI ($rs = 0.474$, $P < 0.001$) rather than aCL ($P = 0.203$). The murine anti-moesin mAbs promote platelet activation and aggregation in vitro, which could be effectively neutralized by moesin-N.

Conclusions: In combination of the detection of aCL and anti-β_2GPI, screening for the presence of anti-moesin-N Abs might has its value in facilitating the laboratory diagnosis of APS. The pathogenic role of anti-moesin-N Abs in the serums of APS patients needs to be further studied.

Introduction

The antiphospholipid syndrome (APS) is an acquired autoimmune disease characterized by clinical manifestations of vascular thrombosis, recurrent pregnancy loss and/or thrombocytopenia, coupled with the persistent laboratory detection of antiphospholipid antibodies (aPL) [1]. aPL antibodies are a heterogeneous group of immunoglobulins, including anti-cardiolipin antibodies (aCL), anti-β_2 glycoprotein I antibodies (anti-β_2GPI), and lupus anticoagulants (LA), which target phospholipids, phospholipid-binding proteins, as well as various serine proteases in hemostasis [2]. Increasing evidences have indicated that aPL antibodies may play a pathogenic role in the development of thrombosis in patients with APS by promoting platelet activation and aggregation or dysregulation of hemostasis [2–4]. However, it is likely that there are a myriad of intravascular and cellular interactions that contribute to the development of thrombosis in APS rather than a single triggering event.

Platelet activation results in formation of filopodia and cell spreading by extension of lamellipodia. Quantitatively dominant ERM (ezrin, radixin and moesin) protein in human leukocytes [5] and the only ERM protein in platelets [6], moesin links the cell membrane and

Abbreviations: APS, the antiphospholipid syndrome; aPL, antiphospholipid antibodies; aCL, anti-cardiolipin antibodies; anti-β_2GPI, anti-β_2 glycoprotein I antibodies; LA, lupus anticoagulants; ERM, ezrin, radixin and moesin; anti-moesin Abs, anti-moesin antibodies; anti-moesin mAbs, monoclonal anti-moesin antibodies; anti-moesin-N Abs, anti-amino (N)-terminal portion of moesin antibodies; anti-moesin-C Abs, anti-carboxyl (C)-terminal portion of moesin antibodies; RA, rheumatoid arthritis; AA, aplastic anemia; ANCA, antineutrophil cytoplasmic antibody; s, seconds; min, minutes; h, hours; RT-PCR, reverse transcription-polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; TBS, Tris buffered saline; BSA, bovine serum albumin; TMB, 3,3,5,5-tetramethylbenzidine; A, absorbance; SD, standard deviation; CO, cut-off value; PBS, phosphate buffer saline; PC5, phycoerythrin cyanin 5; FITC, fluorescein isothiocyanate; Mnx, mean channel number; MFI, mean fluorescence intensity; RGDS, Arg-Gly-Asp-Ser; PAC-1, platelet activator combined-1; GP, glycoprotein; PRP, platelet-rich plasma; PPP, platelet-poor plasma; PAR, platelet aggregation rate; PAIR, platelet aggregation inhibition rate.

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cytoskeleton, thus inducing cytoarchitectural rearrangements and mediating the formation of filopodia and lamellipodia. Platelet moesin, by agonist induced relocation to filopodial extensions, might redistribute and concentrate linked surface receptors, thus contributing to platelet aggregation [7]. Although moesin was thought to be localized inside the cell membrane rather than on the cell surface, increasing evidences revealed that moesin was also detectable on the surface of T cells, NK cells, monocytes as well as macrophages [8–11]. According to Takamatsu et al., moesin was detectable in the exosomes derived from leukemia cell lines. Ingested by antigen-presenting cells, exosomes are known to be capable of presenting antigen, thus leading to the induction of anti-moesin Abs [12].

The presence of anti-moesin antibodies (anti-moesin Abs) was found in some autoimmune diseases, such as rheumatoid arthritis (RA), acquired aplastic anemia (AA) and antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis [13–15]. Although these disorders are not characteristically linked with thrombotic events as APS, aPL has also been detected in patients with AA and RA [16,17]. Moreover, a case–control study on AA conducted by the International Agranulocytosis and Aplastic Anemia Study Group revealed that a past history of RA was significantly associated with the later development of AA [18]. It is therefore possible that APS, AA and RA may share pathogenetic mechanisms leading to a breakdown of immunologic tolerance to moesin. However, no data was available on the existence and the pathogenic effect of anti-moesin Abs in patients with APS.

To identify novel autoantibodies and further study the pathogenesis of thrombophilia disorder in APS, we screened the serums from patients with APS for the presence of anti-moesin Abs recognizing antigens derived from prokaryotic expression system, and investigated the effect of murine monoclonal anti-moesin Abs (anti-moesin mAbs) on platelet activation and aggregation by flow cytometry and platelet aggregation assay in vitro.

Materials and Methods

Purification of Bacterially Expressed Moesin Proteins

The cDNA fragments of human moesin were synthesized by the reverse transcription-polymerase chain reaction (RT-PCR) amplification method. Briefly, cDNA was reverse transcribed from the mRNA of peripheral leukocytes of a healthy individual using SuperScript First-Strand Synthesis (Invitrogen, Carlsbad, CA). Primers used for the amplification of the amino (N)-terminal portion of moesin (1–320 amino acid residues of moesin) termed moesin-N and carboxyl (C)-terminal portion of moesin (494–577 amino acid residues of moesin) termed moesin-C were listed in Table 1. Thirty cycles of PCR were performed and each cycle consisted of denaturation at 94 °C for 30 seconds (s), annealing at 60°Cfor 1 minute (min), and extension at 72 °C for 1 min. PCR products purified from an agarose gel were inserted into the pET32a(+) vector (Novagen, Wisconsin, USA) between the EcoRI and HindIII sites for expression of a His- and Trx-tag fusion protein using BL21 competent cells (TaKaRa, Liaoning, China). Synthesized proteins were purified using glutathione Sepharose 4B (GE Healthcare, Connecticut, USA). Moesin-N and moesin-C protein fragments were released from His-tag moesin proteins using TAGZyme (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. The purity of moesin-N and moesin-C was detected by SDS-PAGE and the immunoreactivity of the recombinant proteins was detected by western blotting with mouse anti-moesin Abs (Clone MSN491; Abcam, Cambridge, UK) and rabbit anti-moesin Abs (Clone EP1863Y; Abcam), respectively. Purified moesin proteins were used as immunogen and screening antigen in the following enzyme-linked immunosorbent assay (ELISA) for the detection of polyclonal anti-moesin Abs in the serums of patients with APS and identification of monoclonal antibodies produced by positive clones.

Table 1
Primers used for the amplification of the amino (N)-terminal and carboxyl (C)-terminal portion of moesin.

Primer	Sequence (5'-3')	Products (bp)
N-F	CGGAATTCGCCTTTGCCGCCACCATGCCC	991
N-R	CCAAGCTTACGCTCCATCTGCTTCTGGTG	
C-F	CGGAATTCCTACGGGCTGATGCTATGGCC	292
C-R	CCAAGCTTTCCCTAGAGGCTGGGTGCCCA	

Detection of anti-moesin Abs, aCL and anti-β_2GPI in Patients with APS

The study was approved by the Ethics Committee of Ruijin Hospital, Shanghai Jiaotong University School of Medicine. After written informed consent was obtained, serum samples were collected from 100 patients clinically diagnosed with APS according to the international consensus classification criteria [1], among which 70 had vascular thrombosis (51 had venous thrombosis, 15 had arterial thrombosis and 4 had both) and 30 had pregnancy morbidity. Another 100 healthy individuals were included as normal controls. The detection of specific anti-moesin-N Abs and anti-moesin-C Abs was carried out by ELISA. Briefly, 96-well high-binding Costar plates were coated with 400 ng purified moesin-N or moesin-C in 100 μl coating buffer (50 mM carbonate/bicarbonate buffer, pH 9.6). After incubation overnight at 4 °C, plates were blocked with Tris buffered saline (TBS) containing 3% bovine serum albumin (BSA) for 2 hours (h) at 37 °C. 100 μl of diluted serum (1:200) from the patients with APS or healthy individuals were added in duplicate and the plates were incubated at 37 °C for 1 h. After a washing with TBS containing 0.05% Tween 20, bound human IgG was detected by HRP-labeled anti-human IgG antibodies (Abcam), and the plates were incubated at 37 °C for another 1 h. Finally, the plates were washed and incubated with 3,3,5,5-tetramethylbenzidine substrate (TMB) at room temperature for 10 min. The absorbance (A) was read at 450 nm. The average A value and the standard deviation (SD) of 100 healthy individuals were calculated. A cut-off value (CO) was defined as an A value equal to the average A value plus three times of SD of 100 healthy individuals. A values > CO were defined as positive, and A values > 3CO were defined as strong positive.

The aCL was detected by Zeus Anti-Cardiolipin IgG/IgA/IgM ELISA Kits (ZEUS Scientific, New Jersey, USA), and the anti-β_2GPI was detected by Anti-beta2 Glycoprotein 1 ELISA Kit IgG/IgA/IgM (Euroimmun Medizinische Labordiagnostika AG, Lübeck, Germany). Both of them were tested on two or more occasions at least 12 weeks apart to confirm the diagnosis. Statistical analyses were performed on GraphPad Prism v5.0 (GraphPad software Inc., CA, USA). The analyses used are indicated in the appropriate sections below. A P-value < 0.05 was considered significant.

Preparation of Murine anti-moesin mAbs

Murine anti-moesin mAbs were raised in our laboratory by hybridoma method first described by Kohler and Milstein [19] by immunizing BALB/C mice with bacterially expressed moesin-N and moesin-C. Briefly, spleen cells from the immunized mice were fused to SP2/0 myeloma cells using standard methodology. The fused cells were selectively harvested using HAT medium. Positive clones were detected by indirect ELISA described above (a moesin-N or moesin-C immune mouse serum served as a positive control and a normal mouse serum served as a negative control). Goat anti-mouse IgG-HRP conjugate (Abcam) was used as a secondary antibody. Positive clones were recloned three times to guarantee monoclonal behavior of the produced immunoglobulins. About 5×10^6 hybridoma cells from the stable clone were intraperitoneally inoculated to a BALB/c mouse for producing high-titer monoclonal antibody. The ascitic fluid was collected after 2 weeks. The class and subclass of mAbs was determined by mouse hybridoma subtyping