A novel strategy to identify autoantigens by proteomic analysis of plasma IgG-bound proteins

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SUMMARY

Autoimmune mechanisms have been hypothesized to underlie a number of human disorders in which both disease pathogenesis and diagnostic biomarkers remain poorly understood. This is partly due to a lack of efficient techniques for identification of plasma autoantibodies associated with specific pathophysiological conditions. We have developed a novel proteomic methodology to comprehensively identify plasma IgG-bound proteins using liquid chromatography tandem mass spectrometry (LC-MS/MS) after denaturing enriched plasma IgG to solubilize and release low molecular weight proteins. In total, we identified 44 proteins using this method that were undetectable in unprocessed plasma, 21 of which were not identified in the Human Plasma Proteome Draft of 2017. Comparison of plasma IgG-bound proteins between healthy subjects and patients with isolated adrenocorticotropic hormone deficiency, a rare endocrine disorder speculated to involve autoimmune mechanisms, revealed several distinct IgG-bound proteins specifically detected in patient plasma but not in healthy subjects. Our results suggest that solubilization of low molecular weight proteins bound to enriched plasma IgG and subsequent proteomic analysis by LC-MS/MS could provide a promising strategy for identification of autoantigens in human peripheral blood.

Key words: autoantigen, differential solubilization, liquid chromatography tandem mass spectrometry, isolated adrenocorticotropic hormone deficiency

INTRODUCTION

The presence of autoantibodies is an important clinical feature of autoimmune diseases, but may also be a serological characteristic of other conditions1. Autoantibodies occur in healthy individuals and could serve to protect against infection as well as to facilitate clearance of oxidized proteins and lipids released from apoptotic cells during inflammation2. Detection of serum autoantibodies is increasingly used in clinical practice, but diagnostic biomarkers for a number of autoimmune diseases remain totally unavailable. Identification of human plasma autoantibodies still remains a major technical challenge3.

Despite the anticipation that plasma proteomics techniques might enable revolutionary advances in the diagnosis and therapy of human diseases, conventional proteomics technology has only identified a handful of autoantigens used in routine clinical diagnosis4. Plasma proteins contain numerous distinct types of immunoglobulins (Igs) and other highly abundant proteins in addition to albumin, which is the largest constituent of plasma by weight. Meanwhile, the rarest proteins and peptides that are currently clinically detectable have plasma concentrations more than 12 orders of magnitude lower. The enormous dynamic concentration range of plasma proteins of interest has made direct proteomic profiling of plasma one of the most challenging tasks faced in contemporary analytical biochemistry5,6. We are already in the process of successfully improving our high-yield plasma extraction technique7. By efficiently depleting highly abundant plasma proteins with minimum loss of low-molecular-weight proteins, our original method, which we called “differential solubilization”, efficiently enriched...
plasma low-molecular weight proteins (LMWPs) including peptides both unbound and bound to carrier proteins7,8. In our original protocol, plasma LMWPs are first denatured, which allows LMWPs bound to carrier proteins to dissociate efficiently. This methodology has enabled detection of low-abundance authentic bioactive peptides that could never have been obtained used conventional methods9).

In an attempt to identify endogenous LMWPs bound to IgG in human plasma, we enriched plasma IgG, extracted LMWPs by denaturing purified IgG using a differential solubilization protocol, digested the resulting extracts with trypsin, and analyzed tryptic peptides using liquid chromatography tandem mass spectrometry (LC-MS/MS). Our expectation was that a successful approach to identify antigens in human plasma could be applied for discovery of autoantibodies in human diseases. We closely compared the sets of plasma antigens detected in healthy volunteers and patients with acquired isolated adrenocorticotropic deficiency (IAD), a rare endocrine disease presumed to be caused by an autoimmune mechanism10, 11). The present study was designed to elucidate whether our new protocol could successfully identify candidate IgG ligands that distinguished patients with IAD from healthy controls.

MATERIALS AND METHODS

Plasma sample collection

Six healthy volunteers (three men and three women, age 37.2±10.9 years) and eight patients with IAD (four men and four women, age 63.3±19.6 years) provided blood samples. None of the healthy volunteers had any current medical problems nor any past history of autoimmunity or allergic disease. The IAD patients were diagnosed by the presence of secondary adrenal insufficiency with diminished cortisol production, normal secretion of pituitary hormones other than adrenocorticotropic hormone, and the absence of structural pituitary defects. Blood samples were collected into vacutainers containing disodium EDTA (1.5 mg/mL) and plasma was separated immediately in a refrigerated centrifuge. Blood and plasma samples were aliquoted and stored at −80°C and −30°C, respectively. The study protocols were approved by the Kitasato University Medical School Ethics Committee (B17-325) and informed consent was obtained from all participants. All study methods were performed in accordance with the relevant guidelines and regulations of Kitasato University Medical School and the Declaration of Helsinki.

Isolation of IgG-bound LMWPs

Sixty μL of human plasma diluted 1:10 with phosphate buffered saline (PBS) was added to 400 μL of pretreated PureProteome™ Protein G Magnet Beads (Merck MILIPORE, Burlington MA, USA) and rotated gently for 1 h at room temperature. The tubes were then placed onto the magnetic stand. After removing and discarding the supernatant, the beads were washed 10 times with PBS. IgG-bound LMWPs were eluted essentially as described7,8 with the following modifications. Eighty μL of a solution of 7 M urea, 2 M thiourea, and 20 mM dithiothreitol (DTT) was added to each tube and vortexed for 1 h. The magnetically-separated eluates were slowly dropped into 2 mL of ice-cold acetone, immediately stirred at 4°C for a few seconds, and then centrifuged at 19,000×g for 15 min at 4°C. The precipitate was resuspended in 400 μL of 70% acetonitrile/ containing 12 mM HCl, mixed at 4°C for 1 h, centrifuged again, and the supernatant was lyophilized and stored at −80°C.

Depletion of high-abundance plasma proteins

High-abundance proteins were removed from human plasma samples using Pierce™ Top 12 Abundant Protein Depletion Spin Columns (Thermo Fisher Scientific, Waltham MA, USA) according to the manufacturer’s instructions. Briefly, 10 μL of plasma was applied to the column and rotated gently at room temperature for 60 min. The columns were then centrifuged at 1,000×g for 2 min. The depleted samples were collected and mixed with an equal volume of 20% trichloroacetic acid, then centrifuged at 19,000×g for 15 min at 4°C. The precipitate was added to 1 mL of 100% acetonitrile, centrifuged at 19,000×g for 15 min at 4°C, and the supernatant was removed and dried.

Tricine-sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE)

Pretreated plasma samples were dissolved in 50 mM Tris-HCl, pH 6.8, containing 50 mM DTT, 0.5% SDS, and 10% glycerol. These samples, along with molecular weight markers (Precision Plus Protein™ Dual Color Standards; BIO RAD, Hercules CA, USA) were applied to tricine-SDS-PAGE gels (Perfect NT Gel, 10–20%; DRC Co., Ltd., Tokyo, Japan) according to the manufacturer’s protocol. After electrophoresis, the gels were stained with silver nitrate (2D-Silver Stain Reagent II, Cosmo Bio Co., Ltd., Tokyo, Japan) or Coomassie Brilliant blue (CBB, PhastGel Blue R; GE Healthcare, Little Chalfont, UK). Gel images were converted to densitograms using ImageJ software (http://rsb.info.nih.gov/ij/)12).

Protein digestion and pretreatment for LC-MS/MS analysis

Three types of pretreated plasma samples were digested: (i) 6 μL of plasma diluted 1:20 with 200 mM triethylammonium bicarbonate/12 mM sodium deoxycholate/12 mM sodium lauryl sulfate13, (ii) LMWP precipitates obtained using protein G beads, and (iii) high abundance protein-depleted plasma dissolved in 40 μL of 200 mM triethylammonium bicarbonate/12 mM sodium deoxycholate/12 mM sodium lauryl sulfate, mixed for 10 min at room temperature and sonicated for 10 min at 4°C. These samples were centrifuged at 19,000×g for 15 min at 4°C, and 20 μL of the su-
pernatant was digested essentially as described\(^\text{13}\) with the following modifications. Two \(\mu\)L of a solution of 200 mM tris (2-carboxylethyl) phosphine hydrochloride and 120 mM triethylammonium bicarbonate were added to samples and incubated at 50°C for 30 min. After adding 2 \(\mu\)L of 375 mM iodoacetamide, the samples were incubated in the dark for 30 min. Two \(\mu\)L of Lys-C (100 ng/\(\mu\)L) and 2 \(\mu\)L of trypsin (100 ng/\(\mu\)L) were added to each sample, which were then incubated at 37°C for 18 h. Thirty \(\mu\)L of 10% acetonitrile and 15 \(\mu\)L of 5% trifluoroacetic acid were added to each digest, which were centrifuged at 19,000 \(\times\) g for 15 min and the supernatants were recovered.

Samples were desalted using stop and go extraction tips (stage tips\(^\text{14}\) filled with Empore\textsuperscript{TM} C18 sealant (Octadecyl) (3M Co., Saint Paul, MN, USA). Peptides were eluted with 80 \(\mu\)L of 70% acetonitrile containing 0.1% trifluoroacetic acid. The sample solution was then lyophilized and re-dissolved in 20 \(\mu\)L of 3% acetonitrile/0.1% formic acid for LC-MS/MS analysis.

**LC-MS/MS analysis and protein identification**

Samples were injected onto a C\(_{18}\) 0.075×120 mm analytical column (Nano HPLC Capillary Column; Nikkyo Technos, Tokyo, Japan) attached to an EASY-nLC 1000 HPLC system (Thermo Fisher). The mobile phase consisted of 0.1% formic acid and 90% acetonitrile (solvent A), and the mobile phase gradient was programmed as follows: 5–25% A (0–135 min), 25–50% A (135–170 min), 50–95% A (170–174 min), and 95% A (174–180 min). Separated peptides were subjected to analysis on a Q-Exactive\textsuperscript{TM} instrument (Thermo Fisher) operated in data-dependent mode to automatically switch between full-scan MS and MS/MS acquisition. Full-scan mass spectra (m/z 350–1000) were acquired on an Orbitrap instrument (Thermo Fisher) with 70,000 resolution at m/z 200 after accumulation of ions to a 1×10\(^6\) target value. The 12 most intense peaks with charge states more than two from the full scan were selected with an isolation window of 2.4 Da, and fragmented in the higher energy collisional dissociation cell with a normalized collision energy of 27%. Tandem mass spectra were acquired on the Orbitrap mass analyzer with a mass resolution of 17,500 at m/z 200 after accumulation of ions to a 5×10\(^6\) target value. The ion selection threshold was 5×10\(^4\) counts, and the maximum allowed ion accumulation times were 120 ms for full MS scans and 200 ms for tandem mass spectra. Typical mass spectrometric conditions were as follows: spray voltage, 2 kV; no sheath and auxiliary gas flow; heated capillary temperature, 250°C; and dynamic exclusion time, 15 s.

Database searches were performed using the SEQUEST algorithm\(^\text{15}\) incorporated in Proteome Discoverer 1.4.0.288 software (Thermo Fisher). The search parameters were as follows: enzyme, trypsin; variable modification, oxidation (M); static modification, carbamidomethyl(C); peptide ion mass tolerance, 6.0 ppm; fragment ion mass tolerance, 0.02 Da. The false discovery rate for peptide identification was set at 1%.

**RESULTS**

**Extraction of IgG-bound proteins**

Commercially-available proteins G beads were used to successfully capture and enrich IgG from human plasma, significantly depleting albumin and other highly abundant plasma proteins in comparison with unprocessed plasma (Fig. 1A, lanes 1 and 2). Both IgG heavy chains and light chains (approximately 50 kDa and 25 kDa, respectively) were clearly visible in protein G-purified fractions when

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**Fig. 1.** Tricine-SDS-PAGE of unprocessed human plasma, abundant protein-depleted plasma and IgG-bound LMWP fractions.

A. Unprocessed human plasma (lanes 1) and human plasma extracted using protein G beads either without (lanes 2) or with subsequent differential solubilization procedure to isolate LMWP fractions (lanes 3) were applied to Tricine-SDS-PAGE gels and subjected to subsequent Coomassie blue and silver staining. M: Precision Plus Protein\textsuperscript{TM} Dual Color Standards. B. Gel images in (A) were converted to densitograms using ImageJ software.
126 proteins were identified from IgG-bound LMWPs after excluding IgG-derived proteins (Table 2).

Comparison of IgG-bound proteins in IAD patients and healthy subjects

We next applied this protocol to extract and identify plasma IgG-bound LMWPs from patients with IAD and healthy volunteers in an attempt to identify circulating autoantibodies associated with IAD. Plasma samples obtained from eight patients with a definite diagnosis of IAD and six healthy subjects were processed using protein G beads, eluted using the differential solubilization procedure and, after proteolytic digestion, subjected to LC-MS/MS. In total, 2372 peptides derived from 497 proteins were identified once all IgG-related and keratin-related proteins were excluded. The majority of these proteins were present separated by tricine-SDS-PAGE and stained with CBB or silver (Fig. 1A, lane 2). Proteins complexed with protein G-adsorbed IgGs were extracted with a denaturing solution consisting of urea, thiourea, and DTT, and the eluates were then dropped onto acetone, which allowed bound proteins to detach and precipitate. By dissolving the precipitate with 70% acetonitrile containing 12 mM HCl, the LMWPs appeared to have been separated from other high molecular weight proteins, consisting mostly of IgG. The yields of LMWPs were evaluated using Tricine-SDS-PAGE, which demonstrated that larger molecular weight proteins had been successfully eliminated by our modified differential solubilization protocol (Fig. 1A, lane 3). Densitometric analysis of Tricine-SDS-PAGE gels confirmed our visual observations of CBB- and silver-stained gels, showing that LMWPs with 15 kDa or less were enriched and extracted (Fig. 1B). Thus, LMWPs complexed with plasma IgG appeared to have been efficiently enriched by this differential solubilization procedure.

Identification of IgG-bound proteins

We proteolytically digested the LMWPs and comprehensively identified IgG-bound plasma proteins using LC-MS/MS. Unprocessed plasma and abundant protein-immunodepleted plasma were similarly digested with trypsin and analyzed by LC-MS/MS. Database searching was performed using SEQUEST. A total of 206 proteins and 6920 peptide-spectrum matches (PSMs) were identified in LMWP fractions, of which 80 proteins and 4902 PSMs were IgG-derived proteins (Table 1). Approximately 70% of these IgG-derived proteins consisted of constant and variable regions of Ig kappa and lambda chains and variable regions of Ig heavy chains with less than 15 kDa. In addition, whole Ig light chains with 25 kDa were also identified. These IgG-related proteins were assumed to represent the largest members of the LMWP fractions observed by tricine-SDS-PAGE (Fig. 1A, faint ~25 kDa band and other proteins in the upper half portion within red rectangle) which escaped removal by our differential solubilization protocol. Excluding all IgG-derived proteins, the proteins identified by LC-MS/MS analysis from unprocessed plasma, abundant protein-immunodepleted plasma, and IgG-bound LMWPs are depicted as a Venn diagram in Fig. 2. In total, 3707 peptides derived from 404 distinct proteins were identified. Among these, 126 proteins were identified from IgG-bound LMWPs after excluding IgG-derived proteins (Table 2).

Comparison of IgG-bound proteins in IAD patients and healthy subjects

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Table 2. List of 126 proteins identified by LC-MS/MS analysis of IgG-binding LMWP fractions isolated using protein G beads and eluted by differential solubilization.

| Protein Name                                      | Uniprot accession | Gene name | Uniprot entry name | MW x 10^-3 | Peptides |
|---------------------------------------------------|-------------------|-----------|--------------------|-------------|----------|
| 39S ribosomal protein L28, mitochondrial           | Q13084            | MRPL28    | RM28_HUMAN         | 30.1        | 1        |
| ADAM DEC1                                         | Q15204            | ADAMDEC1  | ADEC1_HUMAN        | 32.7        | 1        |
| ADAMTS-like protein 4                             | Q6UY14            | ADAMTS4   | ATLA_HUMAN         | 116.5       | 1        |
| Alpha-1,3-mannosyl-glycoprotein 4-beta-N-acetylglucosaminyltransferase A | Q8UM21            | MGAT4A    | MGT4A_HUMAN        | 61.5        | 1        |
| Angiopoietin-related protein 6                     | Q8NI99            | ANGPTL6   | ANGL_HUMAN         | 51.7        | 2        |
| Breast cancer metastasis-suppressor 1             | Q9HC993           | BRMS1     | BRMS1_HUMAN        | 28.4        | 1        |
| Cation-binding protein 26                         | Q8IX18            | CDH126    | CAD26_HUMAN        | 95.2        | 1        |
| Cavin-1                                           | Q13939            | CCIN      | CALL_HUMAN         | 66.5        | 1        |
| Caveolin-associated protein 2                      | Q95810            | CAVE1     | CAVE1_HUMAN        | 47.1        | 3        |
| Caveolin-1-associated protein 2                    | Q13387            | MAPK8I     | JIP2_HUMAN         | 87.9        | 1        |
| Collagen alpha-1(XVIII) chain                     | P90960            | COL18A1   | COL18A1_HUMAN      | 178.1       | 1        |
| Collectin-10                                      | Q6YUZ7            | COLEC10   | COL10_HUMAN        | 30.7        | 1        |
| Dermcidin                                         | P81603            | DCD       | DCD_HUMAN          | 11.3        | 1        |
| Dermolkin                                         | Q6EOU4            | DMKN      | DMKN_HUMAN         | 47.1        | 1        |
| Disintegrin and metalloproteinase domain-containing protein 8 | P78325           | ADAM8    | ADAM8_HUMAN        | 88.7        | 1        |
| DNA excision repair protein ERCC-6                 | Q30468            | ERCC6     | ERCC6_HUMAN        | 168.3       | 0        |
| Fatty acid-binding protein, epidermal              | Q01849            | FABP5     | FABP5_HUMAN        | 15.2        | 1        |
| Heat shock protein beta-1                         | P04792            | HSPB1     | HSPB1_HUMAN        | 22.8        | 1        |
| HERV-H_2q24.1 provirus ancestral Env polypeptide   | Q09258            | no gene name | ENH3_HUMAN     | 60.9        | 1        |
| Hornerin                                          | Q86Y73            | HNR      | HNR_HUMAN          | 282.2       | 2        |
| Inactive serine protease 35                        | Q8N3Z0            | PRSS35    | PRS35_HUMAN        | 47.1        | 1        |
| Insulin-like growth factor-binding protein 4       | P22692            | IGFBP4    | IGBP4_HUMAN        | 27.9        | 3        |
| Insulin-like growth factor-binding protein 7       | Q16270            | IGFBP7    | IGBP7_HUMAN        | 29.1        | 1        |
| Integrator complex subunit 10                     | Q9NVF23           | INTS10    | INTS10_HUMAN       | 82.2        | 1        |
| Interferon regulatory factor 8                     | Q02566            | IRF8      | IRF8_HUMAN         | 48.3        | 1        |
| Kinesin-like protein KIF1C                         | O43896            | KIF1C     | KIF1C_HUMAN        | 122.9       | 1        |
| Leucine--tRNA ligase, cytoplasmic                  | Q9PS15            | LARS      | SYLC_HUMAN         | 134.4       | 1        |
| Matrix-1                                          | Q93G39            | MATN2     | MATN2_HUMAN        | 106.8       | 1        |
| Mediator of RNA polymerase II transcription subunit 30 | Q96HR3           | MED30     | MED30_HUMAN        | 20.3        | 1        |
| Multimerin-1                                       | Q13291            | MMRN1     | MMRN1_HUMAN        | 138         | 1        |
| Neutrophil defensin 1                             | P59665            | DEFA1     | DEFA1_HUMAN        | 10.2        | 1        |
| NHS-like protein 1                                | Q5SYE7            | NHS1     | NHS1_HUMAN         | 170.6       | 1        |
| Protein KIAA0100                                  | Q14667            | KIAA0100  | K0100_HUMAN        | 253.5       | 1        |
| Protein S100-A7                                   | P31151            | S100A7    | S10A7_HUMAN        | 11.5        | 1        |
| Protein S100-A8                                   | P65109            | S100A8    | S10A8_HUMAN        | 10.8        | 1        |
| Regenerating islet-derived protein 3-alpha         | Q06141            | REG3A    | REG3A_HUMAN        | 19.4        | 1        |
| Retikulocitin-1                                   | Q15293            | RCN1     | RCN1_HUMAN         | 38.9        | 1        |
| Serine protease inhibitor Karal-type 5             | Q8NQJ38           | SPINK5    | SPINK5_HUMAN       | 120.6       | 1        |
| Syntaxin-binding protein 1                        | P61704            | STXB1     | STXB1_HUMAN        | 67.5        | 1        |
| Transcriptional adapter 2-beta                     | Q86TJ2            | TAD2B     | TAD2B_HUMAN        | 48.4        | 0        |
| Protein Name                      | Accession | Description          | Msp | Csp | Fsp | CspH | Nsp | Subfamily | Mass | Msp/Human | Csp/Human | Fsp/Human | CspF/Human | FspF/Human | Nsp/Human | Subfamily |
|----------------------------------|-----------|----------------------|-----|-----|-----|------|-----|-----------|------|-----------|-----------|-----------|-------------|-------------|-----------|-----------|
| Transient receptor potential cation channel subfamily M member 7 | Q96QT4    | TRPM7_HUMAN          | 212.6 | 2   | 0   |      |     |           |      | 212.6     | 2          | 0          |             |             |           |           |
| X-linked zinc finger protein 418 | X36ET4    | TRPM7_HUMAN          |   76.6 | 1   | 0   |      |     |           |      | 76.6      | 1          | 0          |             |             |           |           |
| Zinc finger protein 418          | X36ET4    | TRPM7_HUMAN          |   77.8 | 1   | 0   |      |     |           |      | 77.8      | 1          | 0          |             |             |           |           |
| Zyxin                            | X36ET4    | TRPM7_HUMAN          |   61.2 | 2   | 0   |      |     |           |      | 61.2      | 2          | 0          |             |             |           |           |
| X-linked zinc finger protein 418 | X36ET4    | TRPM7_HUMAN          |   58.2 | 2   | 0   |      |     |           |      | 58.2      | 2          | 0          |             |             |           |           |
| Uncharacterized protein C1orf6   | X36ET4    | TRPM7_HUMAN          |   77.2 | 1   | 1   |      |     |           |      | 77.2      | 1          | 1          |             |             |           |           |
| X-linked zinc finger protein 418 | X36ET4    | TRPM7_HUMAN          |   77.2 | 1   | 1   |      |     |           |      | 77.2      | 1          | 1          |             |             |           |           |
UniProt accession code, Gene Name, Uniprot entry name, and approximate molecular weight for each protein are listed together with the number of peptides identified in each protein sequence. The number of peptides identified from unprocessed plasma and abundant protein-immunodepleted plasma is also shown. Peptides identified or not identified by HPPD are denoted by 1 or 0, respectively. *HPPD, Human Plasma Proteome Draft of 2017
in relatively high concentrations and were detected even in unprocessed plasma as well. Thus, it is probable that these proteins were nonspecifically adsorbed by protein G beads. However, we also identified 18 peptides in none of the healthy subjects and in at least four out of eight IAD patients (Table 3).

DISCUSSION

In the present study, we described a novel methodology to isolate and identify IgG-bound LMWPs present in human plasma. We identified 126 proteins, of which 78 were also detected in abundant protein-immunodepleted plasma. Some of these 78 proteins may have non-specifically bound to protein G beads, then eluted and detected in LC-MS/MS analyses. However, others could have been endogenously bound by plasma IgG after acquiring autoantigenicity through cleavage and/or posttranslational modification. The remaining 44 proteins were not identified in unprocessed plasma of the same individuals, and 21 of these proteins were not even identified by the Human Plasma Proteome Project [17]. It is likely that many of these 44 proteins represent either autoantigens or binding partners of IgG non-Fab-fragment regions in healthy human plasma. Thus, the IgG-binding LMWP fractions we isolated using this strategy could contain antigens present in human peripheral circulation.

Analysis of IgG-bound proteins in patients with IAD revealed that 18 tryptic peptides were specifically identified with IAD patients and not in any of the healthy subjects (Table 3). The obtained results suggest that these peptides were parts of LMWP bound to IgG as autoantigen through cleavage and/or posttranslational modification. One example was the 15-residue tryptic peptide, DSDWFFCSDEDWNYK, derived from fibrinogen α chain (residues 49–63) (Table 3, No. 5). This peptide was present at exceptionally high concentrations in four patients with IAD, and its PSMs value exceeded 20. These results suggest the possibility that an autoantibody specific for a cleaved fragment of fibrinogen α chain was present in the plasma of these patients. In the other five peptides exclusively associated with IAD (Table 3, No. 3, 7, and 10–12), a methionine residue was oxidized. In the unprocessed plasma of healthy subjects, identical peptides with methionine oxidation were not detected, although two unoxidized peptides were present at lower concentrations (Table 3, No. 3 and 7). Thus, it is possible that posttranslational modification of plasma proteins could have generated antigenicity and raised autoantibodies in IAD patients’ plasma. IAD is more likely develop in older individuals while the healthy control subjects used in the present study were younger. Therefore, although the IgG-bound LMWPs identified in the current analysis include strong candidates for IAD-specific autoantigens, some of these autoantigens may also be associated with aging.

IAD has been described in combination with autoimmune diseases, including Hashimoto thyroiditis [17,18] and lymphocytic hypophysitis [19]. In IAD, only ACTH-producing cells are impaired in the pituitary anterior lobe tissue in which five distinct types of secretory cells coexist. In addition, autoantibodies against pituitary proteins may often be present in IAD [16,17,18]. Thus, an autoimmune destruction of corticotrophs is implicated as the underlying cause of hormonal deficit in IAD. However, exact pathophysiological significance of such autoantibodies remains unknown and the diagnostic biomarkers unavailable. The current methodology could allow the identification of novel disease-specific antibodies present in human peripheral circulation irrespective of the LMWP/peptides structure.

Identification of proteins in biological samples is a central focus of proteomics. The discovery and validation of biomarkers may be accomplished by mass spectrometry-based proteomics [19,20], but in analyses of mass-limited samples like human plasma, it can be challenging to obtain sufficient enrichment of proteins to generate high-quality mass spectra. In the current analysis, we used three distinct processing methods to extract human plasma prior to LC-MS/MS analysis. For unprocessed plasma samples, 0.01 μL of plasma digested with trypsin was injected onto a column for LC-MS/MS analysis. In the case of abundant protein-depleted plasma and IgG-binding LMWP fractions, the final samples analyzed had been enriched from plasma volume equivalent to 0.2 and 28.5 μL, respectively. Thus, our protocol is advantageous to release and enrich IgG-bound ligands and could comprehensively detect autoantigenic candidate peptides present in human plasma.

In conclusion, we have developed a novel strategy to comprehensively identify plasma IgG-bound proteins using LC-MS/MS after denaturing the enriched plasma IgG and purifying the released LMWPs. Comparison of plasma from IAD patients and healthy volunteers revealed candidate autoantigens associated specifically with this disorder. Our protocol could be applied to identify additional autoantibodies present in human peripheral circulation.

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Table 3. 18 tryptic peptides specifically detected in IAD patients but not in healthy subjects.

| No. | Sequence | UniProt Accessions | Protein Name | Gene Name | Uniprot Entry Name | Start | End | H1 | H2 | H3 | H4 | H5 | H6 | I1 | I2 | I3 | I4 | I5 | I6 | I7 | I8 | PSMs Healthy subjects | PSMs IAD | PSMs Unprocessed plasma | PSMs Top-12-depleted plasma |
|-----|----------|--------------------|--------------|-----------|--------------------|-------|-----|----|----|----|----|----|----|----|----|----|----|----|----|----|------------------|-------------|------------------|------------------------|
| 1   | QLQEELEEVK | Q6Q788 Apolipoprotein A-V | APOA5 | APOA5_HUMAN | 95 104 | 1 1 1 1 1 1 1 | | | | | | | | | | | | | | | |
| 2   | WVQTLSEQVQE ELSSQVTLSEQVQE | P62649 Apolipoprotein E | APOE | APOE_HUMAN | 57 79 | 1 1 1 1 1 1 1 1 1 | | | | | | | | | | | | | | | |
| 3   | SWFPELYEDM(16)QR | P62649 Apolipoprotein E | APOE | APOE_HUMAN | 281 292 | 1 2 2 3 1 | | | | | | | | | | | | | | | |
| 4   | EALIQFLEQVHVQIK | P15169 Carboxypeptidase N catalytic chain | CPN1 | CPN1_HUMAN | 329 343 | 1 1 2 1 3 5 | | | | | | | | | | | | | | | |
| 5   | DSDWPFCSDEDWNYK | P02671 Fibrinogen alpha chain | FGA | FIBA_HUMAN | 49 63 | 20 35 34 23 | | | | | | | | | | | | | | | |
| 6   | MKPVDLVPNFLKSQLQK | P02671 Fibrinogen alpha chain | FGA | FIBA_HUMAN | 236 243 | 2 3 1 2 1 2 | | | | | | | | | | | | | | | |
| 7   | ALTDM(16)PQM(16)R | P02671 Fibrinogen alpha chain | FGA | FIBA_HUMAN | 236 243 | 1 2 2 1 5 5 | | | | | | | | | | | | | | | |
| 8   | FGSYCPITTGIADFLST YQTKVDK | P02679 Fibrinogen gamma chain | FGG | FIBG_HUMAN | 41 64 | 1 1 1 1 | | | | | | | | | | | | | | | | |
| 9   | ANQFLYCEIDGSGN GWTVFQK | P02679 Fibrinogen gamma chain | FGG | FIBG_HUMAN | 200 222 | 1 5 3 5 1 2 2 | | | | | | | | | | | | | | | |
| 10  | FFTSHNMG(16)QSTW DNDKDR | P02679 Fibrinogen gamma chain | FGG | FIBG_HUMAN | 329 347 | 1 3 4 1 2 | | | | | | | | | | | | | | | |
| 11  | LGGSPTSLGTWGSWIG PDHKPSA(16)MK | P14314 Glucosidase 2 subunit beta | PRKSH | GLU2B_HUMAN | 438 464 | 1 1 1 1 2 | | | | | | | | | | | | | | | |
| 12  | EKSM6/PWNYDLSK | Q06543 Hsp90 co-chaperone Cdc37 | CDC37 | CDC37_HUMAN | 109 121 | 1 3 2 1 | | | | | | | | | | | | | | | | |
| 13  | WPEPVPVGR | O00387 Human-binding serine protease 2 | MASP2 | MASP2_HUMAN | 22 29 | 1 1 2 1 | | | | | | | | | | | | | | | | |
| 14  | FYGLQVTK | P04281 Matrix metalloproteinase-14 | MIP14 | MIP14_HUMAN | 71 79 | 1 1 1 1 | | | | | | | | | | | | | | | | |
| 15  | VDIVEFHIFPLMQLTVAK | P0660 Myosin light polypeptide 6 | MYL6 | MYL6_HUMAN | 64 79 | 1 1 3 1 1 | | | | | | | | | | | | | | | |
| 16  | EYGVLPAGSTAVEP LLAGLEAGLR | Q06155 N-acetylmuramoyl-L-alanine amidase | PGPR2 | PGPR2_HUMAN | 107 135 | 1 1 1 1 2 3 | | | | | | | | | | | | | | | |
| 17  | IALGGLFLPSNLIR | P04278 Sex hormone-binding globulin | SHBG | SHBG_HUMAN | 171 183 | 1 1 2 2 1 5 | | | | | | | | | | | | | | | |
| 18  | TLMLNLGLLVAR | P62782 Transglut-2 | TAGLN2 | TAGL2_HUMAN | 128 139 | 1 3 1 1 | | | | | | | | | | | | | | | | |

UniProt accession code, Protein name, Gene name, Uniprot entry name, and identified amino acid sequence number for each peptide are listed together with the number of PSMs identified in each peptide sequences. Number of PSMs identified from unprocessed plasma and abundant protein-immunodepleted plasma, if any, were also shown. *PSMs, peptide-spectrum matches
with accession number PXD012123. No additional external funding was received for this study. We thank Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

**ABBREVIATIONS**

LC-MS/MS, liquid chromatography tandem mass spectrometry; Ig, immunoglobulin; IgG, immunoglobulin G; IAD, isolated ACTH deficiency; ACTH, adrenocorticotrophic hormone; CBB, Coomassie brilliant blue; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PTS, phase transfer surfactant; PSMs, peptide spectrum matches; SDS, sodium dodecyl sulfate.

**CONFLICT OF INTEREST**

The authors declare no competing financial interests.

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