CLINICAL STUDY

Autoantibodies against 21-hydroxylase and side-chain cleavage enzyme in autoimmune Addison’s disease are mainly immunoglobulin G1

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Abstract

Objective: Immunoglobulin G (IgG) antibodies to the steroidogenic enzymes 21-hydroxylase (21OH) and side-chain cleavage enzyme (SCC) are important diagnostic markers for autoimmune Addison’s disease and autoimmune polyendocrine syndromes (APS) types I and II. The characterization of autoantibody (IgG) subclasses may reveal information on how tissue destruction takes place; therefore, IgG subtypes of anti-21OH and anti-SCC antibodies from sera of patients with Addison’s disease, APS I and APS II were determined using recombinant 21OH and SCC.

Methods: SCC \(51-521\) and his-SCC \(51-521\) were expressed by pET-scc in the Escherichia coli strain BL21 Star (DE3) and inclusion bodies were purified. Full-length, human 21OH fused to an N-terminal 6 \(\times\) histidine affinity tag was expressed in insect cells by using the baculovirus expression system bac-to-bac. Western blots were used to investigate the IgG subtype(s) of the autoantibodies against 21OH and SCC in patients and healthy blood donors.

Results: All anti-SCC positive sera (\(n=10\)) contained autoantibodies of the IgG1 subclass, while four out of ten also contained IgG3. All anti-21OH positive sera (\(n=16\)) had autoantibodies exclusively against IgG1. Sera from 20 healthy subjects did not show any reactivity against 21OH or SCC.

Conclusions: The finding of a predominating IgG1 response against 21OH and SCC may suggest that T helper (Th) cells of the Th1 subclass are involved in destruction of the adrenal cortex in patients with autoimmune Addison’s disease.

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Introduction

Autoimmune Addison’s disease can occur as a single disorder or as part of autoimmune polyendocrine syndrome type I (APS I) and type II (APS II). APS I is a monogenic autoimmune disease caused by mutations in the transcriptional regulator gene autoimmune regulator (AIRE) (1, 2). The three main components of APS I are Addison’s disease, hypoparathyroidism and mucocutaneous candidiasis. Several other endocrine and non-endocrine manifestations are also common, among them gonadal failure in females, diabetes mellitus, autoimmune hepatitis, malabsorption, enamel dysplasia and keratopathy (3, 4). APS II is defined as a combination of Addison’s disease with autoimmune thyroid disease and/or type 1 diabetes. An association has been found to the major histocompatibility complex (MHC) class II haplotypes DR3-DQ2 and DR4-DQ8 in both APS II and Addison’s disease, suggesting similar etiology (5).

Patients with Addison’s disease commonly produce autoantibodies against cytochrome P450 enzymes involved in the biosynthesis of steroid hormones, notably 21-hydroxylase (21OH) (6–8), side-chain cleavage enzyme (SCC) (8, 9) and 17-hydroxylase (17OH) (8, 10). All of these enzymes are expressed in the adrenal cortex, while 17OH and SCC are also expressed in the gonads. Anti-21OH is the most common autoantibody found in patients with Addison’s disease and in APS, and assay of anti-21OH is used clinically to determine the etiology of Addison’s disease (5, 11). Furthermore, anti-21OH antibodies can predict the development of adrenal destruction in individuals of families with other organ-specific autoimmune diseases (12). Autoantibodies against SCC seem to be more restricted to APS I (13) and Addison’s disease patients with premature gonadal failure (14).

The identity of the IgG subclasses of antibodies may indicate which T helper (Th) cell subset is involved in
the autoimmune response. In humans, IgG1 and IgG3 indicate Th1 responses, while IgG4 is seen when a Th2 response predominates (15). IgG1, IgG3 and IgG4 are primarily produced in response to protein antigens, while IgG2 is generated in response to polysaccharide antigens. The antibody isotypes stimulated by Th1 cells have high affinity for Fc receptors and are strong inducers of complement activation and antibody-mediated antigen uptake. Human IgG1 and IgG3 support antibody-dependent cellular cytotoxicity and complement-dependent cellular cytotoxicity towards membrane-associated antigens.

The tissue destruction in various experimental animal models for organ-specific autoimmune diseases like type 1 diabetes (16) and thyroid autoimmunity (17) is mediated by Th1 cells. Th2 cells are primarily responsible for allergic responses and asthma. Whether Th2 cells are involved in the development of autoimmune diseases is controversial. In order to find out more about the etiology of autoimmune adrenocortical failure, we have studied the IgG subclass specificity of autoantibodies from patients with Addison’s disease, APS I and APS II against purified, recombinant 21OH and SCC.

Subjects and methods

Patients and sera
Sera from patients with Addison’s disease, APS I and APS II containing autoantibodies against SCC (n = 10) and 21OH (n = 16) were selected for the studies (Tables 1 and 2 respectively). Autoantibodies were analyzed using radioimmunoassay as described below. The diagnosis of Addison’s disease, APS I and APS II was made using established clinical and laboratory test criteria (5, 18). Sera from 20 healthy blood donors were used as controls.

Radioimmunoassay
The expression plasmid pET-scc, containing a cDNA coding for a truncated form of SCC (SCC51-521), was generously provided by Dr Bon-chu Chung (Institute of Molecular Biology, Academia Sinica, Nankang, Taipei 115, Taiwan) (19). The expression plasmid pcDNAII-21OH encoding the full-length human 21OH was a kind gift from Prof. Holger Luthman (Department of Molecular Medicine, Karolinska Institute, Stockholm, Sweden) (11). pET-scc and pcDNAII-21OH were expressed in the cell-free TnT transcription-translation system from Promega in the presence of [15S]-labelled methionine (Amersham) and screened by radioimmunoassay (11). SCC and 21OH antibody reactivity are given as indices relative to a positive (patient serum) and negative control (pool of sera from healthy individuals) (\(\frac{\text{c.p.m. subject} - \text{c.p.m. negative standard}}{\text{c.p.m. positive standard} - \text{c.p.m. negative standard}} \leq 1000\)).

Expression and purification of SCC
SCC51-521 was expressed by pET-scc (see above) in the E. coli strain BL21 Star (DE3) (Invitrogen). Inclusion bodies were purified by the B-PER Bacterial Protein Extraction Reagent (Pierce, New York, USA) according to the manufacturer’s protocol and dissolved in 6 M urea. SCC51-521 was also expressed as a histidine-tagged fusion protein (his-SCC51-521). The truncated SCC was ligated into the Ndel and BamHI restriction sites of pET-19b (Novagen Inc., Madison, USA) by using SCC51-521 cDNA that was PCR-amplified by a forward linker-primer containing an NdeI restriction site (5’-GGAATTCCATATGATCCCCTCTCCTGGTGACAA-3’) and a reverse linker-primer containing a BamHI restriction site (5’-GGCGGATCCTTCCTCCCATGTGGCTGC-3’), his-SCC51-521 was expressed in the E. coli strain BL21 Star (DE3) and inclusion bodies were purified using

<table>
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* Determined by radioimmunoassay.
† Determined by immunofluorescence.
A, Addison’s disease; T, thyroidia; G, gonadal failure; P, pernicious anemia; IF Adrenals, immunofluoresence analysis showed that sera contained IgG that reacted with bovine adrenals.
B-PER Bacterial Protein Extraction Reagent. The inclusion bodies were solubilized in buffer A (2 ml 6 M guanidine-HCl, 20 MM NaPO₄, 500 MM NaCl, pH 7.8) before adding buffer B (4 ml 8 M urea, 20 MM NaPO₄, 500 MM NaCl, pH 7.8). The solution was then centrifuged at 10 000 g and filtered through a 0.45 μm syringe filter followed by his-SCC 51-521 nickel chelate chromatography using the ÄKTAprime chromatography system and HiTrap Chelating HP columns (Amersham) under denaturing conditions. Briefly, 1 ml purified inclusion bodies was loaded onto the column followed by washing with 20 column volumes of buffer B. The protein was eluted by exchanging buffer B with a buffer containing 8 M urea, 20 MM NaPO₄, 500 MM NaCl, pH 4.0 with a gradient from 0 to 100% over 20 column volumes.

Expression and purification of his-21OH

Full-length, human 21OH fused to an N-terminal 6 × histidine affinity tag was expressed in insect cells using the baculovirus expression system bac-to-bac (Gibco BRL). 21OH cDNA was excised from the pcDNAII-21OH construct (see above) by the restriction enzymes BamHI and XbaI and was ligated into the respective restriction sites downstream of a baculovirus polyhedrin promoter of the pFastBac HTc donor plasmid. Recombinant pFastBac HTc plasmids were used to transform competent DH10BAC cells for transposition to the bacmid shuttle vector. Bacmid recombinants were identified by LacZ selection on LB ampicillin plates (Luna-Bertani Broth, Miller, Difco Laboratories, Detroit, USA) containing Bluo-Gal and IPTG (isopropyl-thio-b-D-galactopyranoside, Sigma-Aldrich, Steinheim, Germany). Recombinant bacmid DNA was isolated from selected colonies according to the manufacturer’s instructions, mixed with CELLFECTION reagent (Gibco BRL Life Technologies), and used to transfect monolayers of Sf21 insect cells. Viral supernatant was harvested after 72 h. Stable, high-expressing recombinant virus was selected by one round of plaque purification and a high-titer viral stock (5 × 10⁸ pfu/ml) was generated for further infections and protein expression in Sf9 insect cells in shaker cultures. For protein expression, Sf9 insect cells were infected at a multiple of infection of 10, harvested by centrifugation 72 h post-infection and frozen at −80°C. The protein was extracted by lysing 1 × 10⁷ frozen cells using 5 ml B-PER Bacterial Protein Extraction Reagent. The insoluble fraction was collected by centrifugation and solubilized in 2 ml buffer A and diluted in 4 ml buffer B. The extract was clarified by centrifugation and filtered through a 0.45 μm syringe filter. his-21OH was purified as described for his-SCC 51-521 (see above), except that 20 MM imidazol was added to buffer A to reduce unspecific binding of proteins to the column.

Western blot

Recombinant SCC 51-521/his-SCC 51-521 (approximately 0.4 and 2 μg respectively) and his-21OH (approximately 2 μg) were incubated in 4 × sample buffer and applied to each well on a 10% NuPAGE MOPS Bis-Tris gel (Invitrogen). The proteins were blotted onto a Hybond C extra PVDF membrane (Amersham) and incubated with sera from patients or blood donors, diluted 1:100 in blocking buffer (PBS with 4% fat free dry milk) as primary antibody and alkaline phosphatase-conjugated anti-human IgG (rabbit antitotal IgG 1:3000, Sigma-Aldrich, and monoclonal mouse anti-subclass IgG1–4 1:750 from clones 4E3

**Table 2** Data of patients positive for autoantibodies against 21OH. Titers of autoantibodies higher than 173 are considered as positive values. The results of the subtyping of the autoantibodies against 21OH are also shown.

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</table>

* Determined by radioimmunoassay.

A, Addison's disease; V, vitiligo; T, thyroidea; G, gonadal failure; D, diabetes mellitus; Mal, malabsorption; C, candidiasis.
(anti-IgG1), 31-7-4 (anti-IgG2), HP6050 (anti-IgG3) and HP6025 (anti-IgG4) (20–22), Southern Biotechnology, Birmingham, LA, USA), diluted in blocking buffer, as secondary antibody. The substrate for alkaline phosphatase was 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BioRad). Due to the fact that different patient sera bind with different affinity and avidity to 21OH and SCC on membranes, we applied a high concentration of these proteins to the gels in order to increase the sensitivity at the expense of a relatively high background staining. Anti-human IgG and antibodies to the four human IgG subtypes all reacted with protein A purified IgG from a blood donor, confirming their reactivity (data not shown).

Results

Expression and purification of SCC and 21OH

SCC51-521 and his-SCC51-521 appeared >90% pure as judged by SDS-PAGE and Coomassie blue staining and appeared as proteins with molecular weights of about 55 kDa, which is fairly similar to their calculated molecular weight of 56.1 and 57.7 kDa respectively (Fig. 1). Their identities were verified by Western blot using a SCC-specific rabbit antiserum (9) (not shown). his-21OH appeared >90% pure in polyacrylamide gels stained with Coomassie blue and appeared as a protein with a molecular weight of about 55 kDa, which is fairly similar to its calculated molecular weight of 59.8 kDa (Fig. 1). Its identity was confirmed in Western blots using a patient serum positive for 21OH in radioimmunoassay.

Characterization of SCC-antibodies (Abs)

Figure 2 shows the reactivity of patient sera against his-SCC51-521. All ten sera tested revealed reactivity against the IgG1 subtype (Table 1). In addition, four sera contained SCC-Abs of the IgG3 subtype (two of these appeared only as very weak bands). IgG subtype classification of one of the patients is shown in Fig. 3. This particular APS II patient had both IgG1-Abs and IgG3-Abs (weak signal). None of the control sera from blood donors showed any reactivity against SCC. As a control of the specificity of the autoantibodies, we tested if any of the sera had reactivity against an unrelated protein (a truncated form of platelet-derived growth factor C (PDGF-C), which had been expressed and purified using an identical procedure to that used for SCC51-521. None of the sera detected bands of similar molecular weight to SCC when immunoblotted with PDGF-C (not shown).

Characterization of 21OH-Abs

All 16 patient sera revealed reactivity against 21OH (Fig. 4) and were of the IgG1 subtype (Fig. 5) (Table 2). Sera from 21OH-Ab-positive patients did not detect bands with the same molecular weight as 21OH when using a truncated form of PDGF-C, expressed in E. coli and purified as p-SCC51-521 (not shown), as substrate. None of the control sera from blood donors showed any reactivity against 21OH.
Discussion

The destruction of tissue in organ-specific autoimmune diseases is probably mainly caused by a T-cell-mediated autoimmune inflammation. The hypothesis that T cells are the main effectors of cell destruction in Addison’s disease is widely accepted, but not supported by much data. Published reports have shown T cell reactivity against adrenal proteins (23). Also, expression of the Ia antigen (MHC-class II DR, immune-associated) on T cells in individuals with recent-onset Addison’s disease suggests a T-cell-dependent immunological activation (24). A close association with certain MHC-class II haplotypes has been found in patients with Addison’s disease (25, 26), which further supports the importance of CD4\(^+\) (cluster of differentiation 4) T cells in this disease. Furthermore, knowledge of the mechanisms in related diseases such as type 1 diabetes and autoimmune hypothyroidism supports the involvement of T cells (16, 17).

It has been previously shown that autoantibodies against 21OH and SCC are of the IgG class (6, 9, 27). Here we show for the first time that anti-21OH autoantibodies are of the IgG1 subtype. In a previous study using indirect immunofluorescence, adrenocortical autoantibodies were found to be of the IgG1, IgG2 and IgG4 subtypes (27). Even though 21OH is the major adrenal cortex autoantigen, reactivity to other antigens may have played a role in the previous study. We also confirm that anti-SCC antibodies are predominantly of the IgG1 subtype (28) and that one-third of these patients produce anti-SCC antibodies of the IgG3 subtype. Similar findings have been reported in type 1 diabetes, describing IgG1 antibodies directed against tyrosine phosphatase-like molecule (IA-2) and GAD (29, 30).

The present study was carried out on a mixed cohort of patients. APS I patients have mutations in AIRE while studies on patients with Addison’s disease and APS II suggest multigenetic backgrounds. It cannot be ruled out that the autoantibody subclass distribution differs between groups of patients, although our study showed homogenous results for patients with Addison’s disease, APS I and APS II. In addition, our study was undertaken with denatured antigens. Using native proteins could detect conformational epitopes, and would
possibly identify a wider range of IgG subtypes. Evidence for the existence of conformational epitopes has been reported, e.g., a three-dimensional epitope in the C-terminal region of 21OH (31). Furthermore, even though we used similar concentrations of anti-human IgG subclasses 1–4, they are likely to have different affinities for their antigens. Although they were able to bind their respective IgG subtypes in Western blot of purified human total IgGs, very low levels of certain antigen-specific IgG subtypes might have been detected. Unspecific bands were frequently seen in Western blots using human sera as primary antibodies, especially when using p-SCC51-521, which was purified as inclusion bodies. These bands are likely to be due to contaminating bacterial proteins. Another explanation may be that the smaller bands are products from the proteolysis of proteins.

Our findings are consistent with an antigen-driven, T-cell-dependent antibody response. Even if the antibody responses of Th1 and Th2 immunity in humans are not as distinct as those seen in mice, a dominating IgG1 response against a certain antigen may imply a Th1 profile (32, 33). CD4\(^+\) Th1 cells, which are common effector cells during bacterial and viral infections, are induced by interleukin-12 (IL-12) and type I interferons in humans. They characteristically secrete IL-2, interferon-\(\gamma\) (IFN-\(\gamma\)) and tumor necrosis factor-\(\beta\) (TNF-\(\beta\)) and provide help for production of IgG1 and IgG3 in humans. IgG1 and IgG3 are more potent activators of the classic complement pathway and bind with higher affinity to Fc\(\gamma\) receptors than IgG2 and IgG4.

Unlike autoantibodies to membrane-bound receptors such as in Graves disease and myasthenia gravis, the pathological role of autoantibodies to intracellular proteins in organ-specific autoimmune diseases remains obscure. Since the autoantigens targeted in Addison’s disease are intracellular proteins, it is unknown whether the corresponding autoantibodies and their effector functions play a pathogenic role. A major question is how intracellular adrenal antigens are presented to the immune system in an immunostimulatory context. A possible, yet hypothetical, mechanism, is that the cell expresses the intracellular proteins on the exterior of the cell membrane as a result of stress, possibly during a viral infection. Antibodies may then bind the antigen and natural killer cells can, through Fc receptor binding, cause antibody-dependent cellular cytotoxicity (34). Complement fixing autoantibodies to intracellular proteins may be involved in organ damage if the antibodies bind liberated antigen and form immune complexes that deposit in organs. Another possibility is that autoantibodies to intracellular proteins may cross the plasma membrane and cause cell death (see reference 35 for review). Although antibodies against autoantigens in Addison’s disease have been shown to inhibit or diminish catalytic activity of the antigen/enzyme in vitro (9, 36, 37), they do not affect their function in vivo (38). Furthermore, transfer of sera from patients with Addison’s disease to experimental animals does not transfer disease (39, 40).

Even though autoantibodies to adrenocortical autoantibodies may not be directly pathogenic they may modulate T cell responses to the autoantigens they bind. Efficient complement and Fc receptor-mediated uptake of antigen–antibody complexes by dendritic cells may lead to stimulatory presentation of adrenal autoantigens on MHC class II to CD4\(^+\) T cells and on MHC class I to CD8\(^+\) T cells (41–43). This may boost the CD4\(^+\) T cell responses and initiate CD8\(^+\) T cell responses to the given autoantigen. Some antibodies can, depending on which determinant they bind, boost MHC class II presentation of certain T cell determinants, and might reveal cryptic T cell epitopes involved in autoimmune diseases (see reference 44 for review).

In conclusion, we have shown that autoantibodies against 21OH and SCC are predominantly of the IgG1 subclass. This may indicate that the CD4\(^+\) T cell response in Addison’s disease is dominated by Th1 cells, a finding that is consistent with the clinical findings of inflammation in the adrenal glands of Addison’s patients. Future studies should address the Th1/Th2 profile of patients with Addison’s disease. Hrda and co-workers recently published a report on cytokine levels in sera from patients with autoimmune endocrinopathies (45). They concluded that there were no differences in the concentration of Th1 cytokines and Th2 cytokines between patients and healthy individuals, suggesting that serum levels of cytokines are not good markers for actual T cell activity. The production of cytokines of autoantigen-specific T cells are of more importance, and should be studied in the future.

Acknowledgements

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