Identification of a Novel Autoantigen UACA in Patients with Panuveitis

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To identify the target autoantigens in Vogt-Koyanagi-Harada disease, we made use of an immunoscreening of a bovine uveal cDNA expression library with serum samples obtained from patients with Vogt-Koyanagi-Harada disease. We identified a novel bovine antigen and homologous human autoantigen and designated it as UACA (uveal autoantigen with coiled coil domains and ankyrin repeats). mRNA of human UACA is expressed most abundantly in skeletal muscles and in various human tissues, including choroid, retina, and epidermal melanocytes. IgG autoantibodies were quantitated in an ELISA, using recombinant C-terminal 18.0% fragment of human UACA. The prevalence of IgG anti-UACA autoantibodies in patients with panuveitis (Vogt-Koyanagi-Harada disease, Behc¸et's disease, sarcoidosis) was significantly higher than that in healthy controls (19.6–28.1% vs 0%, P < 0.05) indicating that autoimmunity directed against UACA is a common phenomenon in these diseases.© 2001 Academic Press

Key Words: Vogt-Koyanagi-Harada disease (VKH); Behc¸et's disease (BD); sarcoidosis; serological analysis of recombinant cDNA expression libraries (SEREX);

Vogt-Koyanagi-Harada disease (VKH) is an autoimmune systemic disorder. In VKH, inflammatory disorders occur in multiple organs containing melanocytes, including uvea (resulting in acute bilateral panuveitis), skin (resulting in vitiligo and alopecia), central nervous system (resulting in meningitis) and inner ears (resulting in hearing loss and tinnitus). These inflammatory aspects are attributed to the destruction of melanocytes through immunological mechanisms. Several findings suggest that T helper 1 type autoreactive CD4+ T cells are involved in the development of VKH (1–6). The strong association between HLA-DR4 (DRB1*0405)-DQ4 (DQA1*0302-DQB1*0401) haplotype and the susceptibility to VKH has been noted in the Japanese and Brazilian patients, hence, HLA-linked genetic background is related to the development of VKH (7–10).

Identification of target autoantigen is important to understand the etiology of autoimmune diseases, and for development of antigen-specific immuno-modulation therapy. In experimental autoimmune uveitis, S-antigen and interphotoreceptor retinoid-binding protein are used as autoantigens (11, 12). However, the relationship between these autoantigens and human autoimmune uveitis is uncertain. Studies have been done to elucidate the exact etiology and target autoantigen in VKH, but much remains to be investigated. To identify target autoantigens in autoimmune diseases, serological analysis of recombinant cDNA expression libraries (SEREX) has been done. SEREX is an immunoscreening method which makes use of prokaryotically expressed cDNA libraries prepared from target organs of the autoimmune diseases and sera from patients. This strategy proved effective to identify
disease-related autoantigens, in type 1 diabetes, and systemic lupus erythematosus (13, 14). When we used this approach to identify the target autoantigen of VKH, we identified a novel autoantigen UACA (uveal autoantigen with coiled coil domains and ankyrin repeats). The prevalence of IgG anti-UACA autoantibodies in VKH patients was significantly higher than in healthy control samples. However, these antibodies were observed also in patients with Behçet’s disease (BD) and sarcoidosis known as the major diseases that cause panuveitis in the Japanese population. A novel UACA protein appears to be a possible target autoantigen shared by VKH, BD, and sarcoidosis that cause different types of panuveitis.

MATERIALS AND METHODS

Patients. Forty-six patients (age 19–80 years; 18 men and 28 women) with VKH were studied. All these patients underwent complete ophthalmologic and related examinations to determine the clinical diagnosis. Seven patients (age 30–62; 6 men and 1 woman) with sympathetic ophthalmia, 32 patients (age 15–64 years; 20 men and 12 women) with BD complicated with panuveitis, 19 patients (age 16–67 years; 2 men and 17 women) with sarcoidosis complicated with panuveitis, and 36 healthy unrelated donors (age 22–55 years; 2 men and 17 women) with sarcoidosis were similarly processed. A total of 7.4 plates containing equal numbers of seropositive clones and seronegative phage plaques. Positive recombinant clones were picked up and purified by an additional cycle of plating and screening. To determine the reactivity of other serum samples to positive clones, plates containing equal numbers of sero-positive clones and seronegative control plaques were similarly processed. A total of 7.4 × 10^4 phage plaques were screened, using sera from four VKH patients. Sequence analysis of identified cDNA clones. Immunoreactive phage clones were subjected to in vivo excision of plBluescript phagemids, using the ExAssist helper phage SOLR strain system (Stratagene). Plasmid DNA was purified, using ABI Prism Miniprep Kits (PE Applied Biosystems, Foster, CA). cDNA inserts were sequenced, using an ABI Prism (Perkin-Elmer, Norwalk, CT) automated DNA sequencer and for sequence alignments we used BLAST software (GenomeNet, Japan).

Cloning and sequencing of human UACA cDNA. To isolate the human UACA cDNA, an 815-bp cDNA fragment of human EST clone IMAGE:1527412 (GenBank Accession No. AA916992) digested by BgIII and EcoRI was used as a probe to screen a J urkat cell cDNA library, this human cDNA clone was highly homologous to the isolated bovine immunoreactive cDNA clone (87% identical to nucleotide position 1130–1944 of bovine cDNA clone). One positive clone was isolated and sequenced. The sequence of this human cDNA fragment was highly homologous to the sequence of identified immunoreactive bovine cDNA clone, exhibiting 87% homology at nucleotide sequence level. PCR cloning was also done to isolate the human UACA cDNA, using a human uveal cDNA as a template. Forward primers F-1, F-2 and reverse primers R-1, R-2 corresponding to nucleotide position 4450–4474, 19788–19813, 19728–19753, and 20922–20936 respectively of Homo sapiens chromosome 15 clone RP11-64K10 map 15q24, WORKING DRAFT SEQUENCE (GenBank Accession No. AC010076), and forward primer corresponding to nucleotide position 80–100 of Homo sapiens cDNA clone DKFZp434E2235 (GenBank Accession No. AL044711) were used for DNA amplification. The amplified fragments were subcloned into pEG-T vector (Promega, Madison, WI) and sequenced by using an ABI Prism automated DNA sequencer. Secondary structure analysis was done using the software program COILS (17).

Northern blot analysis and reverse transcription-PCR (RT-PCR). For Northern blot analysis, the mRNA blot from CLONTECH (Palo Alto, CA) was hybridized with the 32P-labeled cDNA fragment of human EST clone IMAGE:1527412 (GenBank Accession No. AA916992) as described in the manufacturer’s instructions. For RT-PCR, total RNA was isolated from human epidermal melanocytes (Cell Systems Corp., Kirkland) and normal human choroidal and retinal tissues, using TRIZOL reagent. Subsequently, poly(A) RNA was purified with the Dynabeads mRNA Purification Kit (DYNAL, Oslo, Sweden). These tissues were obtained from the enucleated eye from a patient with myocytic of supermaxilla. Immunoreactive DNA was purified after explanation of nature of the research was explained following the tenets of the Declaration of Helsinki. Poly(A) RNA was purified from 10 μg of each total RNA subjected to cDNA synthesis, using random hexamer primers and Superscript reverse transcriptase (GIBCO/BRL). Gene-specific PCR primers were designed to amplify fragments of 505 bp and used in the RT-PCR (95°C for 1 min, 58°C for 1 min and 72°C for 1 min, 30 cycles). PCR primers for UACA were forward, 5'-GAGAAAGAAGGTGAATCAATCA-3' and reverse, 5'-TTGTGAGGTTGAGTTGGAAAAG-3'. PCR using cDNA-specific beta-actin primers was done as control (18). Preparation of glutathione-S transferase (GST) fusion protein. A 783-bp DNA fragment digested from Homo sapiens cDNA clone IMAGE 608930 (GenBank Accession No. AA197064) corresponding to nucleotide position 3462–4245 of UACA cDNA was inserted into a pGEX-4T-2 vector to produce glutathione-S-transferase (GST)-UACA fusion protein. This UACA fragment covers C-terminal 261 amino acids (18.0%) of whole UACA consisting of 1449 amino acids (Fig. 1A). Plasmids with this construct were transformed in E. coli (DH 5α) and incubated in 500 ml of Luria broth medium for 8 h at 37°C with shaking. IPTG was added at a final concentration of 0.1 mM and the preparation was incubated for 16 h at 25°C with shaking. This suspension was centrifuged and the pellet was suspended in 20 ml of lysis buffer (50 mM Tris–HCl [pH 7.5], 25% Sucrose). Then we added 100 μl of Nonidet P-40 (10%), MgCl2, (1 M)
The plates were washed in PBS-T and 100 μl of serum samples diluted at 1:50 with 1% skim milk/PBS were added to each well followed by incubation at room temperature for 2 h. The plates were then washed with PBS containing 0.05% Tween 20 and blocked with 5% skim milk/PBS for 2 h at room temperature. The plates were washed with PBS-T and 100 μl of serum samples diluted at 1:150 with 1% skim milk/PBS were added to each well and incubated at 4°C for 15 h at 4°C. GST protein was also coated in different wells, as a control. The plates were washed with PBS-T and blocked with 5% skim milk/PBS for 2 h at 4°C. This suspension was centrifuged and the pellet was resuspended in 2 ml of a slurry of glutathione–Sepharose 4B for 2 h at 4°C. This suspension was centrifuged and the pellet was washed in WE buffer (20 mM Tris-HCl [pH 7.5], 2 mM MgCl₂, 1 mM DTT). The fusion protein was eluted with G buffer (5 mM GSH, 50 mM Tris-HCl [pH 9.6]).

Enzyme-linked immunosorbent assay (ELISA). Detection and titration of antibody to a fragment of UACA were done, using indirect ELISA. GST-UACA fusion protein and GST protein were prepared and used as antigens. Microtiter plates (96-well) (NUNC, Denmark) were coated with GST-UACA fusion protein in PBS (pH 7.4) for 15 h at 4°C. GST protein was also coated in different wells, as a control. The plates were then washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked with 5% skim milk/PBS for 2 h at room temperature. The plates were washed with PBS-T and incubated for 15 h at 4°C with serum samples diluted at 1:150 with 1% skim milk/PBS. The plates were washed in PBS-T and 100 μl of HRP-conjugated mouse anti-human IgG diluted at 1:2000 with 1% skim milk/PBS were added to each well followed by incubation at room temperature for 2 h. The plates were washed with PBS-T, and 100 μl solution of o-phenylenediamine (Sigma Fast; Sigma Chemical Co., St. Louis, MO) was added to each well. After 30 min, the reaction was stopped by adding 50 μl of 3 M H₂SO₄, and OD 490 nm was determined, using a Model 550 microplate reader (Bio-Rad, Hercules, CA). The specific OD of an individual sample was calculated by subtracting the OD value of GST protein coated well from that of GST-UACA fusion protein.

RESULTS

Identification of an immunoreactive bovine cDNA clone, and cloning of its human homologous gene. cDNA expression library was prepared from bovine uvea and 1.0 × 10⁵ to 2.0 × 10⁵ phage plaques were immunoscreened with sera obtained from four VKH patients. Eleven positive immunoreactive clones were identified by serum from one patient HN (age 48 years, woman). These clones were purified and their partial DNA sequences revealed that they were derived from one kind of cDNA. One clone ~6 kb was sequenced and the analysis of this sequence showed an initial ATG preceded by an in-frame stop codon suggesting that the open reading frame (ORF) has been identified. The ORF encodes for a protein of 1405 amino acids with a predicted molecular weight of 161182 daltons (Fig. 1A). A homology search using BLAST software revealed that the sequence of this cDNA shared homology with several human EST clones obtained from various tissues, including melanocytes, retina and cochlea. To isolate a human cDNA homologous to the bovine gene, a cDNA fragment isolated from a human EST clone IMEGE:1527412 (GenBank Accession No. AA916992), one of the human homologous cDNA clones, was used as a probe to screen a Jurkat cell cDNA library. However the sequence of isolated human cDNA did not contain the complete 5’ end of the ORF. Next we did PCR cloning to isolate the complete human cDNA. We found that this sequence encodes for a protein of 1449 amino acids with a predicted molecular weight of 165,778 Da. Analysis of the amino acid sequence showed that this gene contains 6 repeats with the consensus sequence for the ankyrin motif (19), the leucine zipper pattern (20), and coiledcoil domains (Figs. 1A and 1B).

A homology search revealed that the sequence of this human cDNA shares the sequence of a human cDNA clone (mRNA for KIAA1561 protein, GenBank Accession No. AB046781) (21), and several human EST clones, but there was no homology shared with the characterized and published human proteins among reported genes in the database. In other species, the protein sequence encoded by a mRNA that is overexpressed in dog thyroid tissue following TSH stimulation (GenBank Accession No. X99145) was 85% identical to the protein sequence of this human gene (22). Thereby we concluded that the isolated bovine and human cDNAs encode novel proteins and we designated it as UACA (uveal autoantigen with coiled coil domains and ankyrin repeats) (GenBank Accession Nos. AF322915 and AF322916). These two proteins show a 86% amino acid homology. The amino acid sequence of human UACA has 47% similarity (27% identity) with the protein sequence of NOPREG (novel retinal pigment epithelial cell gene) (23) that also contains 6 ankyrin repeats and coiled coil domains, suggesting that there is a structural similarity between these two proteins. The amino acid sequence of UACA also showed similarity with several proteins containing coiled coil domains, including myosin heavy chain (24). The peptide fragment ENKLKKE of UACA was 7/8 identical to the peptide fragment EKSLKKE of segment VP6 of Banna virus (25).

Comparison of the prevalence of IgG anti-human UACA autoantibodies in sera from patients with panuveitis and healthy controls. To determine if autoimmunity directed against human UACA is specifically associated with VKH, serum samples obtained from patients with panuveitis (VKH, BD, sympathetic ophthalmia, sarcoidosis) and healthy controls were tested for their reactivity to a C-terminal 18.0% fragment of recombinant human UACA protein produced as GST fusion protein (Fig. 1A). We evaluated the titer of IgG anti-UACA autoantibody in sera, using ELISA. To exclude the effect of reactivity to GST protein, we used GST-UACA fusion protein and GST protein for ELISA, respectively. Evaluation of IgG anti-UACA autoantibodies was determined by subtracting the reactivities to GST from those to GST-UACA. The ratio of the OD values for GST-UACA fusion protein to GST protein in 22 positive samples was 9.57 ± 0.99 (mean ± SE), and in 118 negative samples was 3.52 ± 0.15 (mean ± SE). Figure 2 shows a scattergram of individual serum titers. The results were expressed as the relative value of OD unit where the OD value of VKH patient HN was defined as 100 OD unit. The cutoff level for positivity of anti-UACA IgG autoantibodies, 99.7 was defined as the mean value plus 3 SD of the autoantibody titers in healthy controls. The prevalence of autoantibodies in
patients with panuveitis and healthy controls is shown in Table 1. We found IgG anti-UACA reactivities in 19.6% (9/46) of VKH patients, and 0% (0/36) of the healthy controls. The prevalence of IgG anti-UACA auto-antibodies in VKH patients was significantly higher than that in healthy controls ($P < 0.005$). Anti-UACA reactivities were found in 0% (0/7) of patients with sympathetic ophthalmia, 28.1% (9/32) of patients with BD and 21.1% (4/19) of patients with sarcoidosis. The differences in prevalence of autoantibodies were also statistically significant between BD and healthy controls ($P < 0.001$), and between sarcoidosis and healthy controls ($P < 0.05$).

Expression of UACA gene in human tissues determined by Northern blot analysis and RT-PCR. To examine the expression of AUCA gene, we performed Northern blot analysis, using Human MTN Blot and Human MTN Blot II (CLONTECH). These membranes were probed with radiolabeled UACA specific cDNA probe which was also used to isolate UACA from a Jurkat cell cDNA library. As shown in Fig. 3, the highest level of expression was observed in skeletal muscle, and all tissues analyzed showed the expression in various levels. As it was impossible in Japan to obtain sufficient amounts of RNA from the human eye to examine Northern blot analysis, we did RT-PCR analysis. As shown in Fig. 4, a 505 bp band corresponding to nucleotide position 3529–4033 of UACA cDNA was observed in all materials, thereby indicating transcription of the UACA gene in human epidermal melanocytes, retina and choroidal tissue.

DISCUSSION

We used the SEREX method to search for autoantigens associated with VKH. It is well established that specific autoantibodies are present in sera from patients with autoimmune diseases, including type-1 diabetes mellitus and multiple sclerosis in which autoreactive T cells play major roles in the pathogenesis (26–33). Therefore, we expected that autoantibody to the target autoantigen may also be present in the VKH patients, although VKH disease is considered to be caused by T helper 1 type T cells recognizing uveal autoantigens. We expected that isotype switches of autoantibodies occur in the presence of antigen specific activated CD4$^+$ T cells, hence we used mouse anti-human IgG as the second antibody to detect IgG autoantibodies.

We identified a novel protein, UACA, as a candidate target autoantigen in VKH. UACA contains 6 ankyrin repeats and coiled coil domains, including a motif of leucine zipper pattern. Ankyrin repeats is a 31–33 amino acid motif present in a number of proteins and it is implicated with protein to protein interactions (19). For example, in GABP$\beta$, ankyrin repeats mediate stable interaction with GABP$\alpha$ and enhance the specificity of the DNA binding activity of the $\alpha$ subunit (34).
And in NF-κB, ankyrin repeats play important roles in protein-protein interactions that regulate localization and activity of this subunit (35). On the other hand, coiled coil domains are involved in self-aggregation or interaction with other proteins and have been noted in many autoantigens (36–39). The leucine zipper motif promotes dimerization through a helical coiled coil formation, and it is able to bind to DNA (20, 40, 41). These observations suggest that UACA have the potential for protein-protein interactions and may interact with other proteins.

The prevalence of IgG anti-UACA autoantibodies in VKH patients was significantly higher than that in healthy controls. The increased incidence of serological response to UACA in VKH patients indicates a possible relationship between the breakdown of immunological tolerance to the molecule and etiology of the disease. In the Japanese population, VKH, BD and sarcoidosis are the major diseases that cause panuveitis. BD, a systemic inflammatory disorder of unknown etiology, is characterized and diagnosed by four major symptoms, oral aphthous ulcers, ocular symptoms, including iritis and chorioretinitis, skin lesion including erythema nodosum, and genital ulcerations. Sarcoidosis is a systemic granulomatous disease of unknown etiology. VKH, BD and sarcoidosis cause different types of panuveitis. The clinical symptoms of eye lesion are mainly granulomatous iritis and serous retinal detachment in VKH, non-granulomatous iritis and chorioretinitis, including retinal vasculitis (arteritis), exudates and hemorrhage in BD, granulomatous iritis, vitreous opacity and periphlebitis in sarcoidosis. Several autoantibodies had been detected in sera from patients with BD and sarcoidosis (42–46) suggesting that the immunological disorder may be concerned with the development of these diseases. In the present study, the presence of anti-UACA IgG antibodies was observed in BD and sarcoidosis patients as well as in VKH patients. This finding suggests that (1) a similar immunological disorder is involved in VKH, BD and sarcoidosis, or (2) the production of anti-UACA IgG antibodies reflects a secondary phenomena related to tissue damage, or (3) the antibodies play different roles in each disease.

Because of difficulty in production of a large recombinant UACA as a whole molecule, we used the C-terminal 18.0% of UACA to detect anti-UACA autoantibodies in patients’ sera. A relatively lower prevalence of anti-UACA autoantibodies in patients may be explained by usage of a small fragment of UACA for ELISA. To evaluate autoimmunity directed against the whole UACA molecule, the production of other fragments of UACA will need to be done.

As the target autoantigen of VKH, such as UACA, is present in various human tissues, it seems unlikely that autoimmunity directed against this protein causes uvea and melanocyte-specific tissue damage. However, many examples are known in which autoantibodies reactive to ubiquitously expresses proteins are de-

### Table 1

<table>
<thead>
<tr>
<th>Disease</th>
<th>Anti-human UACA IgG-positive donors</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vogt-Koyanagi-Harada disease</td>
<td>9/46</td>
<td>0.005</td>
</tr>
<tr>
<td>Sympathetic ophthalmia</td>
<td>0/7</td>
<td>0.001</td>
</tr>
<tr>
<td>Behçet’s disease</td>
<td>9/32</td>
<td>0.05</td>
</tr>
<tr>
<td>Sarcoidosis</td>
<td>4/19</td>
<td>0.0%</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>0/36</td>
<td>0.0%</td>
</tr>
</tbody>
</table>

* Differences in prevalence of IgG anti-UACA autoantibodies are statistically significant between patients and healthy controls.

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**FIG. 3.** Northern blot analysis shows expression of the UACA mRNA in human tissues. A unique transcript of 2.3 kb was detected in all mRNA analyzed. The highest expression was detected in skeletal muscles.

**FIG. 4.** RT-PCR analysis using human UACA-specific primers shows the expression of UACA mRNA in human choroid, retina and epidermal melanocyte.
ected in organ-specific autoimmune diseases (47–52). Mechanisms underlying immune-responses to ubiquitous self proteins in tissue-specific autoimmune diseases remain unclear. A possible explanation is that cryptic epitopes are exposed in target tissues of autoimmunity and activate CD4\(^+\) T cell (53, 54). Cryptic epitopes can be exposed by tissue-specific protein modification, or in the local proteolytic environment, especially in cases of inflammation, then presented to CD4\(^+\) T cells. The activated CD4\(^+\) T cells may provide cognate or cytokine-mediated activation to B cells and cytotoxic T cells, and subsequently inducing antibody production and cytotoxic response. To elucidate the relationship between UACA and VKH and other panuveitis, it will be of interest to determine if experimental animals immunized with UACA develop autoimmune disorders such as VKH, BD or sarcoidosis or not.

In the present study we also showed that the peptide fragment 1029\(^{ENDKLKKLE}\)\(^{1036}\) of UACA was 7/8 identical to the peptide fragment 40\(^{ENAKLKKE}\)\(^{47}\) of segment VP6 of Banna virus (25). The peptide sequence ENA/DKLKK carries the motif for binding with HLA-DR4 (DRB1*0405) (55), the VKH susceptible allelic product DKLKKE carries the motif for binding with HLA-DR4 and UACA and Banna virus suggests that the infection of Banna virus may be a possible trigger of VKH, BD and sarcoidosis that cause different types of panuveitis. To elucidate the relationship between Banna virus and these diseases, it is necessary to do epidemiological studies of Banna virus and immune-responses to ubiquitinated or viral peptides that have sufficient sequence similarity with an immunodominant autoantigen (58) is suggested to be an important event in the induction of autoimmunity. The identity of amino acids sequence between UACA and Banna virus suggests that the infection of Banna virus may be a possible trigger of VKH, BD and sarcoidosis that cause panuveitis. To elucidate the relationship between Banna virus and these diseases, it is necessary to do epidemiological studies of Banna virus, including quantification of anti-Banna virus antibody in patients and healthy controls, and investigate T cell responses directed against these peptides.

In summary, we identified a novel protein UACA, which appears to be a possible target autoantigen in VKH, BD and sarcoidosis that cause different types of panuveitis.

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