Humoral immune response directed against LEDGF in patients with VKH

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Abstract

Vogt–Koyanagi–Harada disease is an autoimmune systemic disorder. In Vogt–Koyanagi–Harada disease, 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. Studies have been carried out to elucidate the exact etiology and target autoantigen in Vogt–Koyanagi–Harada disease, but much remains to be investigated. Identification of target autoantigen is important to understand the etiology of autoimmune diseases, and for development of antigen-specific immuno-modulation therapy. 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 an immunoreactive cDNA clone that encodes bovine lens epithelium derived growth factor. mRNA of human lens epithelium derived growth factor was determined by reverse transcription-polymerase chain reaction and it was expressed in human uvea, retina and melanocytes. Immunoglobulin G (IgG) autoantibodies were quantitated in an enzyme-linked immunosorbent assay, using recombinant human lens epithelium derived growth factor. The prevalence of IgG anti-lens epithelium derived growth factor autoantibodies in patients with Vogt–Koyanagi–Harada disease was significantly higher than that in healthy controls (66.7% versus 21.6%, P < 0.001). On the other hand, the prevalence of the autoantibody in patients with panuveitis of other etiology, Behçet’s disease and sarcoidosis, was almost same as that in healthy controls. These results suggest that the humoral immune response agonist lens epithelium derived growth factor is not a mere secondary phenomena caused by uveal tissue damage. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

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 destruction of melanocytes through immunological mechanisms. In the eyes of VKH patients, T cells infiltrate diffusely throughout the choroid, whereas B lymphocytes are scattered [1]. In the skin lesion, lymphocytic infiltration was observed and infiltrating mononuclear cells primarily consisted of T lymphocytes with a smaller number of B lymphocytes [2]. Melanin-laden macrophages were specifically found in cerebrospinal fluid of patients with early stage VKH [3]. Melanoma-specific and HLA-DR-restricted T helper 1 (Th1) lymphocyte cell lines and CD4+ T cell line recognizing the tyrosinase peptide carrying the HLA-DR4 (DRB1*0405)-binding peptide motif were established from the patients [4,5]. On the other hand, CD8+ T cell clones recognizing a MART-1 peptide in the context of HLA-A2 were also established from the patients [6]. These findings suggest that Th1-type autoreactive T cells may be involved in a development of VKH. 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 may be related to the development of VKH [7–10].

Identification of target autoantigen is important to elucidate 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 pathogenic autoantigens [11,12]. However the relationship between these autoantigens and human autoimmune uveitis is uncertain. Studies have been carried out to elucidate the target autoantigen in VKH. For example, the tyrosinase family proteins were reported as the target autoantigen in VKH because peptides derived from these proteins stimulated proliferation of the lymphocytes from patients with VKH, and immunization of rats with these peptides induced autoimmunity resembled to human VHK [13,14]. But much remains to be investigated to elucidate the exact etiology of VKH. To identify target autoantigens in autoimmune diseases, serological analysis of recombinant cDNA expression libraries (SEREX) had been done. SEREX is an immunoscreening method that makes use of procaryotically expressed cDNA libraries prepared from target organ of the autoimmune disease and sera from patients. This strategy proved effective to identify disease-related autoantigens, including type 1 diabetes, SLE, etc. [15,16]. We used this approach to identify the target autoantigen of VKH and identified autoantigen UACA (uveal autoantigen with coiled coil domains and ankyrin repeats) [17]. But this novel UACA protein appears to be a possible target autoantigen shared by VKH, Behçet's disease (BD) and sarcoidosis that cause different types of panuveitis. In the current study, we used same approach to identify the autoantigen specific to VKH.

2. Materials and methods

2.1. Patients

Thirty-six patients (age 19–80 years; 15 men and 21 women) with VKH were the subjects investigated in this study. All these patients underwent complete ophthalmologic and related examinations to determine the clinical diagnosis. Thirty-two patients (age 15–64 years; 20 men and 12 women) with BD complicated with panuveitis, seven patients (age 30–62 years; six men and one woman) with sympathetic ophthalmia (SO), 16 patients (age 16–67 years; two men and 14 women) with sarcoidosis complicated with panuveitis, and 37 (age 22–55 years; 21 men and 16 women) healthy unrelated donors served as controls. Informed consent was obtained from all subjects prior to blood sampling. This study was performed according to the tenets of the Declaration of Helsinki.

2.2. cDNA library

Total RNA was extracted from bovine uvea using TRIZOL reagent (GIBCO BRL, Rockville, MD, USA), and poly(A)+ RNA was purified with an oligo (dT) column (Oligotex-dT30 〈Super〉 TaKaRa, Kyoto, Japan). First-strand cDNA synthesis was performed using random primers. cDNA was ligated to EcoRI-NotI-BamHI adapters and digested with EcoRI. Double-strand cDNA fragments were cloned into the bacteriophage expression vector λZAPII (Stratagene, La Jolla, CA, USA), then packaged into packaging extract (Stratagene), resulting in 1.5 × 10^6 primary recombinants in this library.

2.3. Immunoscreening

The immunoscreening method used was a modification of published methods (Sahin et al., 1995; Scanlan et al., 1998). Sera were diluted in 1% bovine serum albumin/tris-buffered saline (TBS), and absorbed by Sepharose 4B columns coupled with lysates from *Escherichia coli* Y 1090 and bacteriophage-infected *E. coli* BNN97 (5 Prime 3 Prime, Boulder, CO, USA) to remove antibodies reactive to the vector system. Recombinant phages at a concentration of 1 × 10^4/15 cm plate were amplified for 6 h at 42 °C, covered with nitrocellulose filters, Hybond-c extra (Amersham, Buckinghamshire, UK), pre-treated with isopropyl β-D-thiogalactoside (Wako, Osaka, Japan), and incubated for an additional 3 h at 37 °C to transfer encoded proteins onto the filter membranes. Membranes were then blocked with 5% (w/v) skin milk/TBS. After washing with TBS containing 0.05% Tween 20 (TBS-T), membranes were incubated in prepared sera for 15 h at 4 °C. The membranes washed in TBS-T were
incubated in horseradish peroxidase (HRP)-conjugated mouse anti-human immunoglobulin G (IgG) (Southern Biotechnology Associates, Inc, Birmingham, AL, USA) for 2 h at 4 °C. The membranes were washed in TBS-T, TBS and incubated with ECL RPN 2106 (Amersham) for 1 min, and exposed to autoradiographic film to detect antibody-reactive phage plaques. Positive clones were subcloned and re-tested for serum reactivity, as already described. To determine the reactivity of other serum samples to positive clones, plates containing equal numbers of sero-positive clones and sero-negative control plaques were similarly processed. A total of $6.1 \times 10^5$ phage plaques were screened using sera from four different patients.

2.4. Sequence analysis of identified antigens

Phage clones were subjected to in vivo excision of pBluescript phagemids using the ExAssist helper phage/SOLR strain system (Stratagene). Plasmid DNA was purified using ABI Prism Miniprep Kits (PE Applied Biosystems, Foster, CA, USA). cDNA inserts were sequenced using an ABI Prism (Perkin Elmer, Norwalk, CT, USA) automated DNA sequencer and sequence alignments were performed using BLAST software (GenomeNet, Japan).

2.5. Enzyme-linked immunosorbent assay

Detection and titration of antibody to human lens epithelium derived growth factor (LEDGF) were carried out using indirect enzyme-linked immunosorbent assay (ELISA). Glutathione-S transferase (GST)--human LEDGF fusion protein and GST protein were prepared and used as antigens, as described [18]. Microtiter plates (96-well) (NUNC, Denmark) were coated with GST--LEDGF fusion protein in phosphate-buffered saline (PBS) (pH7.4) for 15 h at 4 °C. For control, GST protein was also coated in different wells. 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 in PBS-T and incubated for 15 h at 4 °C with serum samples diluted 1:100 with 1% skim milk/PBS. The plates were washed in PBS-T, and 100 μL HRP-conjugated mouse anti-human IgG diluted 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, USA) was added to each well. After 30 min, the reaction was stopped by adding 50 μL of 3 M H$_2$SO$_4$ and optical density (OD) at 490 nm was determined using a Model 550 Microplate Reader (BIO-RAD). The specific corrected OD of the individual sample was calculated by subtracting the OD value of the GST protein coated well from that of the GST--human LEDGF fusion protein. Fisher’s exact probability test was used for statistical analysis.

2.6. Reverse transcription-polymerase chain reaction

Total RNA was isolated from human epidermal melanocytes (Cell Systems Corporation, Kirkland), normal human choroid, and retina using TRIZOL reagent. Subsequently, poly(A)$^+$ RNA was purified with the Dynabeads mRNA Purification Kit (DYNAL, Oslo, Sweden). These tissues were obtained from the eye enucleated from the patient with mycosis of super-maxilla. Informed consent was obtained after the nature of the research was explained. The tenets of the Declaration of Helsinki were followed. Poly(A)$^+$ RNA was purified from 10 μg of each total RNA, and subjected to cDNA synthesis, using random hexamer primers and Superscript reverse transcriptase (GIBCO/BRL). Gene-specific polymerase chain reaction (PCR) primers were designed to amplify fragments of 414 bp and used in the reverse transcription (RT)-PCR reaction (95 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min, 30 cycles). PCR primers sequences for LEDGF were: sense, 5’-AACCTGGCTCACCAGGTGTC-3’; and anti-sense, 5’-TCTCTTCACTGGATGGCCTTT-3’. This anti-sense primer sequence was specific for LEDGF/p75 but not p52 [19]. The genomic region between these primers contained four introns (3628, 1037, 4237 and 2595 bp, respectively), according to the genomic sequence of LEDGF [20]. For control, PCR using cDNA-specific beta-actin primers was performed [21].

3. Results

3.1. Identification of immunoreactive cDNA clones

A cDNA expression library (1.5 × 10$^6$ pfu in primary clones) was prepared from bovine uvea, and 1.0 × 10$^5$ to ~ 2.0 × 10$^5$ phage plaques were immunoscreened with each of serum obtained from four different VKH patients. A total of $6.1 \times 10^5$ phage plaques were screened. Twelve positive immunoreactive clones were identified in serum from one patient, JS (age 45 years, female), whereas sera from three other VKH patients did not react at detectable levels. These clones were purified, and their partial DNA sequences revealed that they were derived from 11 different cDNAs. Most of them reacted only to serum from patient JS; however, two clones derived from one cDNA reacted to sera from multiple VKH patients. A homology search using the BLAST software revealed that the partial DNA sequence of this cDNA was the most similar to human lens epithelium derived growth factor [18,20]/human
transcriptional coactivators p52 and p75 [19] among the reported genes, the similarity being 95%. In the area we analyzed, the amino acid sequence of the bovine cDNA clone was completely identical to the human LEDGF (Fig. 1). Therefore, we concluded that the isolated cDNA was bovine LEDGF.

3.2. Comparison of the frequency of IgG anti-human LEDGF autoantibodies in sera from patients with panuveitis and healthy controls

To determine whether humoral immune response directed against human LEDGF is specifically associated with VKH, serum samples obtained from patients with panuveitis (VKH, BD, SO, sarcoidosis) and healthy controls were tested for their reactivity to recombinant human LEDGF protein produced as GST fusion protein. We evaluated the titer of IgG anti-LEDGF autoantibody in sera by ELISA. We measured IgG anti-GST–LEDGF fusion protein and anti-GST protein, respectively. Evaluation of IgG anti-LEDGF autoantibodies was determined by subtracting the reactivities to GST from those to GST–LEDGF. Fig. 2 shows subtracted anti-LEDGF IgG titer in serum samples from patients and healthy controls. The results were indicated as the relative value of OD units where the OD value of VKH patient JS was defined as 100 OD units. The anti-LEDGF IgG autoantibody titers in healthy controls showed a bimodal distribution, and the group with the lower titer autoantibody fitted the normal distribution. The cut-off level for positivity of anti-LEDGF IgG autoantibodies was defined at the mean value plus 3S.D. of the antibody titers in this group, 1.77. The prevalence of autoantibodies in patients with panuveitis and healthy controls is shown in Table 1. We found IgG anti-LEDGF reactivities in 66.7% (24/36) of VKH patients, and 21.6% (8/37) of the healthy controls. The prevalence of IgG anti-LEDGF auto-antibodies in VKH patients’ sera was significantly higher than that in healthy controls (P < 0.001, Fisher’s exact probability test). Anti-LEDGF reactivities were found in 71.4% (5/7) of patients with SO, 34.4% (11/32)
of patients with BD, and 25.0% (4/16) of patients with sarcoidosis. The differences in prevalence of autoantibodies were not statistically significant between SO, BD and healthy controls. But in SO, the distributions of autoantibody titers were similar to those of VKH, whereas the prevalence of autoantibodies in sarcoidosis was as low as in healthy controls (Fig. 2). The age distribution is not significantly different between anti-human LEDGF IgG-positive and -negative donors in each group of patients as well as healthy controls, indicating that ageing itself does not affect the titer of anti-LEDGF antibody (Table 2). Therefore, although the age distribution of healthy controls is younger than that of VKH patients, the difference in prevalence of anti-LEDGF antibody between the two groups is not accounted for by the ageing effect.

### 3.3. RT-PCR determinations of expression of LEDGF in human melanocytes and human uvea

In the previous study, the expression of LEDGF protein was detected in all of the human tissues examined, such as kidney, thymus, and heart [18]. However, the expression in melanocytes or uvea was not examined in the study. To examine the expression of LEDGF gene in human choroid, retina and human melanocytes, we performed RT-PCR analysis. As shown in Fig. 3, a 414 bp band corresponding to nucleotide position 836–1249 of LEDGF cDNA was observed in all materials, thereby indicating the transcription of LEDGF gene in human epidermal melanocytes, human retina and human choroidal tissue. The PCR products of this size are definitely originated from cDNA, because the sequence corresponding to the primer pair are separated by four introns expanding in 11.5 kb region of the genome.

### 4. Discussion

We used the SEREX method to search for autoantigens associated with VKH. As it was impossible to obtain sufficient amounts of RNA from human uvea in Japan and because we expected there to be immunological similarities in protein population between bovine and human uvea, we used mRNA from bovine uvea to generate a cDNA expression library.

It is well established that specific autoantibodies are present in sera of patients with autoimmune diseases in which autoreactive T cells play major roles in the pathogenesis, including type I diabetes mellitus and multiple sclerosis [22–29]. Therefore, we expected that autoantibody to the target autoantigen may also be present in the VKH patients, although VKH disease is suggested to be caused by inflammatory T cells reactive to uveal autoantigens. We expected that isotype switches of autoantibodies occur in the presence of antigen-specific activated CD4+ T cells, and used mouse anti-human IgG as the second antibody to detect IgG autoantibodies. As a result, we identified LEDGF as an autoantigen recognized by multiple patients’ sera. We set up the ELISA to examine serum samples of patients with panuveitis and healthy con-
controls regarding reactivity to recombinant human LEDGF protein. The prevalence of IgG anti-human LEDGF autoantibodies in VKH patients was significantly higher than in healthy control samples. There was no significant association between the titer level of autoantibodies and age, symptoms, and the clinical course of VKH (data not shown).

LEDGF is reported to confer resistance to thermal and oxidative stress in several types of cells, including lens epithelial cells [30,31]. Anti-LEDGF antibody is toxic to lens epithelial cells (LECs), keratinocytes and fibroblasts [18,32], suggesting that LEDGF is an essential factor for survival of various cells. The expression of LEDGF protein was observed in various tissues including liver, lung, spleen, etc., by immunoblot analysis [18]. Transcription coactivators p52 and p75 were isolated from a HeLa cell cDNA library and were expressed in several tissues [19]. In the present study, we found for the first time that the LEDGF gene is expressed in human melanocytes and human uvea.

As LEDGF is present in various human tissues, it might seem 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 expressed proteins are detected in organ-specific autoimmune diseases. Antibodies specific to SS-A/Ro and SS-B/La, which are ubiquitously expressed nuclear proteins, are prevalent in patients with Sjögren’s syndrome, an organ-specific auto-immune disease that destroys salivary glands [33]. Several types of anti-mitochondria antibodies present in subjects with organ-specific autoimmune diseases including autoimmune myocarditis, dilated cardiomyopathy, rheumatoid arthritis, type 1 diabetes and primary biliary cirrhosis [34–39]. The autoimmunity directed against these ubiquitously expressed proteins is considered to have an important role in the development of diseases.

Mechanisms underlying immune responses to ubiquitous self proteins in tissue-specific autoimmune diseases remain unclear. A possible explanation is that cryptic epitopes, to which the immune system does not acquire tolerance, are produced specifically in the target tissues of autoimmunity and activate CD4+ T cells [40,41]. Cryptic epitopes can be produced by tissue-specific protein modification, or in the local proteolytic environment, especially in cases of inflammation, then presented to CD4+ T cells. These activated CD4+ T cells may activate B cells and cytotoxic T cells by cognate or cytokine-mediated interactions, and subsequently antibody production and cytotoxic response are induced. As for LEDGF, because this protein is involved in stress reaction and the production of the protein is likely to be increased in inflammatory lesions, another possible explanation is that the overexpression of LEDGF breaks tolerance in immune systems. If any minor inflammation occurs in tissue containing melanocyte, the expression of LEDGF in melanocytes will be increased and this may break the immunological tolerance to this protein. Subsequently, autoimmunity to LEDGF becomes overt to develop VKH.

SO and VKH share very similar clinical and histopathological features. The strong associations between the HLA-DR4 (DRB1*0405)-DQ4 (DQA1*0302-DQB1*0401) haplotype and the susceptibility to both VKH and SO suggest the prevalence of some common immunogenetic background in these diseases [42]. The increased incidence of serological response to LEDGF in both VKH and SO patients observed in the present study indicates a possible relationship between the breakdown of immunological tolerance to LEDGF and the etiology of the diseases. These findings support the close relationship between these two diseases. However, it remains uncertain whether this autoimmunity is the primary cause of disease or a secondary phenomena related to tissue damage. In sarcoidosis patients, the prevalence of antibodies was as low as in healthy controls. This finding indicates that high prevalence of the anti-LEDGF autoantibodies is not always associated with panuveitis, suggesting that the autoimmunity against LEDGF is not a mere secondary phenomenon caused by tissue damage.

Because anti-LEDGF IgG antibodies are present also in some healthy individuals, the production of autoantibody alone probably does not directly cause VKH. Some events such as local inflammation resulting in infiltration of pathogenic T cells to the uveal tissue may be requisite for the disease development. To elucidate these issues, it will be good to see whether experimental animals immunized with LEDGF develop autoimmune VKH like uveitis or not. In terms of the HLA class II-associated susceptibility to VKH, it is important to investigate whether LEDGF-autoactive CD4+ T cells restricted by disease-susceptible HLA class II molecules exist in VKH patients or not.

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