Thrombosis Research (2004) 114, 347–355

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β_2-glycoprotein I: antiphospholipid syndrome and T-cell reactivity☆

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Received 17 May 2004; received in revised form 21 June 2004; accepted 23 June 2004
Available online 6 August 2004

KEYWORDS
Antiphospholipid syndrome;
Autoimmune disease;
Autoreactive T cell;
β_2-glycoprotein I;
Cryptic epitope

Abstract There is increasing evidence showing that recurrent thrombosis and intrauterine fetal loss in antiphospholipid syndrome (APS) are attributable to antiphospholipid (aPL) antibodies. We have recently identified autoreactive $CD4^+$ T cells to β_2-glycoprotein I (β_2GPI) that promote production of pathogenic antiphospholipid antibodies. β_2GPI-specific $CD4^+$ T cells preferentially recognize the antigenic peptide containing the major phospholipid (PL)-binding site in the context of DR53. T-cell helper activity that stimulates B cells to produce IgG anti-β_2GPI antibodies is mediated through IL-6 and CD40–CD154 interaction. β_2GPI-specific T cells respond to reduced β_2GPI and recombinant β_2GPI fragments produced in a bacterial expression system but not to native β_2GPI, indicating that the epitopes recognized by β_2GPI-specific T cells are 'cryptic' determinants, which are generated at a subthreshold level by the processing of native β_2GPI under normal circumstances. Although β_2GPI-specific T cells are detected in both APS patients and healthy individuals, these autoreactive T cells are activated in vivo in APS patients but not in healthy individuals. These findings indicate activation of β_2GPI-specific T cells and subsequent production of pathogenic anti-β_2GPI antibodies can be induced by the exposure of such T cells to cryptic peptides of β_2GPI efficiently presented by functional antigen-presenting cells (APC). Delineating the mechanisms that induce the efficient processing and presentation of cryptic determinants of β_2GPI as a consequence of antigen processing would clarify the etiology that initiates the autoantibody response in APS.
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☆ Contribution to the Conference XIth International Congress on Antiphospholipid Antibodies, 14–18th November 2004, Sydney, Australia.

Abbreviations: APC, antigen-presenting cells; aPL, antiphospholipid; APS, antiphospholipid syndrome; β_2GPI, β_2-glycoprotein I; PL, phospholipid; TCR, T-cell receptor.

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doi:10.1016/j.thromres.2004.06.029

Introduction

Antiphospholipid syndrome (APS) is an autoimmune disorder characterized by arterial and venous thrombosis as well as recurrent pregnancy loss in association with circulating antiphospholipid (aPL) antibodies [1]. aPL antibodies often target plasma proteins with the capacity to bind anionic phospholipids (PLs) rather than PL itself. β_2-glycoprotein I (β_2GPI) and prothrombin are identified to be the major aPL antigens [2]. β_2GPI-dependent aPL antibodies are strongly associated with thrombosis and other clinical features of APS [3] and thus are included as one of the criteria for the classification of APS [4]. β_2GPI is a plasma glycoprotein that binds various kinds of negatively charged substances, including PLs, lipoproteins, activated platelets and activated endothelial cells [5–7]. β_2GPI possesses five complement control protein repeats (domains I–V), and the major PL-binding site has been identified as a positively charged area located at amino acid positions 281–288 in domain V [8,9]. The physiological function of β_2GPI has not been established to date, but it has been suggested that the protein may play a scavenging role for exposed anionic materials, including PL-expressing foreign particles [10] and oxidized lipoprotein [11]. Pathogenicity of aPL antibodies in APS patients has been demonstrated in the experimental model, into which normal mice are infused with an IgG fraction of APS patients' sera [12,13]. A precise mechanism for thrombophilia caused by aPL antibodies still remains unclear, but many hypotheses have been proposed to date [14]. For example, anti-β_2GPI antibodies bind to endothelial cell surfaces by recognizing the adhered β_2GPI and induce endothelial cell activation, resulting in the up-regulation of procoagulant and inflammatory processes [15]. On the other hand, anti-β_2GPI antibodies bind to oxidized low-density lipoprotein through adhered β_2GPI and promote its uptake by macrophages, resulting in the promotion of atherosclerosis [16].

Although antigenic specificity and functional characteristics of aPL antibodies have been extensively analyzed, little information is available about the cellular mechanisms that induce the production of pathogenic aPL antibodies. However, anti-β_2GPI antibodies in APS patients are mainly of the IgG and IgA isotypes [17], suggesting that its production requires antigen-specific T-cell help. In this regard, adoptive transfer of peripheral blood lymphocytes derived from an APS patient to severe combined immune deficiency mice results in a continuous increase in circulating aPL antibodies and the APS manifestation [18]. Animals immunized with foreign β_2GPI develop manifestations of the APS along with anti-β_2GPI antibody production [19,20]. The APS phenotype can be transferable to naïve mice by infusing bone marrow cells from the β_2GPI-immunized mice [21]. In this model, T cell-depleted bone marrow cells do not induce anti-β_2GPI antibody production in naïve recipients. These findings all together indicate the active involvement of the T cells responsive to β_2GPI in the production of pathogenic anti-β_2GPI antibodies. In this report, we summarize updates on autoreactive T cells to β_2GPI and their pathogenic roles in APS.

Identification of autoreactive CD4$^+$ T cells to β_2GPI

Because β_2GPI is a plasma protein abundantly present in the circulation (~200 µg/ml), antigen-presenting cells (APCs) constantly take up β_2GPI, process it and present the β_2GPI-derived peptides to T cells. Therefore, we hypothesized that T-cell tolerance to β_2GPI is tightly maintained under normal circumstances through negative selection in the thymus as well as through a post-thymic mechanism of peripheral tolerance. To test this hypothesis, we compared the proliferative response of peripheral blood T cells from APS patients in cultures supplemented with 10% fetal bovine serum containing 20 µg/ml bovine β_2GPI and in cultures with 10% fetal bovine serum depleted of bovine β_2GPI and found no difference in the T-cell proliferation between these circumstances [22]. In addition, purified human β_2GPI at concentrations of 1–200 µg/ml did not induce proliferation in T cells cultured in medium containing β_2GPI-free fetal bovine serum. These findings support the theory that T-cell tolerance to the native form of β_2GPI exists even in patients with APS. On the other hand, two groups reported the presence of T cells reactive with native β_2GPI in peripheral blood from patients with SLE or APS. In a report by Davies et al. [23], a borderline T-cell response to native β_2GPI was detected at an antigen concentration of 30 µg/ml (20 µg/ml β_2GPI contained in the culture medium with heat-denatured normal human serum plus 10 µg/ml of purified human β_2GPI), but this result was based on the comparison with a culture with heat-denatured normal human serum containing β_2GPI at a concentration of 20 µg/ml. Visvanathan and McNeil [24] and Visvanathan et al. [25] proposed that T cells reactive with native β_2GPI in APS patients stimulated circulating monocytes to produce tissue factors when stimulated with native

β_2GPI in vitro, whereas little or no tissue factor was induced when T cells from patients without the APS or healthy controls were tested. Later, this investigator group retracted their report because they were unable to reproduce their own findings [26]. These studies lack important data regarding specific recognition of β_2GPI-derived peptides in the context of HLA molecules by the responding T cells.

Previous studies in our laboratory indicate that structural modification of autoantigens could induce expression of antigenic self-determinants recognized by pathogenic autoreactive T cells in other autoimmune diseases [27,28]. According to this idea, we prepared chemically reduced β_2GPI, trypsinized β_2GPI fragments and a series of recombinant β_2GPI fragments produced in a bacterial expression system (rβ_2GPI) and used them to stimulate T cells in bulk peripheral blood mononuclear cell cultures [22]. As a result, we reproducibly detected T-cell responses to reduced β_2GPI and rβ_2GPI, but not to trypsinized β_2GPI fragments, in the majority of APS patients with anti-β_2GPI antibodies. T cells reactive with modified β_2GPI had a $CD4^+$ helper phenotype and were restricted mainly by HLA-DR. In addition, these β_2GPI preparations induced IgG anti-β_2GPI antibody production in peripheral blood mononuclear cell cultures in vitro. Anti-β_2GPI antibodies synthesized in this in vitro culture system had similar features of pathogenic anti-β_2GPI antibodies in APS patients because they recognized β_2GPI immobilized on cardiolipin-coated plastic plates and prolonged diluted activated partial thromboplastin time.

Antigen recognition profiles of β_2GPI-reactive T cells

To further characterize T cells responsive to modified β_2GPI in APS patients, $CD4^+$ T-cell clones specific to rβ_2GPI were generated from APS patients with a high level of serum anti-β_2GPI antibodies [29]. Ten out of 12 β_2GPI-reactive T-cell clones were able to induce production of anti-β_2GPI antibodies from autologous B cells in response to rβ2GPI. As expected, β_2GPI-specific T-cell clones responded to reduced β_2GPI but not to native β_2GPI. These T-cell clones responsive to modified β_2GPI were really β_2GPI-reactive because they specifically recognized synthetic peptides encoding the amino acid sequences of human β_2GPI. Ten of the 14 T-cell clones (71%) recognized a synthetic peptide encompassing amino acid residues 276–290 of β_2GPI (p276–290) containing the major PL-binding site. When a panel of L-cell transfectants expressing a single HLA class II molecule was used as APCs, recognition of p276–290 was restricted by the DRB4*0103 (DR53). This restriction profile may explain the previously reported associations of anti-β_2GPI antibodies with the DR4, DR7 and DR9 haplotypes [30], all of which are in linkage disequilibrium with DR53. On the other hand, a small number of β_2GPI-specific T-cell clones reactive with epitopes in domain I/II, domain IV and p247–261 in domain V was also generated from APS patients, indicating heterogeneous antigenic specificities in β_2GPI-specific T cells. Recently, Ito et al. [31] analyzed the T-cell epitopes on β_2GPI using β_2GPI-specific T-cell lines established from APS patients and healthy individuals by stimulating with a mixture of 40 synthetic peptides covering the entire amino acid sequence of β_2GPI. They identified four distinct epitopes, including p64–83, p154–174, p226–246 and p244–264, but T-cell lines recognizing the peptide containing the major PL-binding site were not generated. It is possible that such peptide was not efficiently presented to T cells in the context of the HLA-DR molecule because synthetic peptides containing the major PL-binding site may be removed by its capacity to bind anionic PLs expressed in abundance on apoptotic cell surfaces in an in vitro culture. Until now, we have generated nearly 100 T-cell lines responsive specifically to rβ_2GPI from APS patients and healthy donors carrying DR53 and found that >90% of them recognize p276–290. As shown in Fig. 1, p276–290 is unique because it contains the entire major PL-binding site (amino acids 281–288) as well as a previously reported DR53-binding motif [32]. Interestingly, Gharavi et al. [33] reported that the immunization of normal mice with a human β_2GPI peptide encompassing amino acid residues 274–288 (termed GDKV peptide), which also contains

Human β_2GPI sequence	276 290
p276-290	KVSFFCKNKEKKCSY
DR53-binding motif	K--I----KI
Major PL-binding site	CKNKEKKC
GDKV peptide (p274-288)	GDKVSFFCKNKEKKC
Mouse β_2GPI sequence	
Mouse p274-288	GDKIHFYCKNKEKKC
H-$2E^d$-binding motif	F--K-L--K

Figure 1 Amino acid sequence of p276–290, a dominant T-cell epitope on β_2GPI. p276–290 contains the previously reported DR53-binding motif and the major PL-binding site. The GDKV peptide was used to immunize normal mice by Gharavi et al. [33]. Mouse version of the GDKV peptide and H2–E^d-binding motif are also shown. A box denotes the major PL-binding site.

the major PL-binding site, induced the production of anti-β_2GPI antibodies possessing thrombotic properties. Production of the IgG isotype of anti-β_2GPI antibodies in their in vivo system indicates that this response is a result of T-cell-dependent isotype switch. Therefore, it is likely that the GDKV peptide binds H2–E^d molecule and prime xeno-reactive T cells to the human sequence. This initial response may subsequently diversify to the mouse sequence by cross-reactivity. We found that many of our T-cell clones responsive to p276–290 generated from APS patients also recognized the GDKV peptide. It is of note that a dominant T-cell epitope on β_2GPI is shared between APS patients and an experimental model for APS.

T cells use clonally unique T-cell receptors (TCRs) to recognize peptide epitopes. β_2GPI-specific $CD4^+$ T-cell clones responsive to p276–290 commonly had the TCR β-chains with a rearranged Vβ7 segment and a TGxxN/Q motif in their CDR3 sequence [34]. This is consistent with another finding obtained from polymerase-chain reaction and single-strand conformation polymorphism analysis combined with in vitro stimulation of peripheral blood T cells with rβ2GPI, in which the Vβ7 segment with the TGxxN/Q motif or minor variations was oligoclonally expanded after stimulation of peripheral blood T cells with rβ_2GPI in the majority of APS patients. Surprisingly, β_2GPI-reactive T cells in three independent APS patients used an identical $V\beta7^+$ TCRβ chain that was shown to recognize p276–290. In addition, the $V\beta8^+$ TCR with a distinct motif PxAxxD/E, which is presumed to recognize the epitope other than p276–290 of β_2GPI, was detected in some APS patients. Depletion of $V\beta7^+$ or $V\beta8^+$ T cells from the peripheral blood mononuclear cell cultures significantly inhibited in vitro anti-β_2GPI antibody production in response to rβ_2GPI, confirming that T cells carrying these TCR β-chains are the predominant repertoire that induces anti-β_2GPI antibody production.

Effector function of β_2GPI-reactive T cells

$CD4^+$ T cells are functionally classified according to their cytokine expression profiles. In this regard, all 12 β_2GPI-reactive T-cell clones produced IFN-γ and had a Th1- or Th0-like cytokine expression profile [29]. It is now known that the Th1/Th2 antagonism in humans is not as strict as reported in mice and that T-cell function is associated with individual cytokine profiles rather than Th1/Th2 phenotypes. When potential associations between cytokine profiles and the capacity to promote anti-β_2GPI antibody production were examined in individual β_2GPI-reactive T-cell clones, an expression level of IL-6 was positively correlated with the helper activity, but expression of IFN-γ, IL-2, IL-4 or IL-10 was not. The mechanisms for the T and B cell collaboration regulating the production of anti-β_2GPI antibodies were further examined using an in vitro assay system consisting of β_2GPI-reactive $CD4^+$ T-cell clones and autologous peripheral blood B cells [29]. In this assay system, anti-IL-6 monoclonal antibody blocked T cell-dependent anti-β_2GPI antibody production, but anti-IFN-γ monoclonal antibody did not, indicating IL-6 as a major B cell-activating factor produced by β_2GPI-specific T

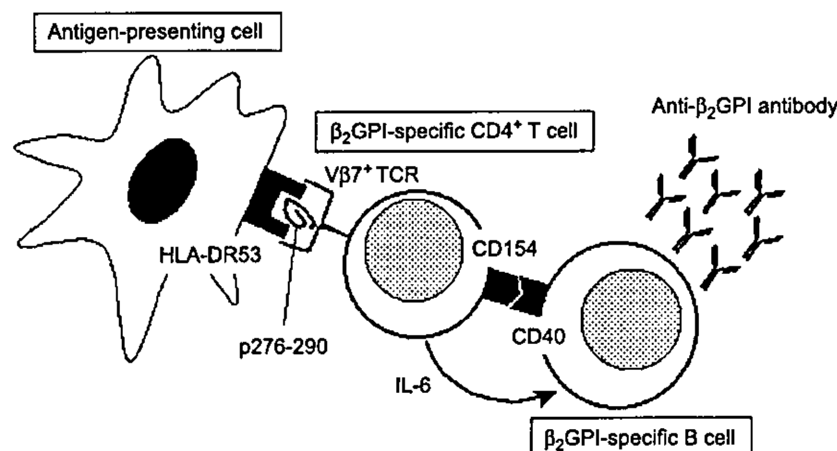

Figure 2 Schematic presentation of the cellular mechanism that induces anti-β_2GPI antibody production in APS patients. β_2GPI-specific $CD4^+$ T cell is activated by the recognition of p276–290 complexed with an HLA-DR53 molecule expressed on APC via $V\beta7^+$ TCR. Activated β_2GPI-specific T cell stimulates specific B cell to produce anti-β_2GPI antibodies through the expression of CD154 and IL-6.

cells. However, the effect of IL-6 required the CD40–CD154 engagement because anti-CD154 monoclonal antibodies almost completely inhibited anti-β_2GPI antibody production induced by β_2GPI-reactive $CD4^+$ T-cell clones. Taken together, $CD4^+$ T cell-dependent B-cell activation depends on two types of stimuli; T cell-derived IL-6 and CD40–CD154 engagement (Fig. 2).

Mechanisms for induction of autoantibody response to β_2GPI

Circulating β_2GPI-reactive T cells are detectable not only in APS patients with serum anti-β_2GPI antibodies but also in some healthy individuals [22,31]. In addition, analysis of TCR β-chains for β_2GPI-reactive T cells indicated that APS patients and healthy responders used similar TCR β-chains [34]. These findings indicate that β_2GPI-reactive T cells are a component of the normal T cell repertoire. To evaluate the activation status of β_2GPI-reactive T cells in APS patients and healthy responders, a precursor frequency of β_2GPI-reactive T cells was determined using limiting-dilution analysis [35]. As shown in Fig. 3, frequency of β_2GPI-reactive T cells in circulating T cells was significantly increased in APS patients compared with healthy individuals, indicating that the production of aPL antibodies in APS patients is a result of the activation of β_2GPI-reactive T cells.

Taken together with the finding that β_2GPI-specific $CD4^+$ T cells responded to reduced β_2GPI and rβ_2GPI but not to native β_2GPI, it is likely that p276–290 and other epitopes recognized by β_2GPI-specific T cells are 'cryptic' determinants. The concept of the 'cryptic' self-determinant was proposed by Lehmann et al. [36] and Sercarz et al. [37] and extended by other investigators

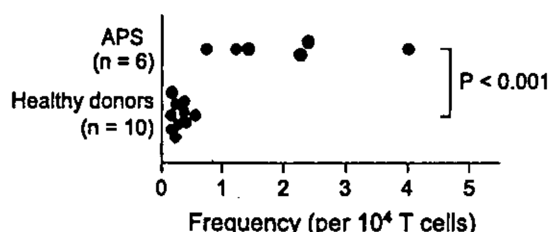

Figure 3 Precursor frequencies of β_2GPI-reactive T cells in peripheral blood from APS patients and healthy donors. The frequency of β_2GPI-reactive T cells was determined by limiting-dilution analysis, and the results are shown as the number of β_2GPI-reactive T cells per 10^4 peripheral blood T cells. All APS patients and healthy donors are responders to rβ_2GPI in bulk peripheral blood T-cell cultures. Comparison was made using Student's *t*-test.

[38,39]. As shown in Fig. 4, extracellular antigens are taken up by APCs and degraded into peptide fragments by endosomal proteases. This process is known as antigen processing. Only the peptides with the capacity to bind HLA class II molecules are expressed on the cell surface in the form of the peptide–HLA class II complex and presented to T cells (a process termed antigen presentation). Of numerous peptides predicted by the amino acid sequences of the autoantigen, a small proportion of peptides is presented to T cells as a consequence of antigen processing and antigen presentation. Those peptides are a representative of the autoantigen and termed dominant self-determinants, whereas the remaining peptides, which are not efficiently presented and are normally hidden from the immune system, are termed cryptic self-determinants. There is increasing interest in the possibility that crypticity is an important characteristic of epitopes recognized by the autoreactive T cells and thus is relevant to autoimmune pathogenesis [37–39]. T cells recognizing dominant self-determinants in APCs undergo deletion in the thymus or inactivation in the periphery. On the other hand, T cells specific for cryptic self-determinants are a component of the normal T-cell repertoire but normally do not encounter antigenic peptides in the periphery. These T cells might become activated and autoaggressive if the previously cryptic self-determinants were presented at a higher concentration. This concept represents the major hypothesis for the pathogenesis of autoimmune diseases. Our findings regarding the T-cell response to β_2GPI in APS patients are consistent with this cryptic epitope model because β_2GPI-reactive T cells recognize the epitope peptides that are not generated from native β_2GPI but are generated from structurally modified β_2GPI, exist in the normal T-cell repertoire and are activated in vivo in APS patients but not in healthy individuals.

The fundamental question is how normally cryptic p276–290 becomes visible to the immune system and elicits a sustained pathogenic response in APS patients. T cells responsive to cryptic self-determinants would not encounter antigenic peptides in the periphery under normal circumstances; it is likely that a pathogenic autoreactive T-cell response is induced by the de novo presentation of a previously cryptic p276–290 under special conditions (Fig. 5). Potential mechanisms that reveal cryptic self-determinants in APCs include modulation of antigen processing and/or increased antigen delivery to the processing compartment [38]. In this regard, reduction of β_2GPI and expression of recombinant β_2GPI in a bacterial expression system induce expression of cryptic peptides that are able

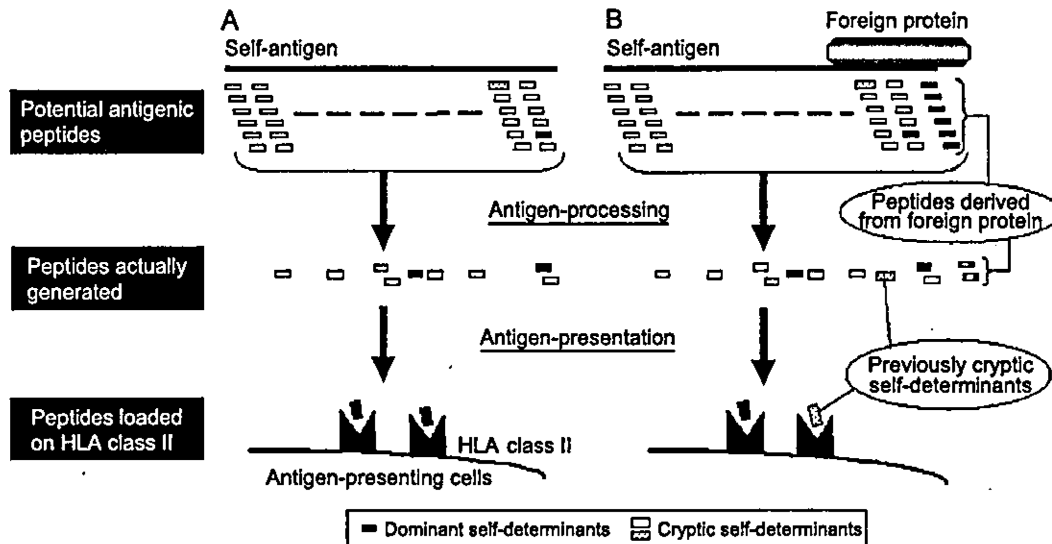

Figure 4 Expression of dominant and cryptic self-determinants as a consequence of the antigen processing and antigen presentation. (A) A self-antigen is taken up by APCs and degraded by endosomal proteases into peptide fragments. Although there are numerous potential antigenic peptides, a small proportion of peptides is actually generated by antigen processing, and only a few peptides with the capacity to bind HLA class II molecules are expressed on the cell surface in the form of the peptide–HLA class II complex and presented to T cells. The peptides expressed on APC membranes are termed dominant self-determinants, whereas the remaining peptides that are normally hidden from the immune system are termed cryptic self-determinants. (B) Modulation of antigen processing by the complex formation of a self-antigen with a foreign protein, one of the potential mechanisms that reveal previously cryptic self-determinants. Antigen processing of a self-antigen complexed with a foreign protein results in the generation of previously cryptic self-determinants as well as peptides derived from the foreign protein. If newly generated cryptic self-peptides were capable of binding HLA class II molecules, those peptides would be expressed on the cell surface in the form of the peptide–HLA class II complex and elicit specific T-cell response.

to stimulate β_2GPI-specific T cells. Although factors that induce the expression of p276–290 as a consequence of antigen processing in APS patients are unknown, several lines of evidence suggest that p276–290 is revealed when β_2GPI is complexed with anionic surfaces. PL-bound β_2GPI, but not PL or β_2GPI alone, induces a high level of anti-β_2GPI antibodies and lupus anticoagulant activity in normal mice without adjuvant [40]. Moreover, immunization of β_2GPI-bound apoptotic cells into normal mice induces the production of anti-β_2GPI antibodies [41]. The major PL-binding site located on the surface of a β_2GPI molecule should be easily accessed by proteases in endocytic compartments during antigen processing, and therefore, the peptides containing the intact major PL-binding site would not be generated from native β_2GPI. In contrast, the binding of β_2GPI to anionic surfaces may protect the major PL-binding site from protease attack by masking the site, resulting in the appearance of the previously cryptic peptide containing the entire major PL-binding site. A similar mechanism is reported by Simitsek et al. [42], who found that antibody-binding to the antigen suppresses the generation of some epitopes and boosts that of others. Remarkably, both suppressed and boosted epitopes were present within a protein domain that was 'fingerprinted' by the antibody, while epitopes that lay outside this domain were not affected. Based on these findings, they speculated that the antibody, by binding and stabilizing a protein domain, might influence the accessibility of

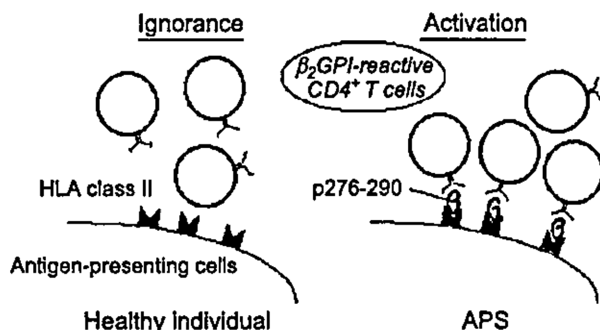

Figure 5 Activation of β_2GPI-reactive CD4$^+$ T cells in the normal T-cell repertoire by the de novo presentation of a previously cryptic p276–290. T cells recognizing cryptic p276–290 would not encounter antigenic peptides in the periphery in healthy individuals (immunologic 'ignorance'), whereas pathogenic β_2GPI-reactive CD4$^+$ T cells are activated upon recognition of a previously cryptic p276–290 expressed on functional APCs in APS patients.

the site to proteases during antigen processing. Taken together, modulation of antigen processing would influence processing of autoantigens that are bound by high-affinity ligands. Because β_2GPI is a plasma protein abundant in the circulation, excessive exposure to anionic surfaces, such as microorganisms and apoptotic cells, may induce the formation of a large quantity of β_2GPIs bound to anionic surfaces in vivo. In this regard, associations between various types of infections and the production of aPL antibodies have been reported [43], and infection is one of the major precipitating factors contributing to the development of catastrophic APS [44]. However, to initiate the pathogenic autoimmune response, additional factors, such as impaired regulatory function and nonspecific inflammation mediated by cytokines and toll-like receptor ligands, are apparently necessary in conjunction with the expression of the previously cryptic epitope.

Another possibility for the presentation of previously cryptic p276–290 is that APS patients might have been exposed to foreign proteins that cross-react with β_2GPI. It has been reported that immunization with foreign β_2GPI induces production of a high titer of anti-β_2GPI antibodies as well as development of clinical features of APS in normal mice [19,20]. In this model, it is presumed that immunization of mice with human β_2GPI first leads to the production of antibody to human (foreign) β_2GPI, followed by the appearance of autoantibodies reactive with murine (self) β_2GPI. The mechanism is thought to be the generation of cross-reactive B cells, which are initially primed by a foreign protein, then bind a self-antigen and process and present self-peptides to T cells [45]. Because B cells that acquire antigen via surface immunoglobulin require approximately 1000- to 10,000-fold less antigen for subsequent T-cell response, B cells may have the ability to efficiently concentrate small quantities of determinants that are previously cryptic [46]. In this regard, Blank et al. [47] reported that bacterial peptides homologous to β_2GPI induced pathogenic anti-β_2GPI antibodies along with the APS manifestation in normal mice.

Summary

Accumulating evidence in APS patients as well as in experimental APS indicates an important role of β_2GPI-specific $CD4^+$ T cells in anti-β_2GPI antibody production and in the pathogenesis of APS. Further studies examining the mechanisms that induce the efficient processing and presentation of cryptic determinants of β_2GPI as a consequence of antigen processing would clarify the etiology of APS. In addition, elimination or inactivation of pathogenic β_2GPI-specific $CD4^+$ T cells could be a therapeutic strategy that inhibits anti-β_2GPI antibody production and prevents thrombosis and fetal loss in patients suffering from APS [48].

Acknowledgements

Supported by grants from the Japanese Ministry of Education, Science, Sports and Culture and the Japanese Ministry of Health, Welfare and Labor.

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ARTHRITIS & RHEUMATISM
Vol. 50, No. 11, November 2004, pp 3658–3662
DOI 10.1002/art.20597
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Autoreactive CD8+ Cytotoxic T Lymphocytes to Major Histocompatibility Complex Class I Chain–Related Gene A in Patients With Behçet's Disease

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Objective. **To detect and characterize the autoreactive CD8+ T cells to major histocompatibility complex class I chain–related gene A (MICA), a stress-inducible antigen preferentially expressed on the epithelium and endothelium, in patients with Behçet's disease (BD).**

Methods. **A candidate for the antigenic MICA peptide was selected based on its predicted binding affinity for HLA–B51 and proteasomal cleavage sites. Peripheral blood T cells from 14 patients with BD and 15 healthy controls were repeatedly stimulated with the MICA peptide, and the specific T cell response was measured by peptide-induced interferon-γ. Cytotoxic T lymphocyte activity was examined by chromium-51 release from an HLA–B51–transfected B cell line in the presence of the MICA peptide.**

Results. **A 9-mer peptide AAAAAIFVI (termed MICA transmembrane [MICA-TM]) was selected as a candidate for the antigenic peptide presented by HLA–B51. A specific T cell response to MICA-TM was detected in 4 patients with BD (29%) but in none of the 15 healthy donors. All 4 responders had HLA–B51 and active disease, and the specific T cell response was lost after the BD-related symptoms disappeared. The MICA-induced T cell response was specifically inhibited by anti–HLA class I antibody or by CD8+ cell depletion. MICA-reactive T cells recognized an HLA–B51–transfected B cell line pulsed with MICA-TM or a B cell line transfected with both HLA–B51 and MICA in the absence of exogenous peptides. Finally, MICA-stimulated T cell lines lysed the HLA–B51–expressing B cell line in the presence of MICA-TM.**

Conclusion. **HLA–B51–restricted cytotoxic T lymphocytes autoreactive to MICA may be involved in the pathogenesis of BD.**

Supported by grants from the Japanese Ministry of Health, Labor, and Welfare, and the Japanese Ministry of Education, Science, Sports and Culture.

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Submitted for publication June 7, 2004; accepted in revised form July 28, 2004.

Behçet's disease (BD) is an inflammatory disorder characterized by uveitis, oral and genital aphthous ulcers, and skin lesions (1). The etiology of BD remains unclear, but BD has several unique epidemiologic and clinical features (1,2), as follows: the high-prevalence area is from southern Europe to Japan along the Silk Route; susceptibility is strongly associated with HLA–B51; and microbial infection, surgical procedures, and mechanical stimulation often precede the disease flare. These findings indicate that both genetic and environmental factors contribute to the pathogenic process of BD. This is supported by the finding that HLA–B51–transgenic mice show enhanced neutrophil function but fail to express the BD phenotype (3). Because a primary role of HLA class I antigens such as HLA–B51 is to present endogenous peptides to CD8+ T cells, the lack of the disease phenotype in this model can be explained by the absence of an HLA–B51–restricted pathogenic peptide that would activate the disease-relevant CD8+ T cells.

Recent advances in the understanding of HLA class I–binding peptide motifs have enabled us to detect and characterize autoreactive CD8+ cytotoxic T lymphocytes (CTLs) involved in the pathogenesis of autoimmune diseases (4). For example, in patients with

primary biliary cirrhosis, disease-relevant CTLs to the E2 component of pyruvate dehydrogenase complexes were detected using synthetic peptides selected according to their binding affinity to HLA–A2 (5). Because CD8+ T cell infiltration is often detected in BD lesions (2), we hypothesized that HLA–B51–restricted CTLs play some role in the BD pathogenesis by targeting a self antigen selectively expressed in the affected tissues. We selected major histocompatibility complex class I chain–related gene A (MICA) as a candidate for this autoantigen, because this protein is preferentially expressed on epithelial and endothelial cells in a stress-dependent manner (6,7). To test our hypothesis, we examined the HLA–B51–restricted CTL response to MICA in patients with BD.

PATIENTS AND METHODS

Patients and controls. We studied 14 patients with BD (5 men and 9 women) who fulfilled the diagnostic criteria defined by the International Study Group (8). Fifteen healthy individuals (9 men and 6 women) served as controls. The patients with BD were grouped as having active disease (6 patients) or inactive disease (8 patients) at the time of blood examination. Active disease was defined as the presence of characteristic BD symptoms, including severe oral/genital ulcers and ocular involvement, that required introduction of treatment with corticosteroids and/or cyclosporine. All samples were obtained after the patients gave their written informed consent, as approved by the institutional review board.

HLA–B51 genotyping. HLA–B51 was detected by polymerase chain reaction using sequence-specific primers and sequence-based typing (9).

Selection and synthesis of MICA peptides. MICA is a polymorphic antigen located near the HLA–B gene on chromosome 6 (10). A potential antigenic MICA peptide derived from the amino acid sequence of MICA*009, which is in strong linkage disequilibrium with HLA–B51 (10), was selected based on the following online resources: a program that ranks peptides based on their predicted half-time dissociation coefficient for HLA class I molecules (http://www.bimas.dcrt.nih.gov/molbio/hla_bind/index.html), and the PAProC program, which predicts proteasomal cleavage sites during antigen processing (http://www.uni-tuebingen.de/uni/kxi/). The selected peptides were synthesized using a solid-phase method based on fluorenylmethoxycarbonyl chemistry and purified using reverse-phase high-performance liquid chromatography. The purity of all peptides was >90%.

B cell lines expressing MICA and HLA–B51. Complementary DNAs encoding HLA–B51 (B*5101) and MICA (MICA*009) were introduced into C1R, a human B cell line deficient in HLA–A and HLA–B, by gene transfer using GenePulser (BioRad, Hercules, CA). The B*5101 and MICA*009 alleles were chosen because this combination is the most common in Japanese patients with BD (10,11). C1R transfectants stably expressing HLA–B51 (C1R/B51) and MICA (C1R/MICA) were generated by limiting dilution. A C1R cell line expressing both MICA and HLA–B51 (C1R/MICA/B51) was generated by the transient expression of HLA–B51 in C1R/MICA by gene transfer. Expression of HLA–B51 and MICA was evaluated by flow cytometry using a monoclonal antibody (mAb) to HLA class I (Sigma, St. Louis, MO) and a polyclonal antibody to MICA (kindly provided by Dr. Minoru Kimura, Tokai University) (12).

Generation of a MICA-stimulated T cell line. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood using Lymphoprep (Nycomed Pharma, Oslo, Norway) density-gradient centrifugation, and resuspended in Iscove's modified Dulbecco's medium containing 10% autologous platelet-poor plasma, 2 m*M* L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. A T cell line was established by the repetitive stimulation of peripheral blood T cells with a potential antigenic MICA peptide or a control peptide as previously described (13), with some modifications. Briefly, PBMCs (3×10^6) were cultured with the peptide (2 μM) in 24-well plates in a humidified atmosphere of 5% CO_2 at 37°C. Interleukin-2 (IL-2) (50 units/ml; Biogen, Cambridge, MA) was added to the culture on day 3. On days 7 and 14, viable cells (3×10^5) were restimulated with 3×10^6 irradiated (60 Gy) autologous PBMCs preincubated with the peptide (2 μM) and IL-2. On day 21, the cells were harvested and used as a MICA-stimulated T cell line.

Interferon-γ (IFNγ) release assay. The antigenic specificity of individual MICA-stimulated T cell lines was examined as previously described (13). Briefly, a MICA-stimulated T cell line (5×10^4) was cultured in duplicate with stimulators (10^5) pulsed with the MICA peptide (2 μM, unless otherwise indicated) and IL-2 (30 units/ml) in 96-well U-bottomed plates. Autologous Epstein-Barr virus–transformed lymphoblastoid B cell line cells (LBLs) or C1R transfectants were used as stimulators. After incubation for 18 hours, the culture supernatants were collected and applied to an enzyme-linked immunosorbent assay to measure the IFNγ concentration. In some experiments, anti–HLA class I, anti–HLA–DR, anti–HLA–DQ, anti–HLA–DP, or an isotype-matched control mAb was added at the initiation of the culture. MICA-stimulated T cell lines depleted of CD4+ or CD8+ cells using a magnetic bead–conjugated anti-CD4 or anti-CD8 mAb (Dynal, Oslo, Norway), respectively, were also used as responders.

Cytotoxicity assay. Cytotoxic activity of the MICA-stimulated T cell line was examined by chromium-51 (^{51}Cr) release assay (13). Briefly, a ^{51}Cr-labeled C1R/B51 cell line was incubated with or without a potential antigenic MICA peptide (2 μM) and cocultured with a MICA-stimulated T cell line at an effector-to-target ratio of 10:1 or 1:1 for 24 hours. Radioactivity in the culture supernatants was counted using a γ counter. All cultures were examined in triplicate, and the percent of specific lysis was calculated as the ^{51}Cr released in association with the peptide stimulation divided by the maximal ^{51}Cr release obtained by lysing cells with 0.5% Triton X.

Statistical analysis. Comparisons between 2 groups were performed using the chi-square test or the Mann-Whitney U test as appropriate.

RESULTS

Selection of MICA peptides. A potential HLA–B51–restricted antigenic MICA peptide was determined

by scanning the amino acid sequence of the MICA*009 protein for a 9-mer peptide containing the HLA–B51–binding motif that would be generated as a consequence of proteasomal cleavage during antigen processing. This analysis identified a peptide encoding a transmembrane region encompassing amino acids 294–302 (AAAAAIFVI; MICA-TM). Notably, MICA-TM includes part of a triplet-repeat microsatellite polymorphism consisting of 6 alanine residues, which is reported to be associated with BD (10). Another peptide encompassing amino acids 70–78 (GKDLRMTLA; MICA70) was selected as a control peptide based on its having a low binding affinity to HLA–B51 and being generated after processing.

Detection of MICA-reactive T cells. Because a MICA-TM–specific T cell response was not detected directly from the peripheral blood of patients with BD, we repeatedly stimulated peripheral blood T cells with MICA-TM before they were subjected to the assay. From a representative BD patient, a T cell line generated by repetitive stimulation with MICA-TM showed a profound response to MICA-TM but not to MICA70 (Figure 1A). This response was specifically blocked by an anti–HLA class I mAb (Figure 1B). When serial concentrations of MICA-TM were used, a significant response was observed at a peptide concentration of 10^{-2} μM or higher (Figure 1C). In contrast, T cell lines repeatedly stimulated with MICA70 failed to respond to MICA70 or MICA-TM. These findings indicate the presence of HLA class I–restricted T cells responsive to MICA-TM in this patient.

The same method was used to screen 14 patients with BD and 15 healthy controls for the MICA-TM–specific T cell response. Figure 2 shows MICA-TM–specific IFNγ production, which was calculated by subtracting the IFNγ concentration obtained for cultures with MICA70 from that obtained for cultures with MICA-TM, in individual subjects. A MICA-TM–specific T cell response was detected in 4 patients with BD (29%) but in no healthy controls ($P = 0.03$). Notably, all the responders were HLA–B51–positive and had active disease. All 6 patients with active disease were not receiving corticosteroids or immunosuppressants at the time of the blood examination, indicating that a difference in the medical therapy did not influence the results. We repeated the assay in 11 patients with BD, including 3 with active disease, and consistent results were obtained regarding the presence or absence of the specific response. In all 4 responders, the MICA-TM–specific T cell response was not detectable at followup, when all of the BD-related symptoms were gone (>6 months after the first evaluation). Thus, a T cell response to MICA-TM was specifically detected in BD patients in association with HLA–B51 and active disease status.

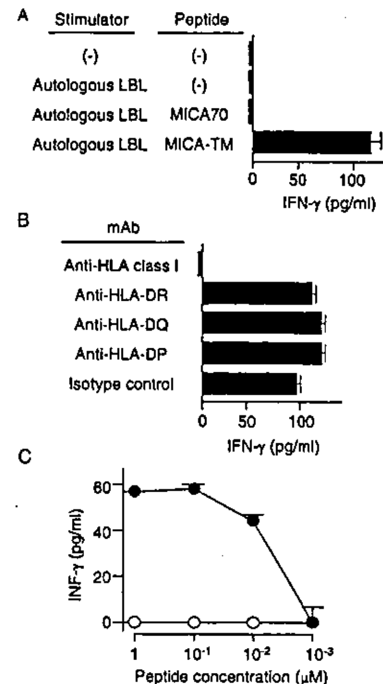

Figure 1. T cell response to major histocompatibility complex class I chain–related gene A (MICA) in a representative patient with active Behçet's disease (BD). **A,** The peptide-specific response of a MICA-stimulated T cell line was assessed by interferon-γ (IFNγ) release in response to autologous lymphoblastoid B cell line cells (LBLs) pulsed with MICA transmembrane (MICA-TM) or MICA70. **B,** A MICA-stimulated T cell line was cultured with MICA-TM in the presence of anti–HLA class I, anti–HLA-DR, anti–HLA-DQ, anti–HLA-DP, or an isotype-matched control monoclonal antibody (mAb). **C,** A MICA-stimulated T cell line was cultured with serial concentrations of MICA-TM (solid circles) or MICA70 (open circles). Results shown are the mean and SD. Analogous findings were obtained from 5 independent experiments using peripheral blood samples from 4 BD responders.

Characterization of MICA-reactive T cells. To examine whether the MICA-TM–induced T cell response was HLA–B51–restricted, a series of C1R transfectants expressing HLA–B51, MICA, or both were used as stimulators in the IFNγ release assay. Wild-type C1R expressed low levels of HLA class I (presumably HLA–C) and MICA, but an up-regulated expression of HLA class I and MICA was detected in C1R/B51 and C1R/MICA, respectively (Figure 3A). At least 10% of the C1R/MICA/B51 cells consistently expressed both MICA and HLA–B51. When MICA-stimulated T cell lines generated from 3 patients with BD were cultured with C1R or C1R/B51 in the presence or absence of

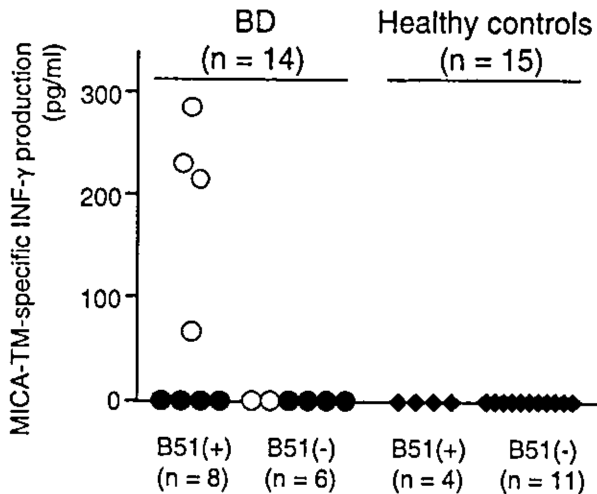

Figure 2. T cell response specific to MICA in 14 patients with BD and 15 healthy controls in the presence or absence of HLA-B51. The MICA-TM-specific IFNγ production of individual MICA-stimulated T cell lines was calculated by subtracting the IFNγ concentration obtained in cultures with MICA70 from that obtained in cultures with MICA-TM. Open circles denote BD patients with active disease; solid circles denote BD patients with inactive disease. See Figure 1 for definitions.

MICA-TM or MICA70, a T cell response was detected solely in the culture with C1R/B51 pulsed with MICA-TM (Figure 3B). C1R/MICA lacking HLA-B51 expression failed to induce the T cell response. The response induced by MICA-TM was abolished by anti-HLA class I mAb. In addition, the depletion of CD8+ T cells, but not CD4+ T cells, from MICA-stimulated T cell lines cancelled the specific T cell response (data not shown). Finally, MICA-stimulated T cell lines responded to C1R/MICA/B51 in the absence of the exogenous peptide, and this response was blocked by anti-HLA class I mAb. These findings together indicate that MICA-reactive CD8+ T cells recognize MICA-TM, which is endogenously generated as a consequence of processing, in the context of HLA-B51.

To evaluate whether MICA-reactive CD8+ T cells have cytotoxic activity, MICA-stimulated T cell lines were cultured with ^{51}Cr-labeled C1R/B51 pulsed with or without MICA-TM (Figure 3C). The target cell lysis was observed specifically in the presence of MICA-TM, indicating that MICA-reactive T cells exhibited CTL activity.

DISCUSSION

Previous studies examining the role of autoimmunity in BD pathogenesis focused on CD4+ T cells and found T cells that were autoreactive to heat shock protein 60 (Hsp60) and retinal S antigen in BD patients, especially those with ocular involvement (2). Interestingly, Hsp60 is a stress-inducible antigen similar to MICA and is overexpressed in the oral mucosa and skin lesions of patients with BD (14). Here, we demonstrate the presence of HLA-B51-restricted CTLs autoreactive to MICA in patients with BD. The relevance of the MICA-reactive CTLs to the pathogenesis is supported

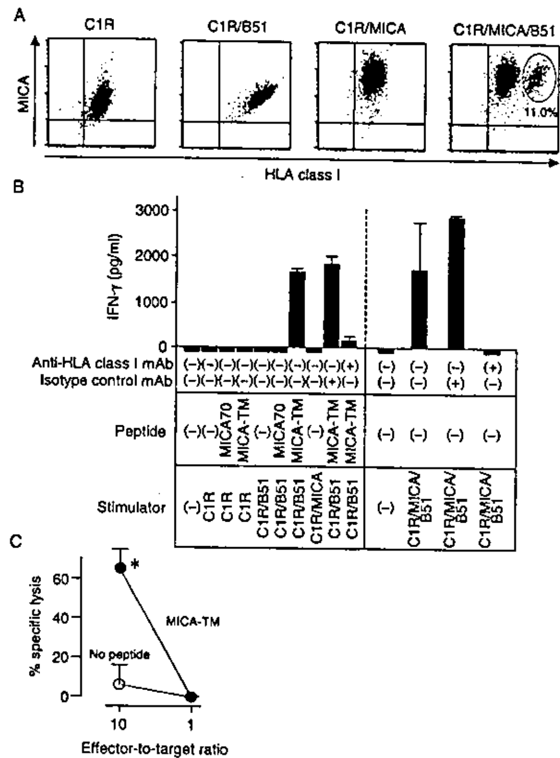

Figure 3. The HLA-B51-restricted antigen recognition profile and cytotoxic activity of MICA-stimulated T cell lines. **A,** Flow cytometric analysis for the expression of HLA class I and MICA in C1R, a C1R transfectant expressing HLA-B51 (C1R/B51), a C1R transfectant expressing MICA (C1R/MICA), and a C1R transfectant expressing both molecules (C1R/MICA/B51). C1R/MICA and C1R/B51 were clones generated by limiting dilution, and C1R/MICA/B51 was a C1R/MICA cell line transiently transfected with HLA-B51. **B,** Responses of a MICA-stimulated T cell line to a series of C1R transfectants in the presence or absence of MICA-TM or MICA70. The T cell response was measured by IFNγ production. Anti-HLA class I monoclonal antibody (mAb) or an isotype-matched control mAb was added at the initiation of some cultures. Results shown are the mean and SD. **C,** Cytotoxic activity of a MICA-stimulated T cell line evaluated by chromium-51 release assay. C1R/B51 preincubated with or without MICA-TM was used as a target. Results shown are the mean and SD. * = $P < 0.05$ by Mann-Whitney U test, versus no peptide at an effector-to-target ratio of 10:1. A representative result of 3 independent experiments is shown. See Figure 1 for definitions.

by their strong association with the active disease status of BD. Moreover, recognition of the endogenously generated MICA peptide by autoreactive CTLs in the context of HLA–B51 may explain why HLA–B51 is a marker of susceptibility and severity in BD (1,2). Thus, MICA is the first "behçetogenic" CD8 autoantigen identified in association with HLA-B51. In BD patients without HLA-B51, it is possible that appropriate MICA-derived peptides are presented in the context of HLA class I molecules other than HLA-B51 and recognized by autoreactive CTLs.

Many extrinsic pathogenic candidates, including bacterial and viral antigens, have been reported in BD (2). An excessive inflammatory response induced by these extrinsic agents is one of the fundamental disease processes of BD (2). Neutrophil hyperfunction and a cross-reactive autoimmune response between microbial and human HSPs are proposed to be responsible for this hyperreactivity (1,14). MICA is a stress-inducible protein that acts as a ligand for a subset of γ/δ T cell receptors (6) and a killer cell–activating receptor, NKG2D, expressed by most cytotoxic cells, including CTLs and natural killer cells (15). Cytotoxic activity is augmented via the engagement of NKG2D to MICA, whose expression is induced on epithelial and endothelial cells under stress conditions. The physiologic role of MICA remains unclear, but it is thought to function as a marker for damaged or infected epithelial and endothelial cells and to facilitate clearance of those injured cells by an immune surveillance system. This process would not normally induce harmful inflammation but may cause tissue injuries in the presence of MICA-reactive CTLs in patients with BD. Namely, after nonspecific minor injuries and microbial infection, MICA-reactive CTLs are activated upon recognition of the MICA-derived peptide presented on the epithelium and endothelium and subsequently lead to excessive and prolonged inflammatory responses at the site of stress by enhancing the MICA-mediated cytotoxicity. This response would be further augmented in the predominant Th1 cytokine environment observed in patients with BD (1,2).

In summary, this report provides the first direct evidence showing the potential involvement of HLA–B51–restricted autoreactive CTLs in the pathogenesis of BD. Moreover, our findings will stimulate further research on MICA as a key molecule in the development of BD. Because this is a preliminary study preformed in a small number of patients with BD, additional studies involving a larger population are necessary to confirm our hypothesis.

ACKNOWLEDGMENTS

We thank Taeko Naruse for assisting in the HLA–B51 genotyping, Toshiharu Sakurai and Tomonobu Fujita for assisting in the peptide synthesis, and Tsuneyo Mimori and Masao Ota for valuable comments.

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Rheumatology 2005;44:192–196
Advance Access publication 27 October 2004
doi:10.1093/rheumatology/keh449

Concise Report

Lack of circulating autoantibodies to bone morphogenetic protein receptor-II or activin receptor-like kinase 1 in mixed connective tissue disease patients with pulmonary arterial hypertension

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Objectives. **To examine whether autoantibodies against bone morphogenetic protein receptor-II (BMPR-II) or activin receptor-like kinase 1 (ALK-1) are associated with pulmonary arterial hypertension (PAH) in patients with mixed connective tissue disease (MCTD).**

Methods. **We studied sera from 37 MCTD patients with or without PAH, six patients with idiopathic PAH, and 30 healthy controls. Circulating anti-BMPR-II and anti-ALK-1 antibodies were detected using immunoprecipitation of recombinant antigens generated by *in vitro* transcription/translation and indirect immunofluorescence of cultured cells that were induced to express these antigens by gene transfer. Anti-BMPR-II antibodies were further examined by immunoprecipitation and immunoblotting using a recombinant fragment of the extracellular domain of BMPR-II.**

Results. **Serum anti-BMPR-II and anti-ALK-1 autoantibodies were not detected in MCTD patients irrespective of the presence or absence of PAH, or in patients with idiopathic PAH.**

Conclusions. **Our finding does not support the hypothesis that autoantibody-mediated dysregulation of signals through BMPR-II or ALK-1 contributes to the development of PAH in patients with connective tissue diseases.**

KEY WORDS: Pulmonary arterial hypertension, Autoantibody, Mixed connective tissue disease, Bone morphogenetic protein receptor-II, Activin receptor-like kinase 1.

Pulmonary arterial hypertension (PAH) is a life-threatening organ involvement in patients with various connective tissue diseases, including scleroderma [1]. PAH also occurs without an underlying disease, and is then termed idiopathic pulmonary arterial hypertension (IPAH; formerly primary pulmonary hypertension) [2]. Recent genetic analyses in IPAH patients have identified germ-line mutations in the gene for bone morphogenetic protein receptor-II (BMPR-II), a type II receptor for transforming growth factor β (TGF-β) superfamilies, in both familial and sporadic cases [3–5]. In addition, families with hereditary haemorrhagic telangiectasia are known to develop PAH frequently, and mutations in the gene for activin receptor-like kinase 1 (ALK-1), a type I receptor for TGF-β, have been identified as a genetic factor responsible for this hereditary disease [6]. Both BMPR-II and ALK-1 are receptors for TGF-β family proteins, with intracellular serine/threonine kinase domains that are preferentially expressed on vascular endothelial cells [7, 8]. Upon ligand binding, these receptors participate in the phosphorylation of a series of Smad proteins that regulate the growth and functional properties of vascular endothelial and smooth muscle cells. It is currently believed that dysregulated Smad signals through BMPR-II and ALK-1 are involved in the pathogenesis of IPAH [9, 10].

IPAH and PAH accompanied by connective tissue diseases have similar characteristics [1], including medial hypertrophy and plexogenic arteriopathy in the pulmonary artery histology; progressive clinical course and poor prognosis; and therapeutic responses to continuous infusion of the prostacyclin analogue and endothelin-1 receptor antagonist. Therefore, we hypothesized that dysfunction in BMPR-II or ALK-1 also contributes to the pathogenesis of PAH in patients with connective tissue diseases. However, it seemed unlikely that genetic mutations would be found as the underlying cause because two independent research groups failed to detect mutations in the BMPR-II gene in patients with scleroderma spectrum disorders and PAH [11, 12]. Another potential mechanism includes the presence of autoantibodies that functionally interfere with ligand binding to BMPR-II or ALK-1, which is reasonably likely, because patients with connective tissue diseases, especially those with systemic lupus erythematosus (SLE), are intrinsically prone to producing autoantibodies. To test this hypothesis, we developed assay systems to detect autoantibodies that react with BMPR-II and ALK-1. Since the majority of Japanese patients with connective tissue disease and PAH are positive for antibodies to U1 small nuclear ribonucleoprotein (U1RNP) and have a diagnosis of mixed connective tissue disease

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Submitted 11 July 2004; revised version accepted 21 September 2004.

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Rheumatology Vol. 44 No. 2 © British Society for Rheumatology 2004; all rights reserved

(MCTD) [13, 14], we examined MCTD patients with or without PAH in this study.

Materials and methods

Patients and samples

We examined 37 patients who were positive for anti-U1RNP antibodies and fulfilled the diagnostic criteria for MCTD proposed by Porter *et al.* [15]. MCTD patients represented heterogeneous clinical characteristics: 21 patients met the American College of Rheumatology (ACR) classification criteria for SLE [16], 24 met the ACR preliminary classification criteria for scleroderma [17], and six met the diagnostic criteria for definite polymyositis or dermatomyositis as proposed by Bohan and Peter [18]. Fourteen patients satisfied two of these three sets of criteria (SLE and scleroderma in nine, and scleroderma and polymyositis/dermatomyositis in five). Echocardiogram was performed on all patients, and those with a mean pulmonary artery pressure ≤25 mmHg at rest were regarded as non-PAH [9]. The remaining patients, with a mean pulmonary artery pressure >25 mmHg, were further divided into two groups: patients with definite PAH in whom the presence of PAH was confirmed by right ventricle catheterization, and those with questionable PAH in whom right ventricle catheterization was not carried out. Serum samples from six patients with IPAH [2] and 30 healthy individuals were used as controls. Written consent was obtained according to the Declaration of Helsinki, and the study design was approved by Keio University Institutional Review Boards.

IgG fraction

The immunoglobulin G (IgG) fraction was isolated from sera by affinity chromatography using a HiTrap™ Protein G HP Column (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's protocol. The protein concentration was measured with the Bradford assay using serial concentrations of bovine serum albumin (BSA) as a standard.

cDNA constructs

Full-length cDNAs encoding human BMPR-II and ALK-1 were kindly provided by Dr Kohei Miyazono (University of Tokyo Graduate School of Medicine, Tokyo, Japan). These cDNAs were subcloned into pcDEF3-FLAG (BMPR-II) or pcDNA3-HA (ALK-1), which add FLAG- and HA-epitope tags, respectively, at the carboxyl terminal end [19, 20].

Immunoprecipitation of recombinant polypeptides generated by in vitro *transcription and translation*

Recombinant BMPR-II-FLAG and ALK-1-HA were produced by *in vitro* transcription and translation of full-length cDNAs using the Single Tube Protein™ System 3 (Novagen, Darmstadt, Germany) in the presence of ^{35}S-labelled methionine (ICN Biomedicals, Irvine, CA, USA). ^{35}S-labelled BMPR-II-FLAG and ALK-1-HA were applied to immunoprecipitation assays as described previously [21]. Mouse monoclonal antibodies (mAbs) to BMPR-II (clone 18; Becton Dickinson, San Jose, CA, USA) and FLAG (clone M2; Sigma-Aldrich, St Louis, MO, USA) were used as positive controls for BMPR-II-FLAG, and rat anti-HA mAb (clone 3F10; Roche Applied Science, Mannheim, Germany) was used as a positive control for ALK-1-HA. Mouse anti-His mAb (Amersham Biosciences) was used as a negative control. The immunoprecipitated materials were separated on sodium dodecyl sulphate (SDS)-6% (for BMPR-II-FLAG) or 8.5% (for ALK-1-HA) polyacrylamide gels, and subjected to autoradiography using a Fuji BAS 5000 Bio-Imaging Analyzer (Fuji Photo Film, Tokyo, Japan).

Immunoprecipitation and immunoblotting using recombinant BMPR-II as an antigen

Anti-BMPR-II antibodies were detected using a recombinant chimeric protein consisting of the extracellular domain of human BMPR-II and the histidine-tagged Fc region of human IgG1 (BMPR-II/Fc chimera; R & D Systems, Minneapolis, MN, USA). Serum samples (10 μl) were incubated with protein A-Sepharose™ CL-4B beads (Amersham Biosciences) for 2 h at room temperature. Goat anti-BMPR-II polyclonal antibodies (R & D systems) and anti-His mAb were used as positive controls, and anti-HA mAb was used as a negative control. The beads were incubated with BMPR-II/Fc chimera (50 ng) for 2 h at 4°C in 10 mM Tris–HCl, 500 mM NaCl, 0.1% Nonidet P-40, pH 8.0, containing 60% fetal bovine serum to block non-specific protein A binding to the Fc region of the BMPR-II/Fc chimera. The immunoprecipitated materials were subsequently separated on SDS–8.5% polyacrylamide gels, and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk, and incubated with anti-BMPR-II polyclonal antibodies and then with alkaline phosphatase-conjugated anti-goat IgG (Sigma-Aldrich). Positive signals were visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Indirect immunofluorescence of cultured cells expressing exogenous genes

COS-7 cells grown on fibronectin-coated eight-well chamber slides (Becton Dickinson Labware, Bedford, MA, USA) were transfected with plasmids harbouring BMPRII-FLAG or ALK-1-HA, using the Effectene transfection reagent (Qiagen, Hilden, Germany). COS-7 cells transfected with the empty vector were used as a control. After a 24-h culture in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, the cells were fixed with cold acetone/methanol for 5 min. Each slide was blocked with 5% BSA, and incubated with purified IgG (40 μg/ml) and then with Alexa Fluor 488-conjugated goat anti-human IgG antibodies (Molecular Probes, Eugene, OR, USA). The cells were further incubated with biotinylated anti-FLAG mAb and streptavidin-conjugated Alexa Fluor 568 (for BMPR-II transfectants) or anti-HA mAb and Alexa Fluor 568-conjugated goat anti-rat IgG antibody (for ALK-1 transfectants). Anti-BMPRII mAb with Alexa Fluor 488-conjugated goat anti-mouse IgG antibodies were used as a positive control for BMPR-II transfectants. Nuclei were counterstained with TO-PRO-3 (Molecular Probes). The cells were observed under a confocal laser fluorescent microscope (LSM5 PASCAL; Carl-Zeiss, Göttingen, Germany).

Results

Of 37 patients with MCTD, 15 and two had definite and questionable PAH, respectively, while 20 did not have PAH. There was no difference in the distribution of clinical diagnosis between patients with PAH and those without. Nine of 15 patients with definite PAH died within 3 yr after the serum sample collection. Autopsy in seven patients revealed a typical medial hypertrophy with or without plexiform lesions in pulmonary arteries. Of the remaining six patients, five are being treated with epoprostenol and one is on bosentan.

Sera from 37 MCTD patients with and without PAH, six patients with IPAH and 30 healthy controls were screened for antibodies to BMPR-II and ALK-1 by immunoprecipitation

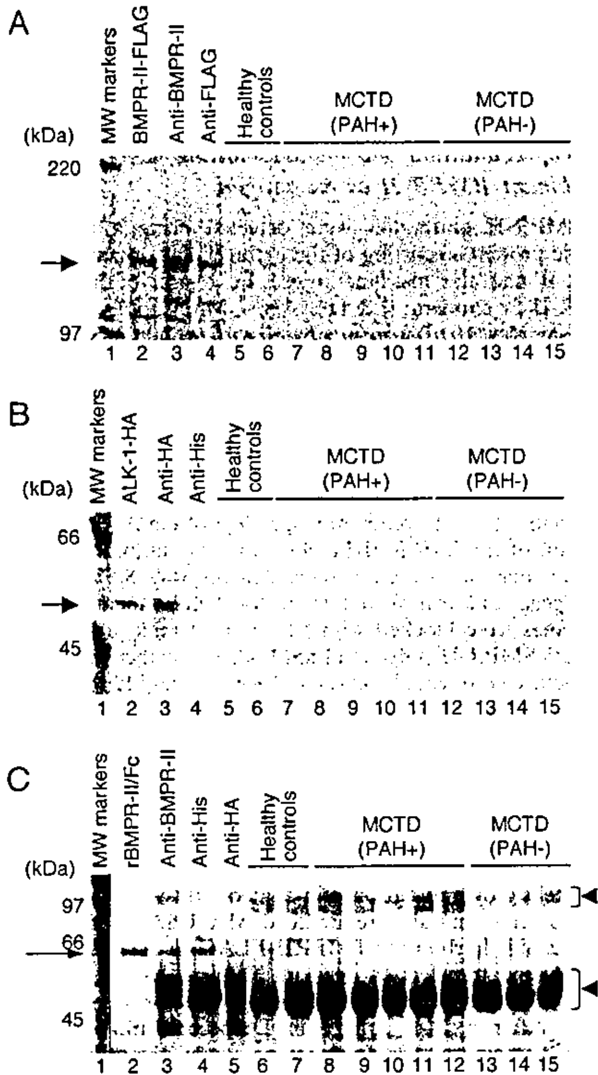

FIG. 1. Screening of anti-BMPR-II and anti-ALK-1 autoantibodies in representative sera from healthy controls and MCTD patients with or without PAH. (A) Detection of anti-BMPR-II antibodies using immunoprecipitation of ^{35}S-labelled BMPR-II-FLAG generated by *in vitro* transcription/translation. Lane 1, ^{14}C-labelled molecular weight markers; lane 2, recombinant BMPR-II-FLAG without immunoprecipitation; lane 3, anti-BMPR-II mAb; lane 4, anti-FLAG mAb; lanes 5 and 6, healthy control sera; lanes 7–11, sera from MCTD patients with definite PAH; lanes 12–15, sera from MCTD patients without PAH. Arrow denotes intact recombinant BMPR-II-FLAG. (B) Detection of anti-ALK-1 antibodies using immunoprecipitation of ^{35}S-labelled ALK-1-HA generated by *in vitro* transcription/translation. Lane 1, ^{14}C-labelled molecular weight markers; lane 2, recombinant ALK-1-HA without immunoprecipitation; lane 3, anti-HA mAb; lane 4, anti-His mAb; lanes 5 and 6, healthy control sera; lanes 7–11, sera from MCTD patients with definite PAH; lanes 12–15, sera from MCTD patients without PAH. Arrow denotes intact recombinant ALK-1-HA. (C) Detection of anti-BMPR-II antibodies by immunoprecipitation and immunoblotting using a recombinant BMPR-II/Fc chimera. Lane 1, molecular weight markers; lane 2, BMPR-II/Fc chimera without immunoprecipitation; lane 3, anti-BMPR-II polyclonal antibodies; lane 4, anti-His mAb; lane 5, anti-HA mAb; lanes 6 and 7, healthy control sera; lanes 8–12, sera from MCTD patients with definite PAH; lanes 13–15, sera from MCTD patients without PAH. Arrow denotes recombinant BMPR-II/Fc chimera and arrowheads denote non-specific IgG bands.

of ^{35}S-labelled BMPR-II-FLAG and ALK-1-HA, respectively. As shown in Fig. 1A, BMPR-II-FLAG produced by *in vitro* transcription and translation represented a 135-kDa intact protein with several degradation products. These proteins were immunoprecipitated by anti-BMPR-II and anti-FLAG mAbs, but recognized by none of the MCTD sera, IPAH sera or by 30 healthy control sera. A 55-kDa ALK-1-HA protein generated by *in vitro* transcription/translation was specifically precipitated by the anti-HA mAb (Fig. 1B). Like the BMPR-II-FLAG, ALK-1-HA was not recognized by any of the MCTD, IPAH or healthy control sera. Anti-BMPR-II or anti-ALK-1 antibodies were not detected when the amount of serum or antigens applied was increased to two-fold in all the MCTD samples.

Anti-BMPR-II autoantibodies were screened in an additional assay using BMPR-II/Fc chimera as an antigen. As shown in Fig. 1C, immunoprecipitation in the presence of an excess of bovine IgG successfully suppressed non-specific protein A binding to the Fc region of the chimeric protein. The BMPR-II/Fc chimera was recognized by anti-BMPR-II polyclonal antibodies and anti-His mAb, but not by an irrelevant anti-HA mAb. None of the sera from the 37 MCTD patients or 10 healthy controls reacted with the BMPR-II/Fc chimera in this assay system.

Because BMPR-II and ALK-1 are transmembrane proteins, recombinant proteins produced in the cell-free systems may have a conformation different from that of the membrane-bound native form. To evaluate antibody binding to membrane-bound BMPR-II and ALK-1, COS-7 cells were induced to transiently express BMPR-II-FLAG or ALK-1-HA by gene transfer, and subjected to an indirect immunofluorescence assay (Fig. 2). At least 10% of the treated cells constantly expressed BMPR-II-FLAG, as assessed by positive staining with anti-BMPR-II (green) and anti-FLAG (red) mAbs. Both mAbs stained principally the same portions of the cell membrane and cytoplasm (yellow). Anti-BMPR-II and anti-FLAG mAbs did not stain cells transfected with a control vector without the cDNA insert. To detect anti-BMPR-II antibodies in MCTD sera, IgG purified from serum samples was used instead of anti-BMPR-II mAb. Figure 2A shows representative results obtained from MCTD sera and a healthy control serum. Both MCTD sera stained nuclei, and one serum additionally stained the cytoplasm of all the cultured cells, but the staining pattern was clearly different from that produced by the anti-FLAG mAb. No staining pattern comparable to that produced by the anti-FLAG mAb was detected in additional IgG samples from four MCTD patients, including three with definite PAH, or from two healthy controls. Similarly, anti-HA mAb specifically stained the cellular membrane and cytoplasm of cells that were induced to express ALK-1-HA (red), but none of the IgG samples purified from the MCTD patients or the healthy controls produced the staining pattern we observed with the anti-HA mAb (Fig. 2B).

Discussion

This is the first report examining the autoantibody response to BMPR-II or ALK-1 in patients with connective tissue disease complicated by PAH and in IPAH patients. Contrary to our expectations, we failed to detect IgG autoantibodies that reacted with BMPR-II or ALK-1 in sera from MCTD patients, irrespective of the presence or absence of PAH. Since the recombinant antigens used in our assays are predicted to have a native conformation, our findings indicate a lack of circulating IgG antibodies capable of binding to BMPR-II or ALK-1 expressed on the endothelium. However, there is no human control serum positive for anti-BMPR-II or anti-ALK-1 antibody, although non-human antibodies to human BMPR-II or tags expressed on the recombinant proteins were used as positive controls in the assays. The possibility that our assays were less sensitive in

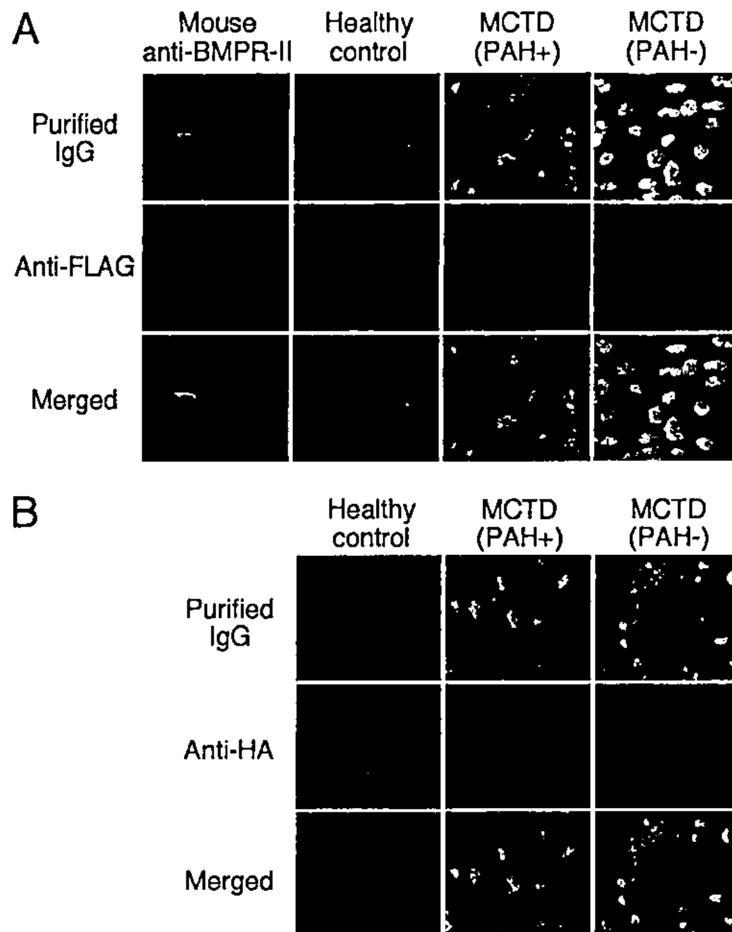

FIG 2. Screening of anti-BMPR-II and anti-ALK-1 autoantibodies using indirect immunofluorescence to observed COS-7 cells induced to express recombinant BMPR-II-FLAG and ALK-1-HA by gene transfer in representative samples from a healthy control, an MCTD patient with definite PAH, and an MCTD patient without PAH. (A) Fixed BMPR-II-FLAG-transfected cells were incubated with anti-BMPR-II mAb or IgG purified from serum samples (green) in combination with anti-FLAG mAb (red). (B) Fixed ALK-1-HA-transfected cells were incubated with IgG purified from serum samples (green) in combination with rat anti-HA mAb (red). The cells were examined with a confocal laser fluorescence microscope. Nuclei were counterstained with TO-PRO-3 (blue). Original magnification, ×40.

detecting human autoantibodies cannot be excluded. In addition, because the endothelium consists of approximately 10^{13} endothelial cells, occupying almost 7 m^2 in adults [22], it is still possible that patients have pathogenic autoantibodies to BMPR-II or ALK-1 that are present on endothelial cell surfaces but not in the circulation.

The mutations in the BMPR-II and ALK-1 genes that were detected in patients with IPAH included frameshifts, deletions, nonsense and missense mutations, and splice-site variations, most of which impaired the proteins' functional properties or transcriptional activities. This is consistent with a model of haploinsufficiency [23] or a dominant-negative effect [24]. The expression level of the BMPR-II protein is in fact reduced in the lungs of patients with severe IPAH, most of whom have an underlying mutation in the BMPR-II gene [25]. Based on these findings, it is widely accepted that a mutation in the BMPR-II and ALK-1 genes presents a major risk of the loss of TGF-β family-mediated growth inhibition of pulmonary arterial endothelial and smooth muscle cells, which leads to the development of the typical IPAH plexiform lesions in the pulmonary arteries [9, 10]. Taken together with the lack of germ-line mutations in the BMPR-II genes of patients with scleroderma spectrum disorders and PAH [11, 12], our findings strongly suggest that the underlying mechanisms for developing PAH are different between patients with connective tissue diseases and those without. This is supported by a recent paper reporting that the endothelial cell proliferation in the plexiform lesions is monoclonal in IPAH and polyclonal in scleroderma [26]. In this regard, the maintenance of the endothelial vascular network is regulated by several factors other than TGF-β superfamily proteins and their signalling pathways, including the angiopoietin-Tie system [27]. It has been reported recently that the expression of angiopoietin-1 and the phosphorylation of Tie-2 are higher than normal in the lungs of patients with non-familial PAH, including IPAH and scleroderma [28]. Further studies are necessary to identify the mechanisms contributing to the development of PAH in patients with connective tissue diseases.

Rheumatology	Key messages
	• Circulating autoantibodies to BMPR-II or ALK-1 were undetectable in connective tissue disease patients irrespective of the presence or absence of PAH.

Acknowledgements

This work was supported by grants from the Japanese Ministry of Health, Labor, and Welfare and the Japanese Ministry of Education, Science, Sports and Culture.

The authors have declared no conflicts of interest.

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Binding of β_2–glycoprotein I to anionic phospholipids facilitates processing and presentation of a cryptic epitope that activates pathogenic autoreactive T cells

Masataka Kuwana, Eiji Matsuura, Kazuko Kobayashi, Yuka Okazaki, Junichi Kaburaki, Yasuo Ikeda, and Yutaka Kawakami

Antiphospholipid syndrome (APS) is an autoimmune prothrombotic disorder in association with autoantibodies to phospholipid (PL)–binding plasma proteins, such as β_2-glycoprotein I (β_2GPI). We have recently found that CD4⁺ T cells autoreactive to β_2GPI in patients with APS preferentially recognize a cryptic peptide encompassing amino acid residues 276-290 (p276-290), which contains the major PL-binding site, in the context of DR53. However, it is not clear how previously cryptic p276-290 becomes visible to the immune system and elicits a pathogenics autoimmune response to β_2GPI. Here we show that presentation of a disease-relevant cryptic T-cell determinant in β_2GPI is induced as a direct consequence of antigen processing from β_2GPI bound to anionic PL. Dendritic cells or macrophages pulsed with PL-bound β_2GPI induced a response of p276-290–specific CD4⁺ T-cell lines generated from the patients in an HLA-DR–restricted and antigen-processing–dependent manner but those with β_2GPI or PL alone did not. In addition, the p276-290–reactive T-cell response was primed by stimulating peripheral blood T cells from DR53-carrying healthy individuals with dendritic cells bearing PL-bound β_2GPI in vitro. Our finding is the first demonstration of an in vitro mechanism eliciting pathogenic autoreactive T-cell responses to β_2GPI and should be useful in clarifying the pathogenesis of APS. (Blood. 2005;105:1552-1557)

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Introduction

Antiphospholipid syndrome (APS) is characterized by arterial and venous thrombosis as well as recurrent intrauterine fetal loss in association with antiphospholipid antibodies.[1] β_2–glycoprotein I (β_2GPI), a plasma protein that binds negatively charged substances including phospholipids (PLs), is the most common target for the antiphospholipid antibody associated with the clinical features of APS.[2] Pathogenicity of the anti-β_2GPI antibody has been demonstrated in animal models, including normal mice immunized with human β_2GPI[3] and severe combined immunodeficiency mice into which peripheral blood lymphocytes from patients with APS were transferred.[4] We recently identified autoreactive CD4⁺ T cells to β_2GPI that promote anti-β_2GPI antibody production in patients with APS.[5-7] β_2GPI–specific CD4⁺ T cells recognize amino acid residues 276-290 (p276-290), which define the immunodominant β_2GPI peptide, in the context of DRB4*0103 (DR53). This epitope is located on domain V and contains the major PL-binding site at amino acids 280-288.[8] The p276-290–reactive T-cell clones did not respond to functional antigen-presenting cells (APCs) bearing native β_2GPI but did to those bearing chemically reduced β_2GPI or recombinant β_2GPI fragments produced in bacteria.[6] Given that β_2GPI-reactive T cells are also detected in some healthy individuals,[5] the p276-290 epitope defined by β_2GPI-specific T cells is "cryptic," since it is generated at a subthreshold level by the processing of native β_2GPI under normal circumstances.[9]

There is increasing interest in the possibility that crypticity is an important characteristic of epitopes recognized by the autoreactive T cells and thus is relevant to autoimmune pathogenesis.[10] T cells recognizing self-determinants generated in sufficient amounts in APCs undergo deletion in the thymus or anergy in the periphery. On the other hand, T cells specific for cryptic self-determinants are a component of the normal T-cell repertoire but normally do not encounter antigenic peptides in the periphery. These T cells might become activated and autoaggressive if the previously cryptic self-determinants were presented at a higher concentration. This concept represents the major hypothesis for the pathogenesis of autoimmune diseases, but the fundamental question is how epitopes that are normally cryptic become visible to the immune system and elicit a sustained pathogenic response. In this study, p276-290–specific T-cell lines generated from patients with APS were used to investigate the mechanisms that induce the efficient processing and presentation of cryptic p276-290 as a consequence of antigen processing.

Patients, materials, and methods

Study subjects

Peripheral blood T cells from 5 Japanese patients with APS were analyzed in this study. All patients fulfilled the preliminary classification criteria for

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Submitted August 13, 2004; accepted October 6, 2004. Prepublished online as *Blood* First Edition Paper, October 14, 2004; DOI 10.1182/blood-2004-08-3145.

Supported by a grant from the Japanese Ministry of Health, Welfare and Labor and by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Sports and Culture.

An Inside *Blood* analysis of this article appears in the front of this issue.

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