ORIGINAL RESEARCH

Peptidic sequence “HSEAETGPP” is recognized by the sera of pars planitis patients

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Abstract

Purpose: HLA class II, p-36 protein, heat shock protein and retinal antigens have been associated with pars planitis (PP), but their participation in the development of the disease are unknown. A search for new molecules related to PP is necessary. This work focused on the identification of peptides recognized by PP patient sera using the phage display method.

Methods: Sera of PP patients were used to isolate peptides fused to M13-phage pIII protein. The response of PP and healthy sera to peptides was determined by ELISA. PCR amplification and sequencing of peptide-encoding fragments from clones with high recognition by PP sera were used to characterize displayed peptides.

Results: One hundred clones were randomly selected from a phage display library after three panning rounds using serum proteins from a PP patient. The immunologic response level of 100 clones selected were determined with a major number of patients, it was found that one clone was recognized stronger in PP patients sera than in healthy sera (PP vs. healthy; $P < 0.05$). The peptide-encoding region of this clone was sequenced and translated. The peptide sequence corresponded to HSEAETGPP. An identical amino acid sequence to HSEAETGPP is found in the human proline-rich transmembrane protein 2 which has not been related with eye diseases.

Conclusion: These results suggest that the peptide HSEAETGPP is associated with PP.

Uveitis, an inflammation in the uveal tract of eye, can be divided into anterior, intermediate and posterior uveitis. Pars planitis (PP) is a sub-type of intermediate uveitis, it is found in 4.3 to 15.4% of uveitis patients. The clinical characteristics of PP are: cellular infiltration in the vitreous, inflammation in the pars plana and the presence of snowbank. The illness is common in children and young people, and is found equally in men and women.

The etiology of PP is unknown but a strong immunological component is involved. Immunological susceptibility of PP is associated with the Human Leukocyte Antigens (HLA) class II. Patients with HLA DR15 from North America have a major susceptibility to PP^{1,2}, and HLA DRB1*0802 has been associated in Mexicans with juvenile PP.³ Other immunological factors present in PP are a cellular immune response and cytokine production. The chronic inflammation in PP is caused by T cells⁴, with a predominance of CD8+ T cells.⁵ T cell lines derived from the vitreous humour produce higher levels of IL-2, interferon-gamma or IL-10 than peripheral blood derived T cell lines in PP patients.⁵ In addition, PP

patients present high levels of IL-6 in the vitreous compared with normal controls.⁶

However, the trigger antigen for the immunologic process present in the PP remains unknown, but some attempts have been made to elucidate this molecule. A 36 kDa protein (p-36) was detected, by western blot, in elevated concentrations in the blood of 81% of the PP patients.⁷ This corresponds to the carboxy-terminal region of a nucleopore complex protein denominated nup36.⁸ However, its putative role in the pathogenesis of PP is unknown. Another protein involved in PP is heat shock protein (HSP). High circulating levels of antibodies to inducible HSP70⁹ and HSP60 of *Yersinia enterocolitica*¹⁰ have been found in the sera from patients with PP. The role of HSP in the PP is not clear, but a mechanism involving molecular mimicry has been suggested.^{9,10} The retinal proteins S-antigen and interphotoreceptor retinoid binding protein give cellular immune response with PP patients¹¹, but this immune response is not specific to PP since patients with other ocular diseases produce cross reactions with these proteins.

The use of new technologies, such as microarrays, differential display, SAGE, etc, to identify genes associated with diseases of unknown etiology has provided new clues to understand the mechanisms involved in the diseases. Recently, phage display methods have allowed the discovery of proteins or peptides related to inflammatory processes. Phage display consists of the production of a library of peptides fused to the pIII protein of the capsid of M13 filamentous phage.¹² These peptides can be recognized by antibodies or proteins capable of binding specifically to them.

The search for new molecules associated with the pathology of PP is important since it will permit better understanding of the mechanisms present in PP. Phage display could be a tool to find new molecules associated to the PP pathology. This work was aimed to identify some peptides recognized by serum antibodies or serum proteins of PP patients using the phage display method.

Materials and Methods

Patients and controls

Human studies were performed following the guidelines of the Research Committees of the Instituto Politécnico Nacional from Mexico City and were approved by the Institutional review board. Informed written consent was obtained from all patients before inclusion in the study.

Sera of 8 PP patients, with an active phase of the disease, were included. Patients with PP were considered to be in the active phase of the disease if they had moderate or severe vitritis (with pars plana snowbanks). Frequently, they had floaters or blurred vision and cystoid macular edema confirmed by biomicroscopy or fluorescein angiography, or they had progression of a posterior subcapsular cataract. The PP patients did not report systemic diseases as multiple sclerosis, Lyme disease or sarcoidosis. None of the patients were taking anti-inflammatory drugs. Healthy subjects with no previous or present ocular disease and no clinical evidence of a systemic pathology were defined as negative controls (n=8). Serum was collected from each subject and stored at -70°C until needed.

Phage display

Serum proteins in PBS (100 mg/mL) from a PP patient were bound to 4 mL maxisorp Nunc-immunotube (Rochester, NY) for 1h at room temperature. After blocking free sites with 2% BSA, 1x10¹⁰ plate forming units (PFU) from the J404 library¹² were added to the immunotube and incubated for an additional hour at room temperature. Tubes were washed ten times with PBS-Tween 0.1 %, followed by ten washes with PBS. Phages were eluted by incubation with 100 mM triethylamine for 10 minutes. After neutralizing with 0.5 mL of 1M tris, pH 7.4, phages were amplified by infection of a logarithmic culture of *Escherichia coli* K91 and titrated using the same *E. coli* strain.

Phages from the first panning were subjected to a second round of selection using serum proteins of a healthy subject. Unbound phages were recovered and amplified by infection of *E. coli* K91. After this subtraction step, a final round of selection using immobilized PP serum proteins was performed and bound phages were eluted, amplified and titrated getting a 2.4×10^3 PFU/mL titer.

Selection of phage clones

Phages from the last panning were used to infect *E. coli* K91 and 100 individual plaques, each corresponding to a peptide displaying M13 clone; were randomly picked and used to infect log phase cultures of *E. coli* K91. Kanamycin (35 µg/mL) was added 20 minutes after phage absorption and cultures were grown overnight at 32°C. Phages from each culture supernatant were concentrated by addition of 30 % Polyethylene glycol, 2.5 M sodium chloride and pelleted in a microcentrifuge. Phages were resuspended in PBS and used as ligands for proteins from healthy and PP sera in ELISA.

ELISA

Direct capture ELISA was used to measure the response of PP and healthy serum to different peptides displayed on phage surface. Polystyrene plates were coated with patient and healthy sera diluted 1:1000 in carbonate (pH= 9.6) solution. Each serum was assayed by triplicate. After overnight incubation at 4°C, plates were washed and blocked with 3% milk in PBS and later 100 µL of phages (2×10^{11} PFU/mL) were added to each well and incubated for 4 hours at 4°C. After washing, plates were incubated with 100 µL of a 1:2500 dilution of peroxidase-coupled goat IgG anti-M13 (Copper Biomedical Inc., West Chester, PA, USA) for 1 hour. Excess conjugate was eliminated with three washes, followed by addition of H₂O₂ and o-phenylene-diamine as peroxidase substrates. Absorbance was measured at 492 nm.

Statistics

Statistical analysis was performed using the Mann-Whitney U test to compare groups. The odds ratio was used to determine the relationship between levels of response to the peptide and the disease. We considered A_{492nm} greater than 1.25 (A_{492nm} mean + 1 SD of the healthy subjects) as a positive response to peptide, and values smaller than 1.25 as negative.

Isolation of phage DNA

Selected plates were used to infect *E. coli* K91. After overnight incubation, replicative form (RF)-DNA of phage was obtained with the Qiagen plasmid minikit (Hilden, Germany). The integrity of RF-DNA was verified by agarose gel electrophoresis.

PCR amplifications

PCR reactions were performed using 0.2 U Taq DNA polymerase (Invitrogen, Carlsbad CA, USA) with 1 µL RF-DNA, 1X buffer, 1 mM MgCl₂, 200 µM of each dNTPs and 0.2 µM of primers (forward: 5' ATT CAC CTC GAA AGC AAG CT 3' and reverse: 5' CAT TCC ACA GAC AAC CCTCA 3'). Optimal PCR conditions were an initial denaturation step of 5 minutes at 94°C followed by 30 cycles of 30 s at 92°C, 30 s at 55°C and 30 s at 72°C. PCR products were analyzed on agarose gels, purified with the high pure PCR product purification kit (Roche Diagnostics, Indianapolis) and sequenced using the Big Dye terminator fluorescence based sequencing method. The sequence of nucleotides was translated into amino acid sequence to establish the correspondent peptide and it was also blasted into the GenBank to find the proteins that contain this peptide.

Results

Phage display

The immune recognition level of the 100 phage display selected clones with the 8 PP and 8 healthy sera were assayed. Clones 26, 84, 86 and 92 showed in-

FIGURE 1. Response levels of PP and Healthy sera to phage display clones. Graphic shows the media A_{492nm} values of 8 PP and 8 healthy subjects. * $P < 0.05$.

consistency in their recognition and only the clones 66, 83, 85, 91, 71 and 10 retained their ability to be recognized by PP sera differentially (Figure 1). From these clones only the clone 83 was recognized stronger in PP patients sera than in healthy sera (PP vs. healthy; $P < 0.05$), we chose this clone for further analyses.

Amplification and sequencing of the peptide encoding region from clone 83

The RF-DNA was obtained from clone 83 (Figure 2; panel A), and the coding region was amplified yielding a PCR product of approximately 300 bp (Figure 2; panel B). The amino acid sequence found for the peptide was HSEAETGPP. This peptide is present at residues 27-35 of human proline-rich transmembrane protein 2 (GenBank Accession number EAW79991) with 100% of homology and with minor percentage of homology (66%) in others proteins such

as: Na⁺/myo-inositol cotransporter, pre-B-cell leukemia homeobox interacting protein 1 and laeverin.

Association of the response of HSEAETGPP peptide with pars planitis

The odds ratio value was 15 (CI_{95%} = 1.03– 218.3, $p < 0.05$) indicating an association between the immune response to the peptide and PP.

Discussion

In this work we found a new peptide associated with PP using the phage display method. We found that a phage clone displaying the peptide HSEAETGPP exhibited the highest recognition levels to PP sera in comparison with healthy sera. A search in the GenBank for human proteins containing this sequence yielded the human proline-rich transmembrane protein 2 with a 100% homology to HSEAETGPP. Some other proteins were also identified but with a minor

FIGURE 2. *Amplification of peptide encoding fragment.* (A) RF-DNA (8651 Kb) of clone 83 (line 2). DNA weight marker II of Roche Applied Science (line 1). (B) amplification by PCR to generate a 300 pb product (line 1) that containing peptidic sequence. DNA weight marker IX of Roche Applied Science (line 2).

homology grade. None of them have been documented to be involved in PP.

The role of human proline-rich transmembrane protein 2 has not been established. This protein has been found in human cDNA libraries¹³⁻¹⁶, and from this library the gene *prrt2*, that encodes this protein, has been identified. The *prrt2* gene contains two exons and it can be translated in five isoforms (A, B, C, D and E isoforms). The peptide isolated in this work is only found in the A, B and C isoforms. The amino acid sequence of the proline-rich transmembrane protein 2 (in the position 265-314) has a homologous region to CD225 protein. CD225 protein is an interferon-inducible transmembrane protein and is associated with interferon-induced cell growth suppression. However, it is not known if the proline-rich transmembrane protein 2 can be induced by interferon, or what tissues express this protein. Its function in the eye is also unknown as is its participation in eye diseases. The Na⁺/myo-inositol cotransporter protein (with 66.6% homology to HSEAETGPP) is expressed in the lens¹⁷ and an increase of its expression is favoured in hypertonic stress.¹⁸ It has been suggested

that it is involved in cataract formation.¹⁹ Other proteins that contain this peptide in a minor homology have not been associated with eye diseases, but we cannot ignore that these proteins could be associated with the PP.

Some studies using phage display techniques to address eye pathologies have been carried out.²⁰⁻²² In one, a cDNA library generated from human eye mRNA was displayed on the surface of bacteriophage T7. Two phages, that contained tribbles homolog 2 (a protein that controlling mitogen-activated protein kinase cascades) and an unknown protein, showed 170- and 42-fold increases in their binding specificities to the patients, respectively.²³ This demonstrates that autoantigens can be identified by phage display using uveitis patient serum. Although phage display has already been employed in the eye and particularly in uveitis^{23, 24}, there are few studies aimed to identify peptides associated with PP.

In summary, using a library of nonapeptides displayed on the surface of M13 bacteriophage, we found that the peptide HSEAETGPP is associated with PP. This peptide is present in human proline-rich transmembrane protein 2 and in others proteins. These have not been linked with PP. More studies are necessary to establish the role of the peptide in PP.

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