Identification of Antigenic Site Within hsc 70 by Serum Autoantibody in Patients with Cancer-associated Retinopathy

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ABSTRACT

In our previous studies, both recoverin and heat shock cognate protein 70 (hsc 70) were found as autoantigens recognized by sera from four patients with cancer-associated retinopathy (CAR). In the present study on the molecular mechanism of antibody generation in CAR, we identified the antigenic site within hsc 70 by the patients' sera using deletion mutants of hsc 73. We expressed a series of deletion mutants of hsc 73 proteins and subjected then to western blot analysis. In western blot analysis, CAR patient's serum reacted with wild type hsp 70, but not with the C-terminal truncated mutants. This data demonstrated that the antigenic site was located within the C-terminus region of hsc 73 as identified by CAR patient's serum.

Key words : Autoantigen, Autoimmunity, Cancer-associated retinopathy, Heat shock protein

INTRODUCTION

Cancer-associated retinopathy (CAR) is an ocular manifestation of a paraneoplastic syndrome, characterized by sudden and progressive visual loss, ring scotoma, photopsia and impairment of dark adaptation[1]. Among the underlying primary cancers, small cell lung carcinoma has been reported most frequently[2-15]. In most cases, CAR is diagnosed before an underlying

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primary cancer is diagnosed. Although the molecular pathophysiology of CAR is not yet fully understood, the findings on serum autoantibodies against retinal antigens suggest that autoimmune responses contribute to retinal degeneration[1,7-15]. In the previous studies, a high-titer of antibodies against a 23-kDa photoreceptor-specific calcium-binding regulatory protein called recoverin was detected in CAR patients[16-18]. Functionally, recoverin is considered to play a major role in adaptation to dark and light processes by regulating rhodopsin phosphorylation in a calcium-dependent manner[19]. Recently, recoverin was identified as existing in the cancer cells of CAR patients[20,21]. Thus autoimmune reactions may be triggered by the host response to recoverin aberrantly expressed in tumor cells, and may mediate retinal degeneration. In addition, it was also reported that other retinal antigens including a 65-kDa protein, a 48-kDa protein[1-9], a 50-kDa protein[10], enolase (a 46-kDa protein)[22], and neurofilaments (58-62-kDa, 145-kDa and 205-kDa proteins)[8] were recognized by CAR patients' sera, either by themselves or along with recoverin. Among these retinal antigens, recoverin alone or a combination of recoverin and 65-kDa protein have most frequently been detected in western blot analysis in previous studies. Most recently, our group identified the 65 kDa protein as heat shock cognate protein 70 (hsc 70)[23]. These observations suggest that both anti-recoverin and anti-hsc70 antibodies are involved in the pathogenesis of CAR.

In this study, for further investigation of the molecular pathogenesis of CAR, especially the molecular mechanism of antibody generation toward hsc 70, we produced a series of deletion mutants of hsc 73 and performed epitope mapping by western blot analysis.

MATERIALS AND METHODS

The studies were performed in accordance with our institution's guidelines and the Declaration of Helsinki on Biomedical Research Involving Human Subjects and the protocols were approved by the institution's Committee for the Protection of Human Subjects.

Patients and serum

A 70 year-old woman (small cell carcinoma in lung) with cancer-associated retinopathy was studied[23]. Serum was separated from peripheral venous blood sample immediately after collection and the sample was stored at -80℃ until use. Anti-bovine recoverin rabbit serum was prepared as previously described[23].

Preparation of the series of hsc70 proteins

The series of deletion mutants of hsc 73 was produced as previously described[24]. Briefly, i.e., pET△S, pET△E, pET△HL, pET△HS, pETSBDce, pETSBDpe and pETHSC70 vector was double digested and inserted into pET21 vector (Novagen, Madison, WI USA).

The constructs were transformed into E. coli BL21(DE3). Bacterial transformants were grown at 37 ℃. Recombinant deletion proteins were induced with 1 mM of isopropyl β-D-thiogalactopyranoside (IPTG) at 37 ℃ for 2 hrs. The IPTG-induced bacterial cell extract were used for western blot analysis.

Western blot

Western blot analysis was carried out as described previously[23]. Briefly, wild type hsc 73 and deletion mutants of hsc 73 were loaded on SDS-PAGE using a 12.5 % polyacrylamide gel, respectively. The proteins on the gel were electrotransferred to polyvinylidene difluoride (PVDF) membranes in 10 mM bistrispropane buffer, pH 8.4 and 10% methanol solution. After blocking nonspecific binding by 5 % skimmed milk in phosphate buffered saline (PBS), the membranes were probed successively with diluted serum and horse radish peroxidase (HRP)-labeled anti-human IgG (Funakoshi Co. Tokyo, Japan). Specific antigen/antibody binding was visualized by the ECL system (Amercharm).

RESULTS

In order to identify the antigenic site within hsc 70, deletion mutants of hsc 73 (Fig.1) were prepared and analyzed by western blot with sera of a

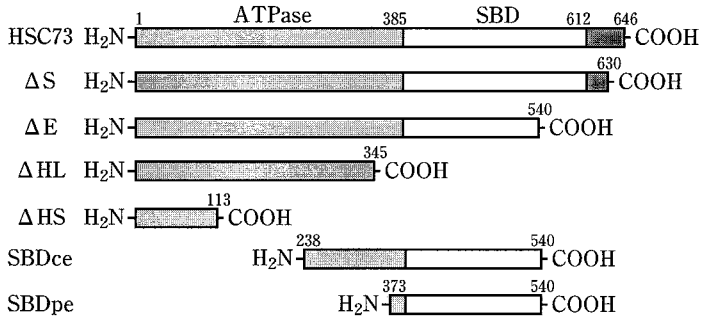

Fig. 1 The deletion constructs of hsc 73

These constructs encoding deleted recombinant hsc 73 proteins were produced by double digestions at certain endonuclease sites of human hsc 73 gene and insertion into pET21 expression vector. The deleted hsc 73 proteins and their retained amino acids were indicated.

CAR patient. As shown in Fig.2, western blot analysis revealed that the sera (1:500 dilution) of the patient with CAR reacted with wild type hsc 73, but not with C-terminal truncated mutants.

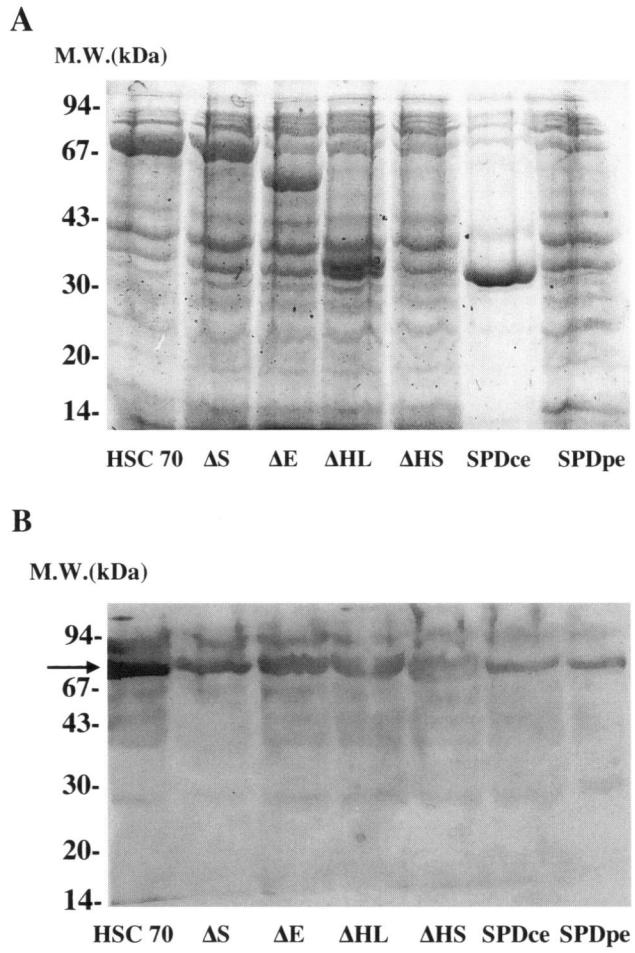

Fig. 2 A. SDS-PAGE and Western blot of a wild type hsc70 and deletion mutants of hsc73

Prepared wild type hsc70 and deletion mutants of hsc73 (75ng per each, see materials and methods) were mixed with the sample buffer (10 μl) and loaded on an SDS-PAGE gel, respectively and stained with Coommassie-blue (A) and immunostained by sera from CAR patient (B). The protocol for immunoblotting is described in Materials and Methods. Only a wild type hsc 70 was probed by the patient's serum (indicated by arrow).

DISCUSSION

So far, more than forty cases of CAR have been reported, and in thirty of these, western blot analysis of the serum has been reported. Both the 23-kDa (recoverin) and the 65-kDa protein were frequently reported[7,11,12,16,20], but other proteins including the 145-kDa/205-kDa[7], 48-kDa[1,9], 50-kDa[10], 62-kDa[14], 34-kDa[15] and 46-kDa[22] proteins have also been recognized as immunoreactive bands on the western blots, but less frequently. Many authors agree that recoverin is a target antigen for the autoimmune response in CAR for the following reasons: 1) recoverin is a retina specific protein, 2) immunoreactivity toward recoverin is unique in CAR patients, and 3) recoverin is expressed in the cancer cells and the cell lines derived from the cancer cells of CAR patients[20,21]. On the other hand, no attention has been paid to the 65-kDa protein, since some authors have reported that such immunoreactivity was detected not only in CAR patients' sera but also in some cancer patients without CAR and in normal subjects at relatively low serum dilutions (100-200 times)[16,20]. Recently, our group reported that both recoverin and the 65-kDa protein were found as autoantigens in all of four patients with CAR, and the 65-kDa protein was identified as hsc 70[23].

Heat shock protein 70 (hsp 70) family proteins are synthesized in response to a variety of cellular stresses, and are also present in normal unstressed cells (hsc 70). Functionally, they play important roles as chaperons: 1) to assist in translocation into organelles, folding and rearrangement of proteins, 2) dissolution of protein aggregates, and 3) protein degradation[25-29]. Interestingly, elevated levels of hsps in peripheral blood mononuclear cells[32] or serum autoantibodies against hsps have been identified in patients with several autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and viral diseases[31-33]. Thus, autoimmune response to hsc 70 in CAR may play a similar role in the pathophysiology of CAR to that in other autoimmune diseases. Taken together, we considered that autoimmune responses toward recoverin might be essentially required for photoreceptor degeneration, while responses toward hsc 70 might weaken hsp-mediated biological defense, which could assist anti-recoverin-mediated retinal degeneration. In fact, among the chaperon proteins mentioned above, the hsp 70 family is known to be one of the most important for protection against stress-induced denaturation.

In the present study, we prepared the series of deletion mutants of hsc 73, performed western blot analysis, and found that the antigenic site is located within the C-terminal region of the hsc 73. It was found that CAR

patients' sera immunoreacted with not only with 65-kDa retinal protein (hsc 70) but also a 65-kDa protein (hsc 70) from tumor specimen. In addition, recent studies suggested that hsp 70 proteins were located not only in cytosol, but also in membranes. If these were true refers specially to hsc 70 proteins location in membranes, we can reasonably speculate that an hsc 70-like molecule expressed on tumor cell surface may trigger autoimmune response. But, it still remains to be clarified as to whether the C-terminus of hsc 70 is expressed on the surface of tumor cells, and what roles hsc 70 antibodies plays in the pathogenesis of CAR. Therefore, further study will be required.

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