行政院國家科學委員會專題研究計畫成果報告

Calreticulin 抗原在自體免疫疾病之研究
The study of calreticulin antigen in autoimmune diseases

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主持人:蔡嘉哲　執行機構及單位名稱:中山醫學院醫學研究所

一.中文摘要

為了瞭解肉狀瘤病人血清所認識的核斑點之自體抗原,我們利用老鼠的肝臟藉由使用 60-100%的硫酸氨沉澱,DE-52 和苯基膠層析法等方法分離並鑑定其特性。

我們的結果顯示:純化的核抗原以 SDS-PAGE 來分析,顯示出 2 條條紋在分子量是 70 和 58 KDa 之處。西方墨點法中,肉狀瘤病人血清只認識58KDa的蛋白質,在 SDS 膠上的 58KDa 染色的條紋被轉移到 PVDF 作為分析氨基酸序列,前面 19 個氨基酸序列來自於 NH-2 端之 59KDa 蛋白質被發現於 100%同種的老鼠 calreticulin。這個發現提出了 calreticulin 為核斑點的新蛋白質,是肉狀瘤病的自體抗原。少數核斑點的蛋白質現在已被知道的包括 Sp100,PML,NDP52,NDP55。Calreticulin 可能扮演一個肉狀瘤病自體免疫反應的角色

關鍵詞:核斑點,肉狀瘤病,自體免疫抗原,Calreticulin

Abstract

To investigate the autoantigen associated with nuclear dots recognized by serum from a patient with sarcoidosis. Rat liver homogenate was used to study the autoantigens by using the combination of 60-100% ammonium sulfate precipitation, DE-52 and phenyl-agarose chromatography. Serum from a patient with sarcoidasis giving nuclear dots staining pattern in immunofluorescence was used for the study. The purified nuclear antigens analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed two bands of molecular weight of 70 and 58kDa. Immunoblotting with the serum from sarcoidosis patient only recognized the 58kDa protein. The stained band of the 58kDa on the SDS-slab gel was transfered to PVDF for the analysis of the amino acid sequence. The first 19 residues of amino acid sequence from NH-2 terminus of the 59kDa protein was found to be 100% homologous to rat calreticulin. These finding suggest that calreticulin, a novel protein of the nuclear dots, is an autoimmune antigen in sarcoidosis. In mammalian cells, several proteins are currently known to colocalize in nuclear dots including Sp100, PML, NDP52, NDP55 and Calreticulin. Calreticulin might play a role in the autoimmune response in sarcoidosis.

Key words: Nuclear dot, Sarcoidosis , autoimmune antigen, Calreticulin

Introduction

The nuclear dots are nuclear organelles whish function are unknown. There are several proteins are known to colocalize in discrete nuclear dots [1-3]. Anti-nuclear dots antibodies have been relevant as a

tool of diagnosis of primary biliary cirrhosis (PBC) [4-5].

Anti-nuclear dots antibody by indirect immunofluoresence (IIF) is characterized by staining of 3 to 20 dots of variable size(about 0.3 to 1um), distributed all over the nucleus, but sparing the nucleoli [6-7]. It produces a characteristic pattern in IIF, which is different from anticentromere antibody. The first component of the nuclear dots, designated Sp100, was characterized using sera from patients with PBC [8]. Later it suggest that Sp100 protein may function as a transcriptional transactivator[9]. The second component of the nuclear dots, designated PML, was identified by several investigators studying the t(15;17) translocation associated with acute promyelocytic leukemia (APL) [10-12]. PML was found to be a tumor suppresser protein involved in development of APL and co-localize with Sp100 in the nuclear dots [13,14]. Recently, it was found that a third protein, designated NDP52, colocalize with Sp100 and PML in nuclear dots [15]. The predicted amino acid sequence of NDP52 contained coiled-coil, lucine zipper, and zinc-finger motif. All three nuclear dots-associated proteins, Sp100, PML,and NDP52, seem to have a coiled-coil domain which may be important for their cellular localization and functions.

Sarcoidosis is a chronic inflammatory disorder that is characterized by the pronounced infiltration of T cells and mononuclear phagocytes into affected organs, such as the lung, lymph nodes and central nervous system. These infiltrations result in the formation of granulomas. The etilology of sarcoidosis is unknown. The possibility that sarcoidosis represents a hypersensitivity response to a single agent or multiple inciting agents has been considered (D1-D2). Abnormalities of immune function as well as autoantibody production, including rheumatoid factors and antinuclear antibodies (ANA). Although ANA can be features of sarcoidosis (D3-D5), it rarely present as nuclear dots patterns stain in IIF. In this study, we used a serum from patient with sarcoidosis giving nuclear dots staining pattern in IIF to investigate the autoantigen recognized by the serum. We shown that the autoantigen recognized by this antinuclear dots antibody is 100 % homologous to rat calreticalin. Calreti-culin colocalize with Sp100, PML, and NDP52 in nuclear dots.

Patients and Methods

Patient

A serum from patient with sarcoidosis giving nuclear dots staining pattern in immunofluoresence (IF) was used for this study. Sarcoidosis was defined as a compatible clinical presentation and the presence of noncaseating granulomatosis on histological examination of patho-logical tissue. Other cases of noncaseating granuloma were excluded, and tissue stains and cultures for mycobacterial and fungal organization were negative.

Purification of the autoantigen

The 60~100% ammonium sulfate fraction of rat nuclear extracts was chromato-graphyed by passing through a DE-52 column (Whatman,10ml size) which was pre-equilibrated with Buffer A(10mM Tris-HCl, pH8.0). After extensively washing with Buffer A and Buffer B(Buffer A containing 0.1M NaCl), the autoantigen was eluted with Buffer C (Buffer A Containing 0.2 M NaCl). Fractions were collected and the solid ammonium sulfate was added to a final concentration of 40%. The resulting sample was chromatographed on a

phenyl-agarose column (Sigma, 1ml size) which was pre-equilibrated with Buffer D(Buffer A containing 40% ammonium sulfate). After extensively washing with Buffer D, the autoantigen was then eluted with Buffer A containing 30% ammonium sulfate. The autoantigen mentioned above was analyzed by immunoblotting using the serum from sarcoidosis patient.

Protein Sequence

The NH2-terminal sequences of the phenyl-agarose purified autoantigen was determined by Edamn degradation in an Applied Biosystem Model 477A amino acid sequencer with version 1.61 software. BLAST from WWW was used for homology search. A 10% SDS-PAGE was used to examine the purity of the protein, and the protein was transferred to PVDF membrane for amino acid sequencing.

Results

The serum from patients with sarcoidosis was used to stain Hep-2 cells in indirect immunofluoresence. Four to 12 nuclear dots of different size were stained within each nucleus leaving to nucleoli unstained The nuclear dots remained dispersed throughout the nucleus during mitosis, which helped distinguish the dots from centromeres.

Total Hela cells and rat liver extracts were examined for the antigenic polypeptides reacting with the serum in immuno-blotting. The serum recognized a 89-87kD doublets protein in the total Hela cells extracts. When rat liver extracts were used as antigen in immunoblotting, the serum reacted with a 60-58 kD doublets protein. Rat liver was chosen as the starting material for the purification of the antigens recognized by the serum giving nuclear dots staining pattern in IIF. The presence of antigens at the different purification steps was monitored by immunoblotting using the same serum. The purified antigen reveled a major band of 58kD and a minor band of 70 kD. The 58 KD band was cut and transferred to PVDF membrane for amino acid sequencing. The first 19 residues of amino acid sequence from NH2-terminus of the 58KDa protein, DPAIYFKEQFLD-GDAWTNR, was found to be identity with rat calreticulin.

Discussion

In this study, serum from a patient with sarcoidosis was used to demonstrate that calreticulin, a novel protein of the nuclear dots, is an autoimmune antigen in sarcoidosis. In human cells, at least four proteins are currently known to colocalize in nuclear dots: SP 100, PML, NDP52, and SP140. These antigens are not well-characterized in its stucture and function. Calreticulin (CR) is the only autoantigen in nuclear dots that has extensively been studied for its role in cell biology. CR is a highly-conserved, calcium-binding protein that was originally identified in the endoplasmic reticulum/ sarcoplasmic reticulum where it is felt to play a role as a calcium storage depot and a molecular chaperone.

Anticalreticulin autoantibodies have been reported in the sera of patients with systemic lupus erythematosus (SLE) and patients with onchocerciasis. Here we report anticalreticulin antibodies are also present in the serum of patient with sarcoidosis. Numerous immunological aberrations can be detected in patients with sarcoidosis. Immunologic abnor-malities associated with sarcoidosis and autoimmune disease suggest a possible common etiopathogenesis. An antigen-driven immune response has been implicated in the pathogensis of

autoimmune disease. Calreticulin is colocalized with SP100, PML, NDP52, and SP140 within nuclear dots. Observations of colocalized antigens in subcellular organelles or subcellular particles are frequent. These autoantigens driving autoimmune responses are likely to be aggregates of molecules forming subcellular particles. The immune response could be induced by several components of such particles. Calreticulin might play a role in the autoimmune response in sarcoidosis.

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