行政院國家科學委員會專題研究計畫　成果報告

利用噬菌體展示技術對肺癌病人特異性抗體以及其相對的 B 細胞抗原決定位的研究

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利用噬菌體展示技術對肺癌病人特異性抗體以及其相對的 B 細胞抗原決定位的研究（I）

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Study of antibodies and their corresponding B cell epitopes specific for patients with lung cancer using phage display technology (I)

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□國際合作研究計畫國外研究報告書一份

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中文摘要

表現在絲狀噬菌體表面的技術已經證明非常適合來辨識特異性的血清中結合抗體的抗原或是尋找抗原特異性的抗體。噬菌體表現系統(phage display system)一個重要的應用為癌症病人體內的免疫反應的研究，因為科學家已經證實對於腫瘤病人的體液性以及細胞性的反應皆存在。事實上，許多可能的腫瘤特異性抗原以及[illegible]，例如 MAGE 以及 NY-ESO-1 已經使用這個技術而被發現到。而且，能夠引發病毒中和性抗體或是刺激潛在的自體免疫性的特異性[illegible]特殊部位（motif）也都陸續被發現。因此，我們利用這種先進技術的優勢來分析肺癌病人的體液性免疫反應。我們先前發現，大約 15％的肺癌病人的胸腔滲出液或是血清能夠辨識一種存在肺癌病灶組織抽取物，而不存在於同一個病人的正常肺組織中的特殊蛋白。我們目前並不清楚這些特異性抗體以及抗原蛋白的重要性，不過，利用純化的疾病特異性抗體來找出在這些病灶組織中的相對抗原或是從隨意[illegible]噬菌體基因庫（random peptide phage libraries）中找出相對的抗原性的特殊部分將是非常重要的。

在這個研究中，我們利用噬菌體抗體表現基因庫來製造以及定性這些腫瘤特異性的抗體，以及調查是否這些存在肺癌病人的抗體能夠被用來富化（enrich）免疫性的細胞蛋白以及用來辨識表現在絲狀噬菌體表面的抗原性特殊部位（噬菌體[illegible]表現基因庫）。這些被找到的腫瘤特異性蛋白以及/或是[illegible]將來在發展對抗癌症的診斷以及治療藥物上會有非常大的幫助。

Abstract

Display on the surface of filamentous phages has been shown to be well suitable for the identification of specific serum antibody-binding antigen. One important application of phage display would be the study of immune response in cancer patients, since both humoral and cellular

responses towards tumor patients have been recognized. Indeed, many potential tumor-specific antigens and peptides such as MAGE and NY-ESO-1 have been discovered using this technique. Moreover, specific peptide motifs capable of eliciting viral neutralizing antibodies or inducing potential autoimmunity were also identified as well. Accordingly, we have taken the advantage of this novel technology to analyze the humoral immune response in patients with lung cancer malignancy. We have previously demonstrated that the pleural influx or sera from approximately 15% of lung cancer patients is able to recognize a novel protein present in the extracts of the lesion tissue of lung cancer, but not in that of the normal lung tissue of the same patient. The significance of these antibodies is presently not known; however, it would be crucial to identify the corresponding antigen in the lesion tissue or antigenic motifs from random peptide phage libraries using the purified disease-specific antibodies.

In the present study, we generated and characterized these cancer-specific antibodies using phage antibody display libraries and investigated whether these antibodies present in lung cancer patients can be used to enrich for immunological cellular proteins and to identify the antigenic motifs expressed on the surface of filamentous phage (phage peptide display libraries). These identified tumor-specific proteins and/or peptides will be extremely helpful in the development of diagnostic and therapeutic agents against cancers in the future.

Introduction

Many novel concepts for the treatment of human cancer try to use the patient's immune system, either by a nonspecific stimulus or by a specific stimulation or vaccination with antigens that are specifically expressed by tumor cells. A prerequisite for the successful application of such tumor vaccines and other immunological interventions for the treatment of cancer is the recognition by the immune system of tumor-specific or tumor-associated antigens, i.e. of molecules that are either specifically expressed or overexpressed in tumor cells when compared with normal cells. In contrast to animal models, the existence of such antigens was not unequivocally demonstrated in human neoplasms until 1991, when Thierry Boon's group described the antigen MAGE-1 in malignant melanoma. Based on the recent advances in our understanding of the molecular mechanisms involved in antigen recognition, extensive efforts have been made to identify tumor-specific antigens in humans by the analysis of the T/B-cell repertoire against tumors.

By implementing molecular cloning techniques into the original strategy of autologous typing, the SEREX approach was

developed; SEREX is an acronym that stands for the serological analysis of autologous tumor antigens by recombinant cDNA expression cloning. It allows an unbiased search for an antibody response and the direct molecular definition of immunological tumor proteins based on their reactivity with autologous patient sera. In the SEREX approach, a cDNA library is constructed from fresh tumor specimens and cloned into 财 phage expression vector; the resulting recombinant phages are used to transfect E. coli. Recombinant proteins which are expressed during lytic infection of the bacteria are transferred onto nitrocellulose paper, which are then incubated with diluted sera from the autologous patients. Clones reactive with high titer IgG antibodies are identified using an enzyme-conjugated secondary antibody specific for human IgG and the nucleotide sequence of the inserted cDNA is then determined. So far, more than 100 different antigens have been identified. These antigens can be divided into three groups; (1) known antigens such as the melanoma antigens MAGE-1 and tyrosinase (2); transcripts that are either identical or highly homologous to known genes that have not been known to elicit immune responses and a large group of previously unknown genes such as HOM-RCC-3.1.1 and HOM-HD-21. Before further consideration as an antigen in a cancer vaccine, the biological significance of each antigen needs to be determined by studying its tissue specificity, the occurrence of natural antibody responses in many cancer patients and by its ability to induce T cell responses in vitro.

The development and application of peptide libraries have become a powerful tool for identifying antigenic epitopes recognized by antibodies and other proteins (2-11). Libraries that have been used so far can be divided into three categories that differ in a way in which these libraries were constructed and/or presented. All types of libraries involve specific screening and affinity selection of peptides that mimic ligands for particular proteins. Libraries that utilize filamentous bacteriophages, such as M13 or fd, are generated by chemically synthesizing a random degenerative oligonucleotide with flanking regions that contain sites for restriction enzymes which allow ligation into cloning vectors. The individual peptides are encoded by millions of random oligonucleotides that are expressed at the N-terminus of the minor protein (pIII) at the tip of the phage or over the entire surface of the phage (10, 11) fused with the major coat protein (pVIII) (12, 13). In both cases, the random peptides are exposed and are therefore available for selection protocols. The selection of antibody motifs from phage

libraries will depend on the genetic diversity of the libraries. Such diversity can be analyzed by examining the distribution of bases at each position within the codons via sequencing of random phage clones. To assess more rigorously the diversity of phage libraries, a modified colony hybridization technique has also been developed. To select phages that bind to particular proteins such as monoclonal antibodies, a biopanning procedure is applied (18). In this technique, the phage library is mixed with a biotin-conjugated ligand. The bound phages can be eluted unspecifically by treatment with low pH or specifically by a competitor (6, 19, 20). Following screening, the peptide sequences displayed by the selected phages can easily be determined by sequencing the coat protein-encoding region of the phages such as the pVIII gene. Peptides can be displayed as linear or cyclic constraints.

Phage libraries have been used to study antibody–antigen and protein–protein interactions as well as to develop novel antibacterial and biologically active peptides (4-6, 20, 21). However, a more general use of the phage libraries would first be the identification of common features of antibodies present in different individuals infected by the same or related pathogens and second the mapping of polyclonal autoantibodies directed against important functional proteins (e.g., cytokines, snRNPs, DNA, and IgG). In the first system, the rationale is that antibodies from patient sera might bind to a phage containing the epitope responsible for initiating the disease. This approach has been used in order to identify putative antigens that are responsible for the initiation and/or perpetuation of the immune response in patients with autoimmune diseases such as rheumatoid arthritis (RA) (22). The humoral response of RA patients toward the selected peptide was found to be higher compared to that of controls (22). A data base search with one overselected peptide (pep1, ADGGAQGTA) identified a significant homology with both the cereal glycine-rich cell wall proteins (GRP) and Epstein–Barr virus nuclear antigen-1 (EBNA-1). Antibody activities against synthetic peptides from the EBNA-1 protein have been found to be elevated in certain autoimmune diseases including RA (23). A significant amino acid homology was found between the GRP 1.8 protein, the EBNA-1 protein, and cytokeratins (24). Antibodies against a synthetic peptide from the GRP 1.8 protein were also found to be significantly increased in RA patients compared to controls.

Taken together, the above examples demonstrate that by using only sera from patients, it is possible to select antibody motifs. Such motifs can be used as probes to identify (auto) antigenthat may play a role in the etiology of rheumatoid arthritis. However, the peptide motifs selected could represent conformational epitopes, since our strategy identifies both continuous and

conformation-dependent epitopes (22, 23). Alternatively, screening of peptide phage libraries with sera from patients who have recovered from diseases may identify peptides for protective antibodies. As mentioned above, patient serum contains millions of different antibody specificities. To increase the possibility of selecting relevant phages, screening of phages with sera from many patients and controls in ELISA experiments is required. To overcome this time-consuming requirement, a rapid immunological screening method has been developed (14). This method allows the direct identification of binding phages following a transfer to nitrocellulose membrane filters. In the past, a similar method has been used in the screening of expression cDNA libraries (22, 23). To improve the screening procedure, in addition to the subtraction step introduced in the biopanning experiments (22), investigators can subtracte two enriched libraries from the screening of sera from RA patients and, in parallel with sera from normal individuals, by colony hybridization in which the probe association is independent of the nature of the sequence.

Materials and methods

Construction of phage peptide-display libraries

To construct the pIII-fused cDNA, PolyA$^+$ RNA was purified from total RNA obtained from lung cancer cell lines or tissues and reverse transcribed using Not I (dT) primer. After reverse transcription, double-stranded cDNA was prepared using standard protocols. The cDNA preparation was precipitated, dissolved in water and then adaptor was added. Following this step, the cDNA was digested with Not I, size purified and then ligated separately into the pComb3 phagemid vectors. The system is based on covalent linkage of cDNA-encoded products to the C terminus of the M13 minor coat protein III. Ligated DNA was precipitated, dissolved in water and subsequently electroporated into *E. coli* XL-1 blue. The size of each library was estimated to be approximately 10^6 to 10^7 independent clones to be satisfactory for subsequent screening. Some gene products, especially high molecular mass proteins, may not be displayed on the surface of the phage. To alleviate this problem, we also constructed a cDNA library using Not I-(N)10-tagged random primer during cDNA synthesis. After the addition of the adaptor, the cDNA was digested with Not I, size-purified, ligated into the pComb3 phagemid vector and then processed as above. All libraries were amplified in *E. coli* XL-1 as described previously. Alternatively, the peptide display libraries can be purchased from

commercials (Pharmacia).

Affinity selection of phage-displayed cDNA by patient IgG

Approximately 10^{10} phagemid particles derived from each amplified library were pooled. The mixture was incubated with 20贡g/ml pooled patient IgG, preincubated with the M13 phage, and then incubated overnight at 4°C. After incubation, biotin-conjugated anti-human IgG, pre-incubated with the M13 phage, was added to the mixture and incubation continued for 3 h with rotation. Streptavidin-coated magnetic beads, preincubated with the M13 phage, was added to the mixture and incubation was continued for 30 min at room temperature. Antibody/phage-binding beads was attracted to a magnet and unbound phages was removed. Beads were washed 10 times with PBS/0.5 % Tween over a period of 1 h and streptavidin-binding phages were acid eluted and neutralized with Tris-base as described elsewhere. *E. coli* XL-1 cells were infected with the eluted phages and then plated on 2x TY agar plates containing 100 g ampicillin/ml, 1 % glucose and incubated overnight at 30°C. Bacterial cells were collected in 10 ml 2x TY medium by scraping. Of these bacteria, 500 L was added to 100 ml 2x TY medium containing 100 g/ml ampicillin and 1 % glucose until the culture reached an OD600 of 0.6. Of this culture 10 ml was infected with M13-KO7 helper phage (Amersham Pharmacia Biotech) and then incubated at 37°C for 30 min without shaking. Following infection, bacteria was spun down at 3000 rpm for 5 min, resuspended in 400 ml 2x TY medium containing 100 贡g/ml ampicillin, 25 贡g/ml kanamycin and 1 mM IPTG and grown overnight at 37°C. Following centrifugation at 8000 rpm for 20 min, supernatant was collected and phage particles was purified by two PEG 8000 precipitations, resuspended in PBS and filtered through a 0.45 μm filter. The final concentration of the phage preparation was estimated to be 10^{12} –10^{13} plaque forming unit (pfu)/ml. Additional rounds was performed as above. The enrichment of specific clones was monitored by immunoscreening experiments as described below.

Immunoscreening

E. coli XL-1 blue cells was infected with unselected and selected phages and grown with shaking at 37°C until the culture reached an OD600 of 0.6. Of this culture 10 ml was infected with the helper phage. Various dilutions of the infected bacteria was plated on 2x TY agar plates containing 100 g/ml ampicillin, 25 贡g/ml

kanamycin and 1 mM IPTG and grown overnight at 37°C. The colonies was transferred to nitrocellulose and then screened using the immunoscreening technique. The membranes was washed twice with the TNT buffer (10 mM Tris-HCl, 150 mM NaCl, 0.05 %Tween-20) for 15 min and then blocked for 1 h with 20 % FCS in TNT buffer. After blocking, membranes were incubated with 25 贡g/ml pooled IgG in FCS/TNT for 2 h and then washed over 1 h. HRP-conjugated anti-human IgG (1/5000) was added to the membranes and incubated for 1h. Following washing, the immunoreactive phage clones were detected by the ECL system and exposed to the hyperfilm (Amersham, Pharmacia Biotech).

Phage preparation

Positive clones were grown and bacterial cells will be pelleted by centrifugation at 8000 rpm for 10 min. Phage particles in the supernatant was precipitated with PEG 8000/NaCl, resuspended in PBS, filtered through 0.45-mm filter and then stored at 4°C. For DNA preparation, the phages were phenol extracted and processed as described.

Enzyme-linked immnosorbent assay (ELISA)

Serum antibodies reacting with phage displayed cDNA products were detected by ELISA. Briefly, 96-well microtiter plates were coated overnight with the phage particles (10^9 pfu) in 100 L PBS. After blocking with 0.5 % BSA in PBS for 2 h at room temperature and subsequent washing, the plates were incubated with serum samples (1/100 dilution in PBS/0.2 % Tween 20) for 1 h at 37°C. After additional washing, plates were incubated with anti-human IgG alkaline phosphatase conjugate (1/5000) for 1 h at 37°C. The immune complexes were detected by adding p-nitrophenyl phosphate as substrate and reading at 405 nm.

Results and Discussion

We have purified the total immunoglobulins using protein A resin from the pleural effusions of several individuals diagnosed as cancer carriers. Of which, 3 representative samples, 427, 502-1 and 510, were analyzed for their purity using SDS-PAGE stained with Coomassie blue dye. All samples showed a major band with 150 Kd molecular weight under non-reducing condition whereas 2 bands with 50 and 25 Kd under reducing condition. The results indicated that the total immunoglobulins were highly purified and may be suitable for the biopanning process. However, we are aware of the interference originated from the majority of other samples. The screening of more than

100 clones has identified 2 major peptide sequences which may be the potential antigenic epitopes specific for B cells existing in the patients with lung cancer. More importantly, the phage containing these specific peptides showed the binding activity to the sera of several patients. A search to the published sequences of both nucleotide and amino acid in the gene bank did not show homology to any known gene or proteins, indicating that these 2 peptides may be derived from novel proteins specific for lung cancer. However, this result could not be verified without the cloning and characterization of the original full-length gene.

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