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REVIEW

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Identification of tumour antigens by serological analysis of cDNA expression cloning

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Abstract The identification of antigens that distinguish normal cells from cancer cells is an important challenge in the field of tumour immunology and immunotherapy. The immunoscreening of cDNA expression libraries constructed from human tumour tissues with antibodies in sera from cancer patents (SEREX: serological identification of antigens by recombinant expression cloning) provides a powerful approach to identify immunogenic tumour antigens. To date, over 2,000 tumour antigens have been identified from a variety of malignancies using SEREX. These antigens can be classified into several categories, of which the cancer/testis (CT) antigens appear to be the most attractive candidates for vaccine development. The SEREX-defined tumour antigens facilitate the identification of epitopes (antigenic peptides) recognised by antigen-specific cytotoxic T lymphocytes (CTLs) and provide a basis for peptide vaccine and gene therapy in a wide variety of human cancers. Moreover, some of these antigens seem to play a functional role in the pathogenesis of cancer.

Keywords Tumour antigen · cDNA cloning · Serological analysis · Antigen identification

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Introduction

The development of antigen-specific cancer vaccines requires the identification of appropriate target antigens, the establishment of effective immunisation strategies, and the ability to circumvent immune escape mechanisms. Therefore the identification and molecular characterisation of tumour antigens that can elicit specific immune response in the tumour-bearing host is one of the major prerequisites for tumour immunotherapy. The genetic approach to identify human tumour antigen has been developed by Boon and his colleagues [1]. It makes use of antigen-loss tumour cell variants or other appropriate target cells. These are transfected with recombinant DNA or cDNA libraries prepared from tumour cell lines. The transfected cells are then tested for their recognition by autologous tumour-specific CTLs. Once the gene is identified, the region encoding the antigenic peptide can be narrowed down by transfecting gene fragments. The deduced amino acid sequence can then be used to produce synthetic oligopeptides which are then tested in a target cell sensitisation assay for recognition by the original tumour-specific CTL clone [2, 3]. The methodology for defining T cell–recognised tumour antigens has depended on stable T cell lines and established tumour cell lines, conditions that are frequently difficult to meet and virtually impossible in the case of certain tumour types. Another strategy uses biochemical approaches to elute antigenic peptides bound to major histocompatibility complex class I molecules of tumour cells which can be recognised by T cells. The peptides are separated by reverse-phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry. The peptide fractions are then tested for their ability to stimulate CTLs when pulsed onto MHC-matched antigen-presenting cells (APCs). After several steps of further fractionation, the sequences of individual peptides recognized by the CTLs are obtained [4, 5, 6, 7].

Antibody-defined tumour antigens

The search for antibodies that can distinguish cancer cells from normal cells is very important in the field of cancer research. Immunisation of experimental animals with human tumour cells and removal of antibodies reactive with normal tissue antigens by serial absorption techniques represents the first approach for serological recognition of tumour antigens. Using this approach, two very useful human tumour antigens were identified: alpha fetoprotein (AFP), a serum marker for hepatic cell carcinoma and germ cell tumour [8], and carcinoembryonic antigen (CEA), a useful marker for colon and other epithelial cancers [9]. The emergence of hybridoma technology opened the floodgates for the identification of new antigens in mice and humans [10]. Some antigens identified by monoclonal antibodies turned out to be quite specific tumour markers [11], such as CA19-9 for human pancreatic cancer [12], CA-125 for ovarian cancer [13] and MGAgs for gastric cancer [14]. These antibody-defined antigens have a role in the diagnosis and follow-up of certain types of human cancers. However, the recognition of tumour-associated molecules by the murine immune system did not enable any conclusions to be drawn concerning the immunogenicity of the tumour antigens in humans.

SEREX approach

It is commonly accepted that immune recognition of tumour antigen is a concerted action between the cellular and humoral immune systems. It would be surprising if cancer antigens induced only a cellular response and no antibodies. Furthermore, the development of high-titre IgG requires CD4 T-cell help. Thus, circulating tumour-associated antibodies may reflect a significant host-tumour interaction and may identify such gene products to which at least cognate T-cell help, but also specific cytotoxic T cells should exist. Based on these rationales, a novel strategy using the antibody repertoire of cancer patients for the molecular definition of antigens was developed by Pfreundschuh and his colleagues Sahin and Türeci [15]. They call their approach SEREX, for "serological identification of antigens by recombinant expression cloning." It allows a systematic and unbiased search for antibody responses and the direct molecular definition of immunogenic tumour proteins based on their reactivity with autologous patient serum. In the SEREX approach, a cDNA expression library is constructed from a fresh tumour specimen, and cloned into λ phage expression vectors. The resulting recombinant phages are then used to transfect *E. coli*. Recombinant proteins expressed during the lytic infection of the bacteria are transferred onto nitrocellulose membranes, which are then incubated with diluted (1:100–1:1,000) extensively preabsorbed serum from the autologous patient. Clones reactive with high-titre antibodies are identified using an enzyme-conjugated secondary antibody specific for human IgG. Positive clones are subcloned to monoclonality, and the nucleotide sequence of the cDNA insert is determined. cDNA sequence analysis via database searching is performed to define whether the cDNA inserts are derived from novel genes or cancer-associated known genes, and the domains and/or motifs for putative function and cellular localisation of the antigens can also be identified.

The SEREX approach is characterized by the following features: the use of fresh tumour specimens restricts the analysis to genes that are expressed by the tumour cells in vivo and circumvents in vitro artefacts associated with short- and long-term tumour cell culture; the use of patient's serum containing polyclonal antibodies as a probe for immunoscreening allows for the identification of multiple antigens with one screening course; the screening is restricted to clones against which the patient's immune system has raised high-titre IgG antibody responses indicating the presence of a concomitant T-helper lymphocyte response in vivo; as both the expressed antigenic protein and the coding cDNA are present in the same plaque of the phage immunoscreening assay, identified antigens can be sequenced immediately. Sequence information of excised cDNA inserts can be directly used to determine the expression spectrum of identified transcripts by Northern blot and reverse transcription polymerase chain reaction (RT-PCR); broad-scale serological surveys of antibodies in normal individuals and patients with cancer to the SEREX-defined antigens can be conducted to evaluate the incidence of humoral immune responses to the respective antigens [16, 17].

Human tumour antigens identified by SEREX

The SEREX approach allows for the identification of an entire profile of antigens using the antibody repertoire of cancer patients and provides a comprehensive view of the immune recognition of human cancer. What is so encouraging about SEREX is that it provides a way to analyse the humoral immune response to intracellular cancer antigens. During the past few years, SEREX has been applied to a wide range of tumour types, including melanoma, renal cancer, astrocytoma, Hodgkin's disease [15], oesophageal cancer [18], gastric cancer [19], colon cancer [20, 21], lung cancer [22, 23], breast cancer [24, 25], prostate cancer [26] and leukaemia [27, 28]. More than 2,000 antigens have been listed in the SEREX database [29]. Besides their role as targets for cancer vaccination, identified antigens may be useful as new molecular markers of malignant disease. The value of each of these markers or a combination of them for diagnostic or prognostic evaluation of cancer patients has to be determined by studies correlating markers with clinical data. Furthermore, the immune system is a sensitive biodetector, which may detect structural and

regulative alterations and may therefore point to gene products with significance for neoplastic transformation or tumour progression [30, 31]. Table 1 lists the categories of human tumour antigens that have been identified by SEREX.

A fascinating category of tumour antigens has been referred to as *cancer/testis* (CT) antigens. These proteins are selectively expressed in a variety of neoplasms (in a lineage-independent manner), but not in normal tissues, except for spermatogonia in the normal testis [32]. As most CT antigens are expressed in various types of cancer, they are ''shared'' tumour antigens. CT antigens are immunogenic and elicit cellular and humoral immune responses in tumour patients. Since their initial identification by T-cell epitope cloning, the list of CT antigens has greatly expanded through serological expression cloning (SEREX) and differential mRNA expression analysis, and approximately 20 CT antigens or antigen families have been identified to date. Characteristics commonly shared by CT antigens, aside from the highly tissue-restricted expression profile, include existence as multigene families, frequent mapping to chromosome X, heterogeneous protein expression in cancer, likely correlation with tumour progression, induction of expression by CpG hypomethylation and/or histone acetylation, and immunogenicity in cancer patients. Spontaneous humoral and cell-mediated immune responses have been demonstrated against several CT antigens, including NY-ESO-1, MAGE-A, SSX and T21 antigens. Since CT antigens are immunogenic and highly restricted to tumours, their discovery has led directly to the development of antigen-specific cancer vaccines [33, 34].

Differentiation antigens are expressed in tumours in a lineage-associated pattern, but also in normal cells of the same origin; examples are tyrosinase and glial fibrillary acidic protein (GFAP), which are antigenic in malignant melanoma and glioma, but are also expressed in melanocytes or brain cells, respectively [15, 35].

Amplified/overexpressed gene products have also been identified by the SEREX approach, demonstrating that antigen overexpression can lead to immunogenicity as it does in the case of HER-2/*neu* [36]. Examples are HOM-RCC-3.1.3, a new carbonic anhydrase which is overexpressed in a fraction of renal cell cancers [37], and MTA1, a metastasis-associated antigen which is highly expressed in cancer tissues compared with matched normal tissues (unpublished data). The overexpression of a transcript may also result from gene amplification as demonstrated for the translation initiation factor eIF-4g in a squamous cell lung cancer [38].

Antigens encoded by mutated genes have been isolated, showing the potential of SEREX to identify aetiologically relevant gene products in cancer. The prime example of a mutational antigen is p53 isolated from a case of colon cancer [39]. For SEREX-cloned antigens, proof of an underlying mutation is technically challenging since antibody responses induced by a mutation may be directed to the wild-type backbone of the molecule and thus detect the wild-type allele. Therefore, sequencing of several independent clones from the same library, as well as exclusion of polymorphisms is required.

Splice variants of known genes were also found to be immunogenic in cancer patients. Examples are the Hodgkin's disease–associated splice variant of restin [15] and gastric cancer–associated splice variants of TACC1 [40].

Cancer-related autoantigens are expressed ubiquitously and at a similar level in healthy and malignant tissues. The encoding genes are not altered in tumour samples; however, they elicit antibody responses in cancer patients and not in healthy individuals. This may result from tumour-associated posttranslational modifications or changes in the antigen processing and/or presentation in tumour cells [41].

We have constructed and analysed cDNA expression libraries by SEREX derived from three different prostate cancers, one gastric cancer, one colon cancer and one breast cancer. In order to identify more CT antigens, we also purchased a cDNA expression library constructed from normal human testes and immunoscreened it with pooled sera from patients with prostate cancer. After screening at least half a million clones per library, multiple reactive clones were identified in each library. According to the sequence analysis and expression patterns in normal and malignant tissues, the gene products of these reactive clones can be classified into different groups. MTA1, a metastasis-associated antigen, was isolated from a prostate cancer cDNA library. MTA1 is highly expressed in tumour tissues and is closely related with invasion and metastasis of cancer [42]. A novel CT

Table 1 Categories of SEREX-identified human tumour antigens

Antigen category	Example	Tumour source
Cancer-testis antigens	NY-ESO-1	Oesophageal carcinoma
	MAGE-1	Melanoma
Differentiation antigens	Tyrosinase	Melanoma
Amplified/overexpressedGene products	Carbonic anhydrase	Renal cancer
	MTA1	Prostate cancer[a]
	eIF-4γ	Lung cancer
Mutated gene products	p53	Colon cancer
Splice variants	Restin	Hodgkin's disease
	TACC1	Gastric cancer
Cancer-related autoantigens	CEBPgamma	Melanoma

[a]Unpublished data

antigen, T21, was identified from a cDNA library constructed from human testes screened with pooled sera from prostate cancer patients. T21 is overexpressed in malignant tissues and normal testes, and not in other normal tissues (unpublished data). Granulin, a growth factor, and Tbdn-1, an orthologue of the mouse acetyltransferase which is associated with blood vessel development were isolated from gastric cancer [19]. Two novel cancer-associated splice variants of transforming acidic coiled-coil (TACC) protein, TACC1-D and TACC1-F, were identified in gastric cancer [40].

Evaluation of humoral responses to the SEREX-identified tumour antigens using allogeneic sera from cancer patients is important for cancer diagnostics, disease monitoring and immunotherapeutic interventions. We have developed an array technique which can be used to rapidly analyse humoral response of multiple SEREX antigens using allogeneic sera. One microlitre of monoclonal positive phages (200–300 pfu/μl) is spotted side by side with nonrecombinant phages directly onto the agar plate premixed with host cells. Following an overnight incubation, ''phage array'' is transfered onto nitrocellulose membrane and screened with preabsorbed and diluted allogeneic sera [19]. We observed that the humoral immune response against some of our SEREX-identified antigens in patients with cancer was higher than that in healthy controls.

Technical challenges and modifications to the standard SEREX approach

The concept behind SEREX is straightforward; however, there were a number of technical challenges that needed to be resolved. Some modifications to the original method have been implemented. The antibodies in human sera that react with bacterial or phage components can be eliminated by preabsorption of the sera with *E. coli*–phage lysate. This is essential because such contaminating antibodies can completely obscure the detection of antibodies specifically reacting with tumour antigens. The presence of B lymphocytes and plasma cells in tumour tissues gives rise to cDNA clones encoding human IgG, which are detected in SEREX. In order to eliminate these false positive clones, filters are prescreened with enzyme-conjugated anti-human IgG prior to incubation with the patient's serum, and reactive clones are detected with the appropriate enzymatic colour reaction buffer and excluded from further study.

In the conventional SEREX approach, the antigens are expressed in bacterial- and phage-based systems, which are generally not capable of naturally folding antigenic proteins and modifying them posttranslationally. The conformation and posttranslational modifications play an important role in the proper function of proteins and also affect their immunogenicity. To overcome this limitation of conventional SEREX, eukaryotic expression systems for SEREX should be developed.

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