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Exploitation of the B cell repertoire for the identification of human tumor antigens

Abstract The screening of tumor-derived expression libraries for antigens that are detected by high-titer immunoglobulin G antibodies from the sera of patients with cancer using serological identification of antigens by recombinant expression cloning (SEREX) allows for the systematic search for antigens of human cancers. SEREX has led to the identification of a plentitude of new tumor antigens in many different tumor entities. These antigens, many of which are encoded by hitherto unknown genes, can be grouped into different classes. Serologically defined human tumor antigens facilitate the identification of antigenic peptides recognized by tumor-specific T lymphocytes, thus providing a molecular basis for polyvalent peptide-based and gene therapeutic vaccine strategies in a wide variety of human neoplasms. Finally, many of the antigens identified using SEREX seem to play a functional role in the pathogenesis of malignant disease.

Key words Tumor antigens · Serology · Immunotherapy

Introduction

A prerequisite for successful immunotherapeutic strategies is the identification of antigenic structures of human tumors which are recognized by the host immune system. By defining the antigenic targets on cultured melanoma cells which are recognized by autologous cytotoxic T lymphocytes (CTLs) a growing number of tumor antigens, including differentiation antigens, mutated antigens, and testicular antigens aberrantly expressed in tumors, have been identified [2, 4, 8, 24, 25]. The difficulties in defining human tumor antigens by T cell responses, due to the tedious establishment and limited availability of precharacterized tumor cell-restricted CTL clones for neoplasms other than malignant melanoma, encouraged us to develop an alternative strategy. Based on the assumption of an integrated immune response that includes the coordinated recruitment of $CD4^+$, $CD8^+$, and B cell responses to a given antigen, we designed a novel procedure to exploit the B cell repertoire using serum from cancer patients for the molecular definition of tumor antigens and subsequent analysis for T cell-recognized epitopes. Serological identification of antigens by recombinant expression cloning (SEREX) was established as a screening method for tumor-derived expression libraries with autologous serum [13]. Applying this method to a number of different tumors, we isolated several new tumor antigens, among them members of the so-called cancer/testis class.

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Materials and methods

Sera and tissues

Sera and tumor tissues were obtained during routine diagnostic or therapeutic procedures. Normal tissues were collected from autopsies of tumor-free patients.

Construction of cDNA expression libraries

$poly(A)^+$ RNA 5–8 μg was prepared using an mRNA isolation kit (Stratagene, Heidelberg, Germany) from total RNA isolated from fresh tumor biopsies [6]. cDNA expression libraries were directionally cloned into a λ-ZAP Express vector using a commercially available adaptor ligation system (Stratagene) according to the manufacturer's instructions. After packaging into phages the

cDNA expression libraries were transfected into XLI MRF bacteria for one round of amplification. In other experiments, a testis transcript-enriched expression library was constructed using a modification of the recently described suppression subtractive hybridization (SSH) technique [7]. In brief, a pool of testis-specific cDNA fragments was amplified by the SSH technique. These cDNA fragments were used to capture their long counterparts from a cDNA phagemid library. Finally, cDNA inserts excised from captured phagemids were cloned into λ phage vectors and used for expression screening. For the construction of testis-specific cDNA, mRNA 2 μg derived from two different testicular tissue specimens were used as a tester probe. The driver cDNA probe was synthesized from mRNA derived from 10 healthy tissues (colon, stomach, brain, resting and activated peripheral blood mononuclear cells, skeletal muscle, liver, kidney, lung, and skin). SSH polymerase chain reaction (PCR) was performed as described [22] after hybridization of tester and driver cDNA. The construction of the phagemid library was performed as described previously [13] using mRNA 5 μg derived from the same testis samples. The cDNA was cloned into λ-ZAP Express phages, resulting in a library with 4.0×10^6 primary clones.

Single-stranded pBK-CMV phagemid DNA was extracted after in vivo mass excision of the phage expression library using standard protocols. The excised single-stranded phagemid cDNA library was hybridized on nitrocellulose membranes (Schleicher & Schüll, Heidelberg, Germany) blotted with the testis cDNA derived from the SSH PCR. After hybridization the nitrocellulose membranes were washed and phagemids bound to immobilized cDNA were eluted. Double-stranded cDNA inserts were synthesized using thermostable polymerase from *Pyrococcus furiosus* (Stratagene) and (Reverse, Universe) flanking vector-specific primers. The double-stranded cDNA inserts were excised by restriction enzyme digestion from the phagemids and were religated into a precut dephosphorylated λ-ZAP Express vector. The ligation product was packaged into λ phage particles and used for transfection and library amplification.

Immunoscreening of transfectants

XLI MRF′ bacteria transfected with recombinant λ-ZAP Express phages were plated onto Luria-Bertani agar plates. Expression of recombinant proteins in lytic phage plaques on the bacterial lawn was induced with isopropyl β-d-thiogalactoside. Plates were incubated at 37 °C until plaques were visible and then blotted onto nitrocellulose membranes. The membranes were blocked with 5% low-fat milk in Tris-buffered saline and incubated with a 1:500 dilution of the patient serum which had been preabsorbed with transfected *Escherichia coli*. Serum antibodies binding to recombinant proteins expressed in lytic plaques were detected by incubation with alkaline phosphatase-conjugated goat anti-human immunoglobulin G (IgG) and visualized by staining with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. For screening of testis transcript-specific libraries, allogeneic sera of patients with different tumor types were used. Positive clones were monoclonalized by plaque elution.

Screening for allogeneic antibodies

Monoclonalized phages from positive clones were mixed with nonreactive phages of the cDNA library as internal negative controls at a ratio of 1:10 and used to transfect bacteria. IgG antibodies in the 1:200 diluted *E. coli*-preabsorbed sera from allogeneic patients and healthy controls were tested with the immunoscreening assay described above to assess tumor-associated antibody responses.

Sequence analysis of identified antigens

Positive clones were subcloned to monoclonality and subjected to in vivo excision of pBK-CMV phagemids. The nucleotide sequence of cDNA inserts was determined using an Excel cycle sequencing kit (Epicentre, Madison, WI, USA) on a LICOR (Lincoln, NE, USA) automatic sequencer. Sequencing was performed according to the manufacturer's instructions starting with the vector-specific primers. Insert-specific primers were designed as the sequencing proceeded. Sequence alignments were performed with DNASIS (Pharmacia Biotech, Uppsala, Sweden) and BLAST software (www.mcbi.mlm.nih.gov/BLAST) on the EMBL, Genbank, and PROSITE databases.

Northern blot analysis

Northern blotting was performed with RNA extracted from tumors and normal tissues. The integrity of RNA was checked by electrophoresis in formalin/MOPS gels. Gels containing RNA 10 μg per lane were blotted onto nylon membranes. After prehybridization the membranes were incubated with the specific ^{32}P-labeled insert-specific full-length cDNA probes overnight at 42 °C in hybridization solution (50% formamide, $6 \times$ SSC buffer, $5 \times$ Denhardt solution, 0.2% sodium dodecyl sulfate [SDS]). The membranes were then washed at progressively higher stringency, with the final wash in $1 \times$ SSC and 0.2% SDS at 65 °C. Autoradiography was conducted at −70 °C for up to 7 days using Kodak X-OMAT-AR film (Kodak, Stuttgart, Germany) and intensifying screen. Northern blots were used to assess semiquantitatively the expression levels of specific transcripts in a panel of normal tissues and tumors of various origins.

Reverse-transcription PCR

Total cellular RNA was extracted and primed with a mixture of dT(18) oligonucleotide and random hexamer primers and reverse-transcribed with Superscript RT (Life Technologies, Gaithersburg, MD, USA). cDNA thus obtained was tested for integrity by amplification of β-actin transcripts in a 30-cycle PCR reaction. For each clone positive in SEREX screening, specific primers located in different exons were designed and a panel of normal tissues was screened for expression.

Southern blot analysis

Southern blotting was performed according to standard protocols with EcoR1- or HaeIII-digested DNA extracted from tissue specimens. Equal loading of samples was confirmed by staining with ethidium bromide and visualization of DNA under ultraviolet light. Hybridization with [^{32}P]dCTP-radiolabeled insert-specific probes was carried out in buffer containing $6 \times$ SSC buffer, $4 \times$ Denhardt solution, and 0.5% SDS. Washing and autoradiography were performed as described for Northern blot analysis.

Results

Summary of SEREX screening

Expression libraries were constructed and analyzed using SEREX from a variety of different neoplasms including three different renal cell carcinomas of the clear cell type, two melanomas, one ovarian carcinoma, one hepatocarcinoma, three astrocytomas, one colorectal cancer, one pancreatic cancer, one breast cancer, two Hodgkin lymphomas, and two acute T cell leukemias. Established primary libraries from tumors consisted of at least 1×10^6 independent clones. By screening the respective libraries, multiple reactive clones were found in each, of which some appeared several times because they were

abundantly expressed. Testis transcript-enriched libraries as surrogate libraries were screened with tumor patient sera to bias for antigens of the cancer/testis class which show coexpression in testis and in tumors in a pantumoral rather than tumor type-specific fashion.

Molecular characterization of identified antigens

Because of the plentitude of positive clones, a three-step procedure was used to narrow the number of clones to be selected for further investigation. In the first step, sequence data were compared with databases to reveal identity or homologies with known genes and proteins and to identify domains or motifs informative for putative function or cellular localization. In the second, expression in normal tissues and tumors was determined by reverse-transcription (RT)-PCR and by analysis in a database containing expressed sequence tags (electronic Northern), and quantified by Northern blot hybridization with specific probes. Finally, each clone was tested with sera from healthy controls and allogeneic tumor patients to evaluate the incidence of serum antibodies to the respective antigen. Sequences were checked for similarities in sequence databases (Genbank, EMBL) using the BLAST software program.

Using this alignment information, identified antigens were subdivided into four groups. The first group consists of tumor antigens originally defined by their T cell reactivity, such as the melanoma antigens MAGE-1 and MAGE-4a, and tyrosinase. These examples demonstrate that at least some of the serologically identified antigens are also targets for CTLs. The second group of antigens represents known classical autoantigens for which immunogenicity is associated with autoimmune diseases, such as anti-mitochondrial antibodies or antibodies to U1-snRNP. The incidence of such autoantibodies is $<1\%$ when sera from tumor patients without obvious autoimmune disorders are used for SEREX analysis. The third group of tumor antigens detected by SEREX consists of transcripts that are either identical or highly homologous to known genes, but have previously not been known to be antigenic, i.e., to elicit immune responses in humans. Examples of the latter group are specifically expressed molecules such as restin, which had originally been described by a murine monoclonal antibody specific for Hodgkin and Reed-Sternberg cells [1], and lactate dehydrogenase, a molecule expressed by many benign and malignant tissues and often elevated in the sera of patients with malignant disease. The fourth and by number the largest group (30–40%) of serologically defined antigens, however, represents products of previously unknown genes.

Expression patterns of human tumor antigens

The expression pattern for each clone was assessed by RT-PCR with transcript-specific oligonucleotides chosen to hybridize with two different exons. Northern blot hybridization with labeled cDNA inserts as probes was performed to determine the quantitative level of mRNA abundance. Different expression patterns of human tumor antigens were observed. The first pattern was selective expression in tumors. An intriguing observation was that the respective antigens were not expressed in normal tissues except for testis, but were expressed in malignant tissues of a wide spectrum of histogenetic origin, rather than in a tumor type-specific fashion. In a melanoma library we detected HOM-MEL-40/SSX-2 [23] and showed that it is a member of a family of highly homologous genes that are selectively expressed in tumors [9, 15, 18, 23]. Screening libraries enriched for testis-specific transcripts with tumor patient sera rather than the autologous tumor/serum combination biased for the detection of this type of antigen. Among the newly defined antigens is HOM-TES-14/SCP-1, which represents the synaptonemal complex protein 1 (SCP-1) [22]. SCP-1 was originally described to be specific for the prophase of meiosis I [10, 12].

A second expression pattern was shown by differentiation antigens. Differentiation antigens display lineage-specific expression in tumors, but also in normal cells of the same origin at a given stage of differentiation. Tyrosinase is an example of such a differentiation antigen. Tyrosinase was originally described as a target for CTLs. It is expressed in melanocytes and melanoma cells. Other examples of differentiation antigens detected by SEREX are HOM-HD-21/galectin-9 [19], which is expressed in dendritic cells and Hodgkin and Reed-Sternberg cells, and HOM-GLIO-2.4/glial fibrillary protein, which we found in an astrocytoma library using SEREX.

A third expression pattern of antigens detected by SEREX are overexpressed antigens, which are expressed in tumors at much higher levels than in their normal counterpart tissue. An example is HOM-RCC-3.1.3/CAXII. It is encoded by the gene for human carbonic anhydrase XII. CAXII is expressed ubiquitously and overexpressed in 10% of renal cell carcinomas of the clear cell type compared to the normal kidney parenchyma in the same patient [20].

A fourth group of antigens is ubiquitously expressed and shows no obvious difference in expression level between tumors and normal tissue.

Antibody responses against SEREX antigens

Antibodies against cancer/testis antigens [5, 11] and some differentiation antigens, but also against some autoantigens, the expression of which is not restricted to tumors, were exclusively found in the sera from cancer patients, e.g., antibodies against HOMHD-21/galectin-9, HOM-MEL-40/SSX-2, of HOM-TES-14/SCP-1. On the other hand, antibody responses against other differentiation antigens, such as restin, and the majority of autoantigens were found in cancer patients and in

Table 1 Specificity of tumor antigens detected by SEREX

Specificity	Example	First detected in
Shared tumor/cancer testis antigen	HOM-MEL-40/SSX-2	Melanoma
Differentiation antigens	HOM-MEL-55 (tyrosinase)	Melanoma
Mutated gene product	NY-COL-2 (p53)	Colorectal carcinoma
Splice-variant product	NY-COL-38	Colorectal carcinoma
Viral antigens	HOM-RCC-1.14 (HERV-K10)	Renal cell cancer
Overexpressed antigens	HOM-RCC-3.1 (CA XIII)	Renal cell cancer
Underexpressed antigens	HOM-HCC-8.1	Hepatocellular carcinoma
Amplified gene product	HOM-NSCLC-11 (eIF-4γ)	Lung cancer
Cancer-related autoantigens	HOM-MEL-2.4 (CEBP)	Melanoma
Cancer-independent autoantigens	NY-ESO-1 (Ul-snRNP)	Esophageal carcinoma

healthy controls at similar rates. Finally, against some antigens, antibody responses were found only in the serum of a patient which had been used for SEREX analysis.

Discussion

At least four different antigens in each tumor analyzed could be detected by SEREX. This demonstrates that many if not all human tumors express multiple antigens that elicit an immune response in the autologous host. Meanwhile SEREX has been extended in a joint effort with several groups from all over the world to define the whole spectrum of antigens expressed by human tumors, the so-called human tumor immunome. To date, >1000 different antigens have been identified, but only some of them have been characterized beyond a preliminary status. The cumulative information on these antigens has been gathered in an International SEREX Data Bank which is accessible to the public via the Ludwig Institute of Cancer Research, Lausanne, Switzerland.

Different types of antigen specificity have emerged (Table 1). In addition to the cancer/testis antigens and differentiation antigens described above and the autoantigens with a cancer-restricted and commonly found antibody response, mutations have been found to be the mechanism most likely underlying the immunogenicity of the respective antigens. An example is p53. A viral antigen detected by SEREX is the human endogenous retrovirus K10 (HERV-K10), which was detected by autologous screening of a patient with a renal cell cancer and a seminoma. Overexpressed genes are known to elicit immune responses by overriding thresholds critical for the maintenance of tolerance. Many SEREX antigens are not strictly tumor specific, but are overexpressed in tumors, like HOM-RCC-3.1.3, a new carbonic anhydrase. Gene amplification was the underlying mechanism for overexpression of the translation initiation factor eIF-4g in a squamous cell lung cancer [3]. Splice variants as the likely immunogenic trigger have been shown for NY-COL-38 [16].

Cancer-related autoantigens are expressed ubiquitously and at a similar level in neoplastic and normal tissues (e.g., HOM-MEL-2.4 which represents the CCAAT enhancer binding protein), but elicit antibody responses only in cancer patients. This might result from tumor-associated posttranslational modifications as well as from changes in antigen processing and/or presentation in tumor cells [17]. Cancer-independent autoantigens, of which autoimmunity to and antigen expression of are not related to neoplastic disease, have also been identified, for example, HOM-RCC-10 which represents mitochondrial DNA and HOM-TES-11 which is identical to pericentriol material-1.

Finally, an autologous antibody response was detected by SEREX in a patient with hepatocellular carcinoma against an antigen which was underexpressed in the carcinoma compared to normal liver. This together with immune responses against nontumor-associated antigens in cancer patients demonstrates that antitumor immunity and autoimmunity are two faces of the same coin [14, 21] and that the context in which a molecule is presented (e.g., inflammation or "danger") is more decisive for its immunogenicity or the breakdown of autotolerance than its more or less restricted expression pattern.

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