Systematic search and molecular characterization of the antigenic targets of myeloma immunoglobulins: A monoclonal IgA from a female patient targeting sperm-specific cylicin II

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Abstract

The identification of the antigenic stimuli of B-cell neoplasms might be of considerable importance since a causal relationship between these neoplasms and antigenic stimulation has been suggested. To date the identification of such antigens has been erratic and accidental. For a systematic search and molecular characterization of human proteins that are antigenic target structures of myeloma-associated immunoglobulins, we applied SEREX (serological analysis of antigens by recombinant cDNA expression cloning) using a testis cDNA expression library and myeloma proteins from 42 patients. A monoclonal IgA from a female patient was shown to target sperm-specific cylicin II. The specificity of the reaction was confirmed by the characteristic staining of the equatorial belt of human sperm heads by the patient's myeloma protein. Serological analysis of recombinantly expressed cDNAs is a straightforward and high throughput approach for the molecular characterization of the targets of myeloma-associated immunoglobulins. The analysis of the antigenic spectrum of immunoglobulins associated with B-cell neoplasms will provide valuable information for the understanding of the pathogenesis of these diseases.

Introduction

A causal relationship between B-cell neoplasms and antigenic stimulation has been suggested (1,2), hence the identification of the antigenic stimuli of B-cell neoplasms might be of considerable importance. Since the recognition that human myeloma-associated paraproteins are structurally normal immunoglobulins with functional antibody activity, several target structures of the paraproteins associated with myeloma and Waldenström's macroglobulinemia have been reported. The antigenic targets of the respective paraproteins were discovered under three circumstances: (i) accidentally, due to clinical symptoms caused by the paraprotein, e.g. chronic cold agglutinin disease or cryoglobulinemia (3) or bleeding disorder (4); (ii) because of interference of the paraprotein with laboratory tests ordered for the clinical work-up of the patient, e.g. HIV-1 p24 antigen in an HIV-infected patient with myeloma (5); and (iii) screening for paraprotein activity against defined antigens (e.g. anti-streptolysin, anti-DNA, anti-IgG; 3,6). Systematic searches covering a broad spectrum of antigens have not been

reported to date.

With the availability of recombinant expression cloning techniques, it has become possible to systematically screen for putative antibody-antigen interactions, even if neither the antigen nor the antibody of putative immune responses are known (7). Using a cDNA library derived from normal human testis and human myeloma proteins as the source of antibody, we identified human cylicin II, a specific component of the cytoskeleton of sperm heads (8) as the target structure of an IgA-kappa myeloma protein from a female patient. This suggests that the sperm-driven immune response played a role in the pathogenesis of the patient's plasmacytoma and demonstrates the usefulness of this strategy for the systematic identification of plasmacytoma immunoglobulin targets.

Results

Detection and characterization of paraprotein-binding clones

We screened 1×10^5 clones of the testis cDNA expression library with sera diluted $1:1 \times 10^6$ from 42 myeloma patients with myeloma protein in the serum, 26 IgG (14 IgG-kappa, 12 IgG-lambda) and 16 IgA (8 IgA-kappa, 8 IgA-lambda). Of these, two clones, HOM-MM-1 and HOM-MM-2 reacted with serum from patient TW. Sequence analysis showed that the clones contained identical inserts with a high content of charged amino acids, an abundance of Lys-Lys-Asp tripeptides, and repetitive units of presumably alpha-helical configuration. A search in sequence databases confirmed the identity of HOM-MM-1 and HOM-MM-2 to be human cylicin II (EMBL Database, Accession No. Z46788), a basic protein specifically expressed in the sperm head cytoskeleton (8).

Expression spectrum of human cylicin II

The expression of human cylicin II in normal tissues and tumor specimens was analyzed by RT-PCR. As can be seen in Table 1, no cylicin II expression was detectable in any healthy adult tissue, except for testis. Of a panel of 113 human neoplasms tested, only 4 (3.5 %) expressed cylicin II (1/13 cancers of the kidney, 1/9 endometrial carcinomas, 1/19 lymphomas and 1/1 acute lymphoblastic leukemia of the T-cell type).

Table 1. Expression of cylicin II in normal human tissues and neoplasms.

Normal tissues	# positive / # tested	Neoplasms	# positive / # tested
Breast	0/3	Bladder carcinoma	0/1
Colon	0/2	Breast Cancer	0/14
Intestine	0/1	Colorectal carcinoma	0/7
Myocardium	0/3	**Endometrial carcinoma**	**1/9**

Liver	0/2	Gastric cancer	0/2
Lymph node	0/5	Glioma	0/9
Lung	0/3	Leiomyoma	0/1
Kidney	0/2	Lung cancer	0/11
Ovary	0/1	**Lymphoma**	**1/19**
Pancreas	0/1	Melanoma	0/5
PBL	0/1	Meningioma	0/4
PBL activated	0/1	Neuroblastoma	0/1
Skeletal muscle	0/1	Ovarian Cancer	0/14
Spleen	0/4	Pancreatic carcinoma	0/1
Stomach	0/1	Prostate cancer	0/1
Testis	**2/2**	**Renal carcinoma**	**1/13**
Thyroid	0/2	**T-ALL (spleen)**	**1/1**
Tonsil	0/5		

Prevalence of anti-cylicin II antibodies

The maximum dilution of the myeloma patient's serum to give a positive reaction in the phage assay was 1:1 x 10^8. To check for the prevalence of low titer anti-cylicin II antibodies, a panel of human sera was tested in the phage assay at a dilution 6 logs lower (1:100). None of the sera from 51 other myeloma patients, 20 infertile men

with known anti-sperm antibodies and 20 healthy controls had detectable anti-cylicin II antibodies.

Staining of human sperm cells with anti-cylicin II myeloma protein

Subcellular localization of cylicin II was studied by immunofluorescence microscopy of sperm cells after staining with the serum from myeloma patient TW. The serum, diluted 1:1000, stained human sperm heads in the form of an equatorial belt (Fig. 1), a staining pattern identical to that obtained with a murine anti-human cylicin II antibody. The isotype-matched sera of three other patients with IgA-lambda myeloma did not stain human sperm cells, even at a dilution of 1:10.
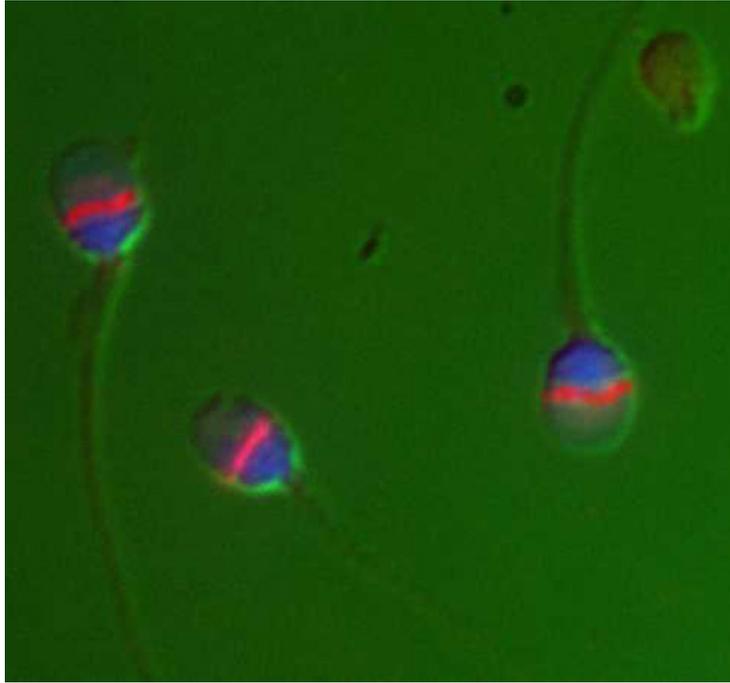

Figure 1. Localization of cylicin II in sperm cells. Calyx structures of human spermatozoa visualized by immunofluorescence microscopy using the serum of a multiple myeloma patient with a paraprotein specific for human cylicin II. The serum intensively decorates cylicin II, forming an equatorial belt around the sperm heads (red). The DNA dye DAPI was used to counterstain the nuclei.

Discussion

An expression library derived from human testis was chosen to investigate whether myeloma proteins target unmutated human protein antigens. Testis is a suitable source of tissue for an expression library that is to contain as many expressed genes as possible, because (similar to many malignant tumors) the chromatin of normal testis is characterized by hypomethylation (9). While one might have expected primarily autoantigens as targets for human myeloma proteins, the only protein expressed in the testis expression library and bound by a myeloma protein was cylicin II, a specific component of the sperm head cytoskeleton and as such hardly an autoantigen in a female patient.

The limited amount of serum available from the deceased patient made purification of the myeloma protein impossible. Nevertheless, several lines of evidence exclude the possibility that the observed reactivity with cylicin II was unspecific and/or not due to the paraprotein: (i) the reactivity was observed with dilutions of up to 1:1 x

10^8; this is about 3 logs stronger than any other reactivity that we and others have observed with a human serum using the described screening assay; (ii) crossreactivity with other antigens is unlikely because, of the 1×10^5 clones screened, one third can be expected to express a recombinant protein. This, together with the observation that a given insert is represented on average less than twice in the 1×10^5 clones, means that at least 15000 other proteins served as internal negative controls for cylicin II; (iii) the patient's serum was devoid of proteins reactive against common autoantigens, such as anti-nuclear antibodies, rheumatoid factors and others; and (iv) immunocytology with the patient's serum stained the equatorial belt of human sperm heads in the same way as (murine) monoclonal anti-cylicin II antibodies ([8]).

While we could confirm the expression of cylicin II in normal human tissue to be restricted to testis, we found aberrant expression of cylicin II in rare cases of malignant tumors. With its expression restricted to human testis (sperm) and malignant tumors, cylicin II fulfills the criteria of a cancer-testis antigen or CTA ([10]). Because the expression of cylicin II in malignant human tumors is very rare, stimulation of patient TW's immune system by a clinically undetected neoplasm is very unlikely, even though it cannot be definitely excluded. It is more likely that the malignant transformation in this female patient occurred in a B-cell clone that was stimulated by the presentation of an exogenous human antigen expressed by the spermatozoa of the patient's sexual partner.

There have been previous reports of myeloma paraproteins directed against a wide variety of infectious agents, including bacteria ([3],[6],[11],[12]) and the p24 antigen of the human immunodeficiency virus-1 ([5]). In another case, a patient developed a serum M-component specific for horse alpha2-macroglobulin 30 years after receiving passive serotherapy with horse antiserum to tetanus ([13]). These and other findings ([1],[2]) suggest the existence of a causal relationship between myeloma and antigenic stimulation. According to a multi-step hypothesis, the antigenic stimulation in a susceptible host is the first hit, giving rise to a benign monoclone. Stromal cells in the bone marrow, e.g. dendritic cells after infection with HHV-8, may provide additional growth-promoting and anti-apoptotic signals until a second hit, postulated to be a mutagenic or transforming event such as a switch translocation involving an oncogene, gives rise to myeloma from the expanded monoclonal B-cell population ([14]). As our patient had died before we started our study, we were unable to pursue such obvious additional investigations, e.g. the patient's T-cell reactivity towards cylicin II. With respect to the patient's medical and social history, our only sources are the patient's chart and an a posteriori interview with the patient's son. We found no clues as to conditions that might have boosted the immunogenicity of sperms in patient TW (e.g. chronic pelvic inflammation).

Previous reports on antibody targets of myeloma proteins have been extremely biased since they have been directed by suggestive clinical symptoms, focusing on predefined antigens or interference with laboratory tests performed for other reasons; it is therefore impossible to draw an objective picture as to the spectrum and frequency of the antigenic targets of myeloma proteins. Expression cloning, as performed in this study, is less biased especially if it covers a large spectrum of the expressed genome of a species as is the case for human testis cDNA expression libraries. It is intriguing that cylicin has an abundance of Lys-Lys-Asp tripeptides and repetitive units of presumably alpha-helical configuration, characteristics which might make cylicin II a T-cell independent antigen.

The approach pursued in this study allows for the high throughput screening of large numbers of myeloma proteins. Since only minute amounts of myeloma serum are needed for this type of antigen search, large serum banks of myeloma proteins can be rapidly screened and the molecular nature of the myeloma target can be determined practically instantaneously, because our approach allows for the immediate cloning and sequencing of the target antigen. The use of other human, animal or plant tissues as sources of cDNA expression libraries and, in particular, of eukaryotic expression systems that enable the screening of glycosylated antigens will give a more complete picture of auto-, allo-, and possibly xenoantigens that are the target of myeloma proteins and hence might have functioned as the original stimulus of a B-cell clone prone to malignant transformation. Moreover, this approach can be extended to other B-cell neoplasms by using recombinantly expressed immunoglobulins of B-cell lymphomas. Delineating the spectrum of the antigenic targets of these B-cell neoplasms will answer the question as to whether antigens stimulating the respective B-cell clones are random

or whether they share common features that contribute to their propensity to malignant transformation. With these molecular tools at hand, it should be possible to define more precisely the role that the target structures play in the process of the malignant transformation of a B-cell into a lymphoma or myeloma.

Acknowledgements

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Materials and methods

Materials

The study was approved by the local ethical review board ("Ethikkommission der Ärztekammer des Saarlandes"). Recombinant DNA work was performed with the official permission and according to the rules of the State Government of Saarland. Sera, semen and human tumors were obtained during routine diagnostic or therapeutic procedures after obtaining written informed consent. Sera were stored at -80°C until use. Normal tissues were collected from autopsies of tumor-free patients. The myeloma protein reacting with cylicin II was obtained from a 70-year-old who had never been seriously sick and mother of one son. The diagnosis of multiple myeloma of the IgA-lambda type had been made in September 98 and she had received 6 courses of chemotherapy with melphalan and prednisone with a good response. In September 99 she had a rapidly progressing relapse. Before salvage chemotherapy could be implemented, the patient developed sepsis and died of cardiorespiratory failure. Nearly 2.0 ml of serum obtained a few days before the patient's demise were retrieved for immune fixation and used for this study.

Construction of the cDNA expression library and immunoscreening for myeloma paraprotein-binding human antigens

Normal testis tissue was obtained from an orchiectomy performed as a hormone-ablative measure in a patient with metastatic prostate carcinoma. Fresh testis tissue was used to construct a cDNA expression library as described for malignant tissues (7). Immunoscreening of the testis-derived transfectants by the phage assay (7) was performed with the following modifications: Sera from 42 patients with myeloma-associated paraprotein (26 IgG, 16 IgA) were probed at a dilution of $1:1 \times 10^6$ to detect IgG and IgA myeloma proteins binding to recombinant proteins expressed in lytic plaques by incubating with alkaline phosphatase-conjugated goat anti-human IgG and IgA respectively and visualization following staining with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. Positive clones were subcloned to monoclonality and the nucleotide sequence of cDNA inserts determined on a LICOR automatic sequencer using an Excel™ II cycle sequencing kit (Epicentre) as described previously. Sequence alignments were performed with DNASIS (Pharmacia Biotech) and BLAST software on EMBL, GenBank and PROSITE databases.

Determination of antigen expression profile

The expression profile of myeloma paraprotein-binding clones in a spectrum of normal and malignant human tissues was determined by RT-PCR as described (15) using the primers 5'GAG TCT AAG AAG GAC GCC ACG 3' (sense) and 5'AAG CCT GAG ATC ATG TCA CTG 3' (anti-sense). For the PCR reaction, an annealing temperature of 65°C was used and 35 cycles of PCR performed, yielding a product 921 bp in length.

Immunofluorescence microscopy analysis

Subcellular localization of cylicin II was studied by immunofluorescence microscopy of sperm cells following staining with the anti-cylicin II positive myeloma patient's serum diluted 1:1000. Anti-cylicin-negative myeloma serum of the same isotype was used as a negative control at dilutions of 1:10 and 1:100. Ejaculates of donors with normal semen characteristics were obtained from the Department of Gynecology of Saarland University *in vitro* fertilization clinics. Cytospins of PBS-washed sperm cells on glass slides were air-dried, fixed for 10 min in methanol at -20°C, washed three times in PBS and incubated with different myeloma paraproteins. Staining of fixed cells was performed by sequential incubation with myeloma serum and CY-3-labeled rabbit anti-human immunoglobulin (Dianova, Hamburg, Germany). Nuclei were counterstained with DAPI and slides were embedded in anti-fade mounting medium before fluorescence microscopy.

Detection of anti-cylicin II antibodies in control sera

For the detection of antibodies against cylicin II in the sera from healthy controls, other myeloma patients and infertile men with known anti-sperm antibodies, phages from positive clone HOM-MM-1 were mixed with non-reactive phages of the cDNA library (internal negative controls) at a ratio of 1:10 and used to transfect bacteria. *E. coli*-absorbed sera and sera diluted 1:100 were tested for anti-cylicin II antibodies using the immunoscreening phage assay described previously.

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