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Analysis of the antibody repertoire of patients with mantle cell lymphoma directed against mantle cell lymphoma-associated antigens

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Abstract Treatment results of mantle cell lymphomas (MCL) are not satisfactory and novel therapeutic approaches are warranted. Because “shared” tumor antigens like the group of cancer testis antigens are only rarely expressed in MCL, we applied serological analysis of antigens using recombinant expression cloning (SEREX) to a complementary DNA library derived from five cases of MCL using the sera of eight patients with MCL in order to define MCL-associated antigens that are immunogenic in these patients and might be used as vaccines for patients with MCL. Five antigens were detected by SEREX. Four of the five detected antigens (hypothetical protein FLK10233, recombining binding protein suppressor, a chromosomal sequence, and interleukin-1 receptor associated kinase) are also expressed by a wide spectrum of normal human cells, excluding their use as vaccines. In contrast, the expression of CD52, which was detected by antibodies in the serum of an MCL patient, is restricted to hematopoietic cells. Interestingly, anti-CD52 antibodies were detected in this patient before and >2 years after allogeneic transplantation, indicating that both the autologous as well as the allogeneic immune system recognized CD52. Since the anti-CD52 monoclonal antibody alemtuzumab has shown activity in MCL, a vaccine consisting of recombinant CD52 alone or combined with passive

immunotherapy using alemtuzumab warrants further clinical and immunological evaluation in MCL.

Keywords Mantle cell lymphoma · Lymphoma-associated antigens · CD52 · Vaccine development

Introduction

Mantle cell lymphomas (MCL) make up 8% of the lymphomas and have a poor prognosis because of their eventual refractoriness to many chemotherapy regimens. Monoclonal antibodies, particularly rituximab, have activity in MCL patients [1–3], and chemotherapy combined with rituximab and followed by high-dose therapy with auto-transplant may be the best treatment for these patients [3]. For older patients, no standard treatment is available, and allogeneic transplantation is the only, yet toxic approach, to which a curative potential is ascribed.

The demonstration of responsiveness to the monoclonal anti-CD20 antibody rituximab and evidence for a graft-versus-MCL effect after allogeneic transplantation [4] support the notion that MCL are amenable to immunotherapy. Immunotherapy holds promise as an alternative approach for the prolongation of remissions and reduction of relapses. Vaccine approaches using dendritic cells fused with MCL cells met with some success in vitro and in a pre-clinical model in vivo by inducing T cells with specific cytotoxicity against MCL cells [5]. However, the identification of specific antigenic targets on MCL cells is cumbersome because cytotoxic T-cell-response-based approaches to the identification of tumor antigens are hampered by the inherent problems of establishing and characterizing [6] specific CTL clones. These problems are

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the main reason why no tumor-specific antigens have been defined to date that are frequently and specifically expressed in MCL. While the clonal immunoglobulin idiotype that is expressed by the malignant MCL cells of a given patient represents a specific antigen, and idiotype vaccines have been shown to induce remission in follicular lymphomas [7], their production is extremely expensive because they must be individually tailored, which has prohibited their widespread use [8]. Of the antigens with a broader expression spectrum, the PRAME antigen has been reported to be frequently expressed in MCL [9]. However, PRAME does not seem to be a good vaccine candidate because it is frequently expressed in normal tissues, albeit at a lower level than in certain neoplastic tissues [10].

Our group has developed an alternative approach for the identification and molecular characterization of specific antigens of human malignancies. The experience with SEREX, an acronym for “serological analysis of antigens by recombinant expression cloning” which uses antibodies in patients’ sera and expression cloning techniques, has taught us that many, if not all human malignancies elicit responses in the patient’s own immune system. For a growing number of these antigens, which were identified by antibodies, i.e., by the patient’s humoral immune system, a T-cellular immune response has been demonstrated. This supports the hypothesis that there is an integrated immune response against tumors in tumor-bearing patients which involves both B and T cells. The most promising vaccine candidates are the so-called cancer testis antigens (CTA); however, a survey by our group on the expression of cancer testis antigens in lymphoma revealed that MCL cells express CTA quite rarely [11].

Therefore, the identification of additional antigens that are specifically and frequently expressed in MCL is warranted. To identify antigens which might serve as the target structures for a broadly applicable vaccine, we applied a SEREX analysis to MCL.

Materials and methods

Patients, sera, and tissues

The study had been approved of by the local ethical review board (“Ethikkommission der Ärztekammer des Saarlandes”). Recombinant DNA work was done with the official permission and according to the rules of the State Government of Saarland. Sera and tissues were obtained during routine diagnostic or therapeutic procedures performed at Saarland University Medical School, Homburg, Germany and stored at -80°C until use after obtaining written informed consent by the patient. Normal tissues were collected from autopsies of tumor-free patients. Tumor

samples used for SEREX and reverse transcription polymerase chain reaction (RT-PCR) analysis were checked microscopically for the presence of neoplastic tissue and absence of contaminating normal tissues. Normal tissues were collected from autopsies of tumor-free patients.

Identification of MCL-associated antigens by SEREX

Fresh biopsy material obtained from five patients with MCL was used to establish complementary DNA (cDNA) libraries as described by us [12]. Poly(A)⁺ RNA was prepared with a mRNA isolation kit (Stratagene, La Jolla, USA) out of total RNA isolated from fresh tumor biopsies [13]. cDNA expression libraries were constructed by starting with 5 to 8 μg of poly(A) RNA. First-strand synthesis was performed using an oligo(dT) primer with an internal *Xho*I site and 5-methylCTP. cDNA was ligated to *Eco*RI adaptors and digested with *Xho*I. cDNA fragments were cloned in sense direction with respect to the lacZ promoter into the bacteriophage expression vector λ -ZAP Express using a commercially available adaptor ligation system according to the manufacturer’s instructions (Stratagene). After packaging into phage particles, these cDNA expression libraries were used to transfect *Escherichia coli*. The sera from patients with untreated MCL which had been preabsorbed with transformed *E. coli* lysates were screened for IgG reactivity against transfectants derived from the cDNA expression library using the phage assay described by us in detail previously [12]. 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 IPTG. 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 1:250 dilution of the patient’s serum, which had been preabsorbed with transfected *E. coli* lysates. Serum antibodies binding to recombinant proteins expressed in lytic plaques were detected by incubation with alkaline phosphatase-conjugated goat anti-human IgG and visualization by staining with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. Positive clones were subcloned to monoclonality and submitted to in vivo excision of pBK-CMV phagemids. The nucleotide sequence of cDNA inserts was determined using Excel cycle sequencing kit (Epicentre Technologies, Madison, WI, USA) on a LICOR sequencer. Sequencing was performed according to the manufacturers’ instructions starting with the vector-specific primers. Insert-specific primers were designed as the sequencing proceeded. Sequence alignments were performed with DNASIS (Pharmacia Biotech, Freiburg, Germany) and BLAST software on EMBL, Genbank, and PROSITE databases.

For the demonstration of antibodies against defined antigens, monoclonalized phages from clones that were reactive with the patient serum used for the immunoscreening were mixed with non-reactive phages of the cDNA library as internal negative controls at a ratio of 1:10 and used to transfect bacteria. IgG antibodies in the 1:100 diluted *E. coli* pre-absorbed sera from other patients and healthy controls were tested with the phage assay to assess antibody responses against the respective antigen.

Reverse transcription PCR

Total cellular RNA was extracted from frozen tissues, primed with a dT(18) oligonucleotide and reverse-transcribed with Superscript II (Gibco BRL, Eggenstein, Germany) according to the manufacturer's instructions. cDNA thus obtained was tested for integrity by amplification of β -actin transcripts in a 30-cycle PCR as described elsewhere [14]. For PCR analysis of the expression of individual gene transcripts, 1 μ g first-strand cDNA was amplified with transcript-specific oligonucleotides (10 pM) using 2 U AmpliTaq Gold (Perkin Elmer, Weiterstadt, Germany), 10 nM of each dNTP (dATP, dTTP, dCTP, dGTP), and 1.5 mM MgCl₂ in a 30- μ l reaction. The primers specific for the respective cDNA were designed to amplify coding sequences of the respective gene and were obtained commercially (MWG Biotech, Ebersberg, Germany). Presence of specific transcripts was checked by amplification with specific primer pairs, followed by electrophoresis.

Results

The patients from whom the sera had been taken had no obvious other diseases apart from their mantle cell lymphoma; in particular, the sera were negative for antibodies against common autoantigens such as antinuclear antibodies or rheumatoid factor.

Antigens detected by SEREX

Approximately 1×10^6 recombinant clones represented in the MCL-derived cDNA expression library expressed in *E. coli* were screened by the SEREX approach using the

1:1,000 diluted sera from eight patients with untreated MCL. The summary of the results of the SEREX analysis with these sera is shown in Table 1. Eighteen clones encoded by five different genes reacted with IgG antibodies in the 1:1,000 diluted sera from the MCL patients. The antigens were designated HOM-MCL-1 to HOM-MCL-5. Except for one of them (HOM-MCL-3), they were represented by several clones with overlapping sequences. All antigens detected represented known transcripts.

HOM-MCL-1 is identical to hypothetical protein FLJ 10233 and HOM-MCL-2 to recombining protein suppressor. HOM-MCL-3 is identical to CD52, HOM-MCL-4 represented chromosomal sequences, and HOM-MCL-5 is identical with interleukin-1 associated kinase 4.

Specificity of the detected antigens

For antigens HOM-MCL-1, HOM-MCL-2, HOM-MCL-4, and HOM-MCL-5, there was information from the published literature or from the GenBank that they are ubiquitously expressed in human tissues. HOM-MCL-3 is identical with CD52. CD52 is expressed by B and T lymphocytes, natural killer cells, monocytes and macrophages, and some dendritic cells, but not by granulocytes, red blood cells, platelets, or hematopoietic progenitor cells [15, 16].

Survey of antibody reactivity against antigens identified by screening with sera from MCL patients

To determine whether the immune responses against the antigens identified in this study were MCL-related, the sera from eight patients with MCL were tested against the SEREX antigens expressed in *E. coli*. Sera from healthy donors served as controls. As can be seen from Table 2, at a serum dilution of 1:100, antibodies against HOM-MCL-2 and HOM-MCL-5 were also detected in the sera of healthy controls. MCL-1, MCL-3, and MCL-4 were not detected by antibodies in the sera of healthy controls, but only by 2/8, 1/8, and 1/8 patients with MCL, respectively. The patient with antibodies against MCL-3/CD52 was tested for anti-CD52 antibodies 2 years before (during her second relapse) and after allogeneic transplantation, while in continuous complete posttransplant remission. The anti-CD52 antibody titer

Table 1 MCL-derived antigens detected by SEREX with IgG antibodies in the sera of eight MCL patients

Antigen	Length (bp)	Encoding gene
HOM-MCL-1	2,100	Hypothetical protein FLK10233
HOM-MCL-2	1,400	Recombining binding protein suppressor
HOM-MCL-3	450	CD52
HOM-MCL-4	1,300	Chromosomal sequence
HOM-MCL-5	650	Interleukin-1 receptor associated kinase

Table 2 Antibody reactivities against MCL-derived antigens in the sera of MCL patients and healthy controls

Antigen	MCL sera	Healthy controls
HOM-MCL-1	2/8	0/9
HOM-MCL-2	2/8	1/9
HOM-MCL-3/CD52	1/8	0/9
HOM-MCL-4	1/8	0/9
HOM-MCL-5	2/8	3/9

was 1:10,000 during relapse and 1:500 while in complete remission 2 years after transplant. The patient's stem cell donor (her sister) was negative for anti-CD52 antibodies.

Discussion

In previous studies [11], it had been shown that a common group of “shared” antigens, the cancer-testis or cancer-germline antigens are only rarely expressed in malignant lymphomas with the exception of T-NHL and diffuse large B-cell lymphomas. Furthermore, the frequency of expressed antigens reactive with the sera from NHL patients was low compared to studies with the sera of patients with solid tumors, suggesting that humoral immune responses in NHL patients against antigenic structures expressed by their tumors are less frequent, even though antinuclear autoantibodies have been quite often found in patients with NHL [17]. The rare antitumor immune responses in patients with MCL and other lymphomas might be partially due to the lack of the expression of costimulatory molecules by lymphoma cells, hampering the induction of an efficient immune response and partially to an immunodeficiency which is often associated with lymphomas.

The results of this study show that mantle cell lymphomas express several structures that are immunogenic in patients with MCL. However, most immunogenic structures are widely expressed in normal and malignant human cells therefore prohibiting their use as specific vaccines in MCL. In contrast, the expression pattern of CD52, which can function as a costimulatory molecule for the induction of CD4⁺ regulatory T cells [18], is more restricted: CD52 is expressed by B and T lymphocytes, natural killer cells, monocytes, macrophages, red blood cells, platelets, hematopoietic progenitor cells [15, 16], and mast cells [19]. Among hematologic neoplasms, the expression of CD52 is heterogeneous [20], but MCL cells express CD52 consistently [20–22] rendering MCL a promising target for therapy with the monoclonal antibody alemtuzumab, a humanized monoclonal antibody against CD52, even though experience with the anti-CD52 antibody in MCL is limited [23, 24]. Notably, the MCL patient

with serum anti-CD52 antibodies in our study had never been treated with alemtuzumab, excluding the possibility that the immune response in her serum might have had its origin in an idiotypic anti-alemtuzumab immune response. She had a very strong (1:10,000) anti-CD52 antibody response while in her first relapse 2 years before allogeneic transplantation. After allogeneic stem cell transplantation, a new immune system derived from the stem cell donor was established, and the serum taken 2 years after the transplantation had an anti-CD52 antibody titer of 1:500. That this antibody response was a neo-response is shown by the fact, that the stem cell donor (the patient's HLA identical sister) was always negative for anti-CD52 antibodies. This indicates that both the patient's former and her new immune system recognized CD52 as an immunostimulatory molecule and raised an immune response against it. Since the donor was negative for anti-CD52 antibodies, we must assume that CD52-expressing mantle cells persisting after allogeneic transplantation have induced the neo-immune response in the transplanted immune system by presenting the immunogenic CD52 molecule the context of “danger” [25]. Therefore, passive immunotherapy of MCL with anti-CD52 antibodies might work not only by a direct attack of the monoclonal antibody against the MCL cells, but may be complemented by an additional immune response against this MCL surface structure induced, i.e., by malignant MCL cells. In light of the considerable toxicity of the humanized anti-CD52 monoclonal antibody alemtuzumab [26], it is noteworthy that the presence of the endogenous anti-CD52 antibody in the patient was not associated with neutropenia and lymphopenia and the patient did not suffer from opportunistic infections. This may be due to the fact that the endogenous antibody bound to an epitope different from the one which is recognized by the humanized antibody alemtuzumab (data not shown) and/or due to the possibility that the immune response is restricted to mantle lymphoma cells that present CD52 in the context of danger [25].

The finding that CD52 can be immunogenic in MCL patients supports additional clinical trials using CD52 as a vaccine alone or together with alemtuzumab. Apart from measuring the anti-lymphoma effects of alemtuzumab, such studies should be designed to evaluate if and to which degree this therapy is associated with or induces host anti-CD52 responses that could complement the direct effects of the monoclonal CD52 antibody. If a cellular anti-CD52 immune response induced by a CD52 vaccine would lack the toxicity of alemtuzumab, it can only be investigated in a carefully designed prospective study.

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