

SHORT REPORT

Identification of antigenic targets of paraproteins by expression cloning does not support a causal role of chronic antigenic stimulation in the pathogenesis of multiple myeloma and MGUS

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Antigenic targets of monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (MM) paraproteins have been suggested to play an important role as growth stimulators in the pathogenesis of these neoplasms. To identify such targets, we screened cDNA libraries from human testis, lung and breast cancer, bovine and porcine muscle and wheat germ for reactivity with paraproteins in the sera from 115 patients with MGUS and MM. Of $>6 \times 10^8$ paraprotein–antigen interactions screened, an IgA paraprotein from a female patient bound to sperm-specific cylicin-2, and 3 IgG paraproteins bound to tripeptidyl-peptidase-II (TPP-2), insulin-like growth-factor binding-protein-2 (IGFBP-2) and porcine kinesin. Specificity was confirmed by reverse Western blots using recombinant antigens. The broad spectrum of auto-, allo- and heteroantigens as targets of human paraproteins in patients without signs of chronic antigenic stimulation renders a causal role of the antigenic stimulus in the pathogenesis of MGUS and MM unlikely.

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The identification of the antigenic stimuli of B-cell neoplasms might have considerable impact, because a causal relationship between these neoplasms and antigenic stimulation has been suggested.^{1,2} To date, antigenic targets of paraproteins were discovered accidentally due to clinical symptoms caused by the paraprotein (e.g., chronic cold agglutinin disease or cryoglobulinemia³ or bleeding disorder⁴), 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 myeloma⁵) or by screening paraproteins against predefined antigens (e.g., anti-streptolysin, anti-DNA, anti-IgG³). Systematic searches covering a broad spectrum of potential antigens have not been reported to date.

SEREX (serological identification of antigens by expression cloning) allows for the systematic screening of putative antibody–antigen interactions, even if neither the antigen nor the antibody are known.⁶ We therefore used SEREX for the identification of antigenic targets of paraproteins in expressed cDNA libraries derived from human, animal and plant tissues.

Patients, material and methods

Patients

The study was approved by the local ethical review board (“Ethikkommission der Ärztekammer des Saarlandes”) and conducted according to the Declaration of Helsinki. Recombinant DNA work was performed with permission and according to the regulations of local authorities (Government of Saarland). Human materials were obtained during routine diagnostic or therapeutic procedures after obtaining written informed consent and stored at -80°C .

SEREX

cDNA expression libraries were established as described.⁶ A wheat germ cDNA library was obtained from Sylvia de Pater

(Utrecht, Netherlands). The phage assay⁶ was used for the screening of sera from 115 patients with (multiple myeloma) MM or (monoclonal gammopathy of undetermined significance) MGUS at a dilution of 1:1,000,000. Positive clones were sub-cloned to monoclonality and the nucleotide sequence of cDNA inserts was determined as described before.⁶ Antibodies against the identified antigens were probed in 1:100 diluted control sera.

Reverse western blot

Patient’s serum was subjected to electrophoresis and transferred to nitrocellulose membranes. Membranes were incubated with recombinant GST-TPP-2 fusion protein that had been produced by cloning TPP2 into a pGEX4T1 vector according to the manufacturer’s (Pharmacia, Uppsala, Sweden) recommendations. Recombinant GST-SCP-1 fusion protein served as negative and a mouse antibody against the respective light chain (Dianova, Hamburg, Germany) as positive control.

Immunohistochemistry

Deparaffinized bone marrow sections were incubated with recombinant GST-TPP-2, followed by mouse anti-GST antibody and visualization by the APAAP technique according to the manufacturer’s (Dako-Cytomation, Glostrup, Denmark) recommendations.

Results and discussion

Identification of paraprotein-binding clones

At least 1×10^6 clones from each cDNA expression library derived from human testis, nonsmall cell lung cancer, breast cancer, bovine and porcine muscle as well as wheat germ were screened with 1:1,000,000 diluted sera from 115 patients with MGUS or MM (55 IgG_κ, 33 IgG_λ and 11 IgA_κ, 9 IgA_λ, 5 IgG_{κ+λ}, 1 IgA_λ + IgG_λ, 1 IgA_κ + IgG_κ; male: 69, female: 46, median age: 65 years, range: 33–90 years). A testis library was chosen because a large proportion of the human genome is expressed in testis due to its genome-wide hypomethylation. Non-small-cell lung and breast cancer libraries served as sources for putative tumor-associated, and bovine and porcine muscle (beef and pork), as well as wheat germ as sources for food-associated antigens.

The screening of $>6 \times 10^8$ potential paraprotein/antigen interactions revealed 39 positive clones, 1 reacting with an IgA and all others reacting with IgG paraproteins. The positive clones coded for cylicin-2 (target of an IgA paraprotein), SOX6, metalloproteinase 17, tripeptidyl-peptidase-II (TPP2), insulin-like growth-factor

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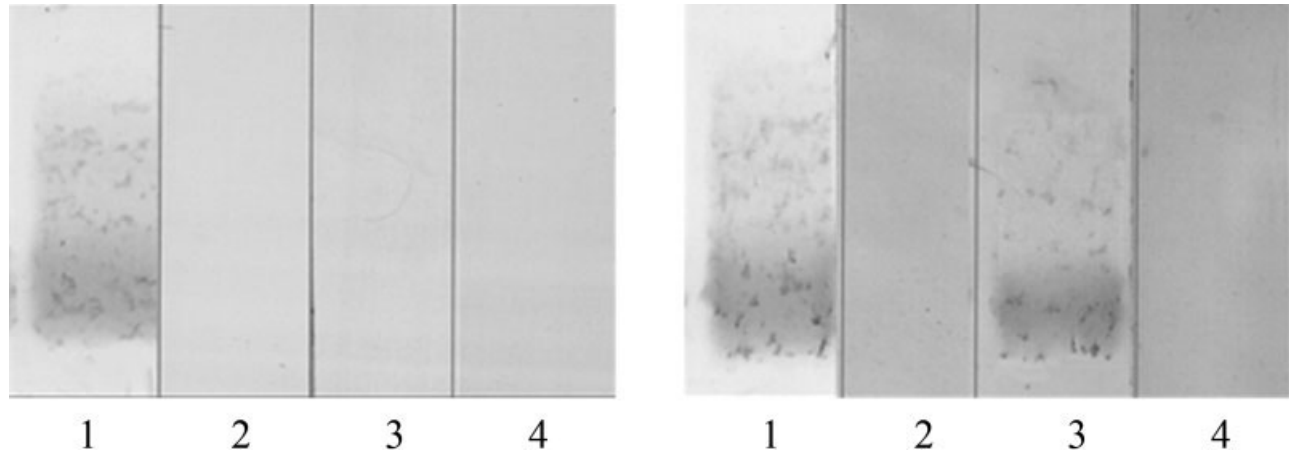


FIGURE 1 – Demonstration of the paraprotein-mediated reactivity against identified antigens. Western blot using recombinant GST-TPP-2. Left panel: serum from an MGUS patient with an IgG κ paraprotein without anti-TPP-2 reactivity in the phage assay; right panel: serum from the patient with TPP-2 reactive IgG κ paraprotein; 1: anti-human-IgG; 2: anti-mouse-IgG; 3: TPP-2-GST fusion protein (500 μ g/ml); 4: GST-SCP-1 fusion protein (500 μ g/ml).

binding-protein-2 (IGFBP-2), porcine kinesin and 33 unknown sequences or proteins with unknown function.

Demonstration of paraprotein-mediated reactivity

Reactivities at high dilutions of the respective paraprotein-containing sera were detected against TPP-2 ($1:10^{10}$), IGFBP-2 ($1:10^9$), porcine kinesin ($1:10^9$) and cylicin-2 ($1:10^9$, as previously described⁷). In contrast, only low-titered reactivities ($<1:10^7$) were observed against the latter 3 antigens, and no reactivity at all against cylicin-2 in normal male and female sera. The reactivities being mediated by paraproteins was confirmed by Western blotting with the recombinant GST fusion antigens (Fig. 1). The antigen-binding bands were identical with the bands detected by the anti-sera used for the demonstration of monoclonality of the paraproteins by immunofixation. Moreover, recombinant GST-TPP-2 stained plasma cells in the bone marrow of the MGUS patient with the anti-TPP-2 reactive paraprotein (Fig. 2). All other antigens reacted with MM and MGUS sera at dilutions $<1:10^5$, and antibodies against these antigens were detected in normal sera up to dilutions of $1:10^4$, making a paraprotein-mediated reactivity unlikely.

TPP-2, detected in a testis-derived cDNA and IGFBP-2, which was detected in a lung-cancer derived cDNA, are widely expressed human autoantigens. Cylicin-2, which was also detected in a testis-derived cDNA, is a specific component of the sperm head cytoskeleton and as such an alloantigen for the female patient with the reactive paraprotein.⁷ Porcine kinesin meets the definition of a heteroantigen. Remarkably, despite the high homology between human, bovine and porcine kinesin, the patient's paraprotein did not cross-react with the recombinant human or bovine homologue (data not shown). Thus, 2 autoantigens, 1 allo-antigen and 1 hetero- or food-associated antigen were identified in this study as targets of more than 100 paraproteins tested. Notably, none of the 4 patients had clinical signs of chronic antigenic stimulation or associated morbidities. The female patient with the antibodies against the sperm-head associated cylicin-2 had given birth to 2 children, and the patient with the anti-porcine kinesin did not have any symptoms of hypersensitivity reactions against pork or other porcine products.

Several previous reports have identified myeloma paraproteins directed against various infectious agents, including bacteria³ and the p24 antigen of the human immunodeficiency virus-1.⁵ In another case, a patient developed a serum M-component with specificity for horse α_2 -macroglobulin 30 years after receiving passive serotherapy with horse antiserum to tetanus.⁸ Even though

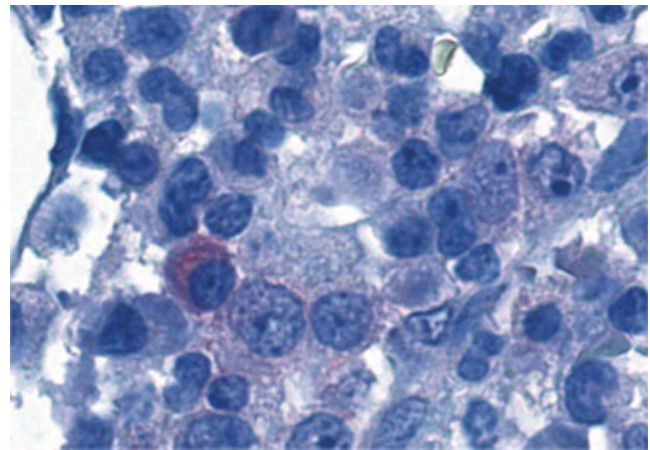


FIGURE 2 – Immunohistologic demonstration of intracytoplasmic anti-TPP-2 antibodies using GST-TPP-2. A plasma cell in the bone marrow of the MGUS patient with the anti-TPP-2 reactive paraprotein stains positive with recombinant GST-TPP-2. GST-TPP2 stained cells were confirmed as plasma cells by staining an adjacent section with the respective anti-light chain antibodies. APAAP stain, Fast Red; ca. $\times 400$ magnification.

few of these studies convincingly proved that the observed phenomena were indeed caused by binding of the respective paraprotein to its specific antigenic target (*e.g.*, by “reverse Western” blotting or sequencing of the target antigen as done in this study), these selective observations are often cited to support a causal relationship between the development of an MGUS or MM clone and chronic antigenic stimulation.^{1-5,9-16} In contrast to these previous reports, our unbiased systematic study did not only convincingly prove the paraprotein-mediated reactivity against the identified antigens, it also revealed a broad spectrum of antigenic targets of paraproteins. The fact that of $>6 \times 10^8$ possible paraprotein/target-antigen interactions screened only 4 were identified as paraprotein-mediated indicates that the antigenic sources tested (which included a large proportion of the expressed human genome as possible sources for autoantigens as well as the major sources for food allergens) represent only a small part of the entire antigenic target spectrum of human paraproteins.

We have identified autoantigens in patients without signs of chronic antigenic stimulation, and heteroantigens in patients without

animal allergies. Thus, this unbiased systematic study lends support to the view that the role of chronic antigenic stimulation in the pathogenesis of MGUS and MM has probably been overestimated in the past. The view that many—if not any—auto-, allo- and hetero-antigen-reactive B-cell clones can become the random target of malignant transformation is also supported by the fact that there is no convincing evidence for the preferential use of V genes in MGUS and MM,^{17,18} which contrasts with the selective immunoglobulin V gene use in other B-cell malignancies, *e.g.*, marginal zone lymphomas associated with chronic infection or autoimmunity.¹⁹ The knowledge of the antigenic target structures of paraproteins allows

to address in more detail tumor-host interactions in the presence and absence of specific antigens in the respective patients, and to study more specifically the role of immunoregulatory deficiencies, such as the recently reported dysfunction of regulatory T cells²⁰ in patients with MGUS and multiple myeloma.

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