CML28 Is a Broadly Immunogenic Antigen, Which Is Overexpressed in Tumor Cells

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ABSTRACT

Donor lymphocyte infusion (DLI) reliably induces durable remission in 75–80% of patients with relapsed chronic myelogenous leukemia (CML) after allogeneic hematopoietic stem cell transplantation. To identify immunological targets of the graft-versus-leukemia response (GVL) after DLI, we used CML post-DLI responder sera to screen a CML cDNA expression library. One of the antigens identified in this screen is a 28,000 protein, termed CML28. CML28 is identical to hRrp46p, a component of the human exosome, a multiprotein complex involved in the 3’ processing of RNA. Components of the human exosome include known autoantigens, such as PMSc1-100, an autoantibody target in patients with polymyositis, scleroderma, or polymyositis-scleroderma overlap syndrome. Recombinant CML28-GST fusion protein was purified, and used in Western blot and ELISA to demonstrate the development of a high-titer CML28-specific IgG antibody response in a patient with relapsed CML who responded to DLI. Northern blotting demonstrated that CML28 is highly expressed in a variety of hematopoietic and epithelial tumor cell lines, but not in normal hematopoietic tissues or other normal tissue, with the exception of testsis. Purified recombinant CML28 was used to generate a CML28-specific murine monoclonal antibody. Western blotting with CML28 monoclonal antibody against whole-cell lysates derived from blood and marrow of normal donors and patients with leukemia revealed high expression of this antigen in tumor but not in normal samples. Because CML28 was highly expressed in epithelial tumor cell lines, anti-CML28 responses were also examined in patients with solid tumors. By ELISA, we found specific serological responses in 10–33% of patients with lung cancer, melanoma, and prostate cancer. Our studies suggest that immunogenicity of CML28 is likely because of overexpression of this antigen in tumor cells. Moreover, given its expression and immunogenicity in a wide variety of malignancies, CML28 merits additional evaluation as a target for antigen-specific immunotherapy.

INTRODUCTION

The immune response induced by DLI 4 presents a unique opportunity to identify the components of an effective antitumor response. In patients with CML who have relapsed after BMT, infusion of donor lymphocytes induces durable remission in 70–80% of patients (1–3). Despite the effectiveness of DLI, the target antigens of this immune response have not been well characterized. In recent experiments we specifically examined whether this potent immune response was directed against CML-specific antigens such as bcr-abl breakpoint peptides (4). Although T-cell clones specific for bcr-abl breakpoint peptides could be generated from patient lymphocytes in vitro, we could not demonstrate reactivity directed against bcr-abl breakpoint peptides in vivo. This suggests that the immune response induced by DLI may be directed at tumor antigens that are more broadly expressed. This has been confirmed by our recent demonstration that responding patients develop a coordinated immune response involving B cells as well as T cells. By using serum from DLI responders to screen a CML cDNA expression library, we isolated a panel of 13 candidate tumor-associated antigens (5). Each of these antigens was found to elicit a specific and high titer antibody response in the recipient, appearing only after infusion of donor lymphocytes and temporally correlated with the elimination of leukemia cells in vivo. Additional characterization of individual candidate antigens, such as CML66, has revealed that at least some of these antigens are expressed by solid tumors as well as leukemia, and are immunogenic in a wide array of cancers, thus validating our cloning strategy as a method to identify tumor-associated antigens (6).

One of the antigens identified as a potential tumor-associated target in DLI responders is a 28,000 protein, termed CML28. CML28 is identical to hRrp46p, a component of the human exosome, which is a multiprotein complex involved in the 3’ processing and degradation of RNA (7). Components of the human exosome include known autoantigens, such as PMSc1-100, an autoantibody target in patients with PM, Scl, or PMSc1 overlap syndrome (8). Herein, we demonstrate that CML28 mRNA is highly expressed in different tumors, but high-level expression in normal tissues is restricted to testsis. Moreover, we found CML28 protein to be highly expressed in highly proliferative tumors such as AML and blast crisis CML. In contrast, CML28 protein was undetectable or present at very low levels in normal bone marrow and peripheral blood. By ELISA, the development of high titer antibody correlated well with the cytogenetic remission induced by DLI. IgG antibodies specific for CML28 were also found in 10–33% of patients with melanoma, lung, and prostate cancer. These observations demonstrate that CML28 antigen is immunogenic in patients with a variety of different solid tumors, and this response is not restricted to patients after allogeneic bone marrow transplantation. The broad immunogenicity of CML28 and its association with effective antitumor immunity in CML suggest that CML28 may be a widely applicable target for the immunotherapy of cancer.

MATERIALS AND METHODS

Patient Samples. Serum was obtained at various time points before and after DLI in patients enrolled on a clinical trial of CD4+ DLI for treatment of relapse after allogeneic BMT (9). Serum samples were also obtained from patients with metastatic melanoma or non-small cell lung carcinoma on enrollment into Institutional Review Board-approved tumor cell vaccine trials (10, 11). Serum samples were obtained from patients with prostate cancer enrolled in the genitourinary cancer clinic at Dana-Farber Cancer Institute.

CML cDNA Library and Human Testis cDNA Library Screening. CML cDNA library construction and screening were performed previously (5). Briefly, mRNA was extracted from PBMCs from 3 patients with CML, 1 with accelerated phase and 2 with stable-phase disease using standard methods, and
CML28 IS OVEREXPRESSED IN TUMOR CELLS

pooled to create a representational CML expression library in a λ bacteriophage expression vector. Filters with recombinant phage were then incubated with post-DLI patient serum at 1:500 dilution followed by alkaline phosphatase-conjugated antihuman IgG.

A human testis cDNA library (1 × 10⁹ phage) derived from normal whole human testes pooled from 11 males (Clontech, Palo Alto, CA) was screened with a 0.5 kb 3²P-labeled CML28 cDNA probe, as described previously (6). After three rounds of phage plaque purification, five positive clones were identified, converted into plasmid pTriplEx by cre-lox mediated excision, and sequenced in both strands. Sequence homology searches were performed using the GenBank databases (National Center for Biotechnology Information). CML28 cDNA sequence has been submitted to GenBank (accession no. AF285785).

Generation of GST Fusion Proteins. A cDNA fragment encoding CML28 with EcoRI restriction site on both ends was generated by PCR using high-fidelity enzyme Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA) and primers 59E (5‘- CGGAAGATGGACGAGAAGTTCTGACGCCCA- AATC-3’) and 59 F (5‘- CGGAAGATTTCTGCTGCTGAGTACG- CCT-3’). The underlined sequences in these primers were designed for subcloning into EcoRI site of GST fusion vector pGEX-3X (Amersham-Pharmacia, Piscataway, NJ). A CML28 fragment was fused in frame to the COOH terminus of GST protein after cloning into the EcoRI site of the GST expression vector pGEX-3X and was additionally examined by DNA sequencing before transformation into the BL-21 strain of Escherichia coli. The fusion protein GST-CML28 was purified with B-Per Bacterial Protein Extraction Reagent (Pierce, Rockford, IL). Recombinant GST protein (without fusion protein) was purified by standard techniques and used as a control.

Detection of CML28-specific Antibody in Patient Sera by ELISA Assay. Each serum sample was tested against CML28-GST fusion protein and GST protein alone. To perform this assay, ELISA plates (VWR Scientific, Bridgeport, NJ) were coated with 50 μl of purified recombinant CML28-GST protein or GST protein alone at 2 μg/ml in coating buffer [carbonate buffer (pH 9.5)] overnight at 4°C (5, 6). Plates were washed with PBS with 0.05% Tween X-100 blocked overnight at 4°C with 200 μl/well of 2% nonfat milk with 0.05% Tween X-100. Fifty μl/well patient sera was added to a final dilution of 1:1000 and incubated at room temperature for 3 h. The procedure for detection of specific IgG antibody has been described previously (5, 6). Immunoglobulin A cDNA fragment encoding CML28. GenBank accession no. AF285785.

Fig. 1. Complete nucleotide sequence of CML28-cDNA. GenBank accession no. AF285785.

Generation of CML28-specific Monoclonal Antibody. Monoclonal antibody to CML28 was generated by serially immunizing mice with 50 μg, 25 μg, and 25 μg of purified recombinant protein at 2-week intervals in the presence of Freund’s adjuvant. Mice were bled 10 days after the last immunization, and serum was tested for reactivity against CML28-recombinant protein by ELISA. Spleens were harvested from the mice with highest titer antibody and fused with SP20 myeloma cells using standard procedures. Hyridoma clones were selected by testing supernatants for specificity against CML28-GST but not for FAK-GST by ELISA. Specificity was additionally confirmed on Western blot. Clone 21F.38 (IgG1, κ light chain) was subcloned and expanded. This monoclonal antibody was purified and concentrated using a Protein G column (HiTrap affinity column; Amersham Pharmacia) to a protein concentration of 20 μg/ml. For all of the Western blotting experiments, this antibody was used at 1:100 dilution.

Western Blotting. Whole-cell lysates were generated from various tumor cell lines or from patient samples by lysis with radioimmunoprecipitation assay buffer (1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) in the presence of protease inhibitors. Lysate (20–30 μg/lane) or recombinant proteins expressed in transformed E. coli were subjected to protein gel electrophoresis using 10–12% SDS-PAGE and transferred onto nitrocellulose filters in 20% methanol in Tris-glycine buffer. Proteins on the blots were visualized as described previously (6). Antibody to β-actin (Sigma, St. Louis, MO) was used as a control to ensure equal loading of lanes.

RESULTS

Cloning of CML28 cDNA. We identified previously a 0.9-kb cDNA clone after antibody-based screening of a CML expression cDNA library using sera from patients with relapsed CML who achieved a complete response after DLI (5). The 0.9-kb clone contained a 700-bp incomplete ORF with a missing 5' end. Using this clone to screen a normal human testis cDNA library, we identified a 1.126-kb full-length clone (containing a 55-bp 5' UTR, a 804-bp ORF encoding 268 amino acids, and a 264-bp 3' UTR). The DNA sequence at the start codon in the ORF contained a Kozak consensus sequence (A/GNNATGG) for high efficiency protein translation (12). In vitro transcription and translation experiments confirmed that this ORF encoded a Mₙ 28,000 protein (Fig. 1), which correlated with the predicted molecular weight. A polyadenylation signal (AAATAAA) was found in the 3' UTR, suggesting that this transcript had a complete 3' UTR sequence upstream of the polyadenylate tail. Because this antigen was Mₙ 28,000 in size and was originally isolated from a CML library, it was termed CML28. Comparison of the sequences of CML28 isolated from the normal testis and CML libraries demonstrated them to be identical in the 0.9-kb 3' overlapping region. GenBank analysis of the full-length sequence revealed it to be...
identical to the recently identified hRrp46p, a component of the human exosome, which is a multiprotein complex involved in the 3’ processing of RNA. Comparisons with the published sequence of hRrp46p reveal that the 5’ coding region of CML28 is 33 amino acids longer than hRrp46p. No other differences in nucleotide sequence compared with hRrp46p were found. The cDNA sequence of CML28 was additionally confirmed by examination of the genomic DNA sequence on human chromosome 19 in the GenBank database.

High Titer Antibody Response to CML28 Correlates with Response to DLI. To establish that CML28 is a target of the immune response after DLI, recombinant GST-CML28 fusion protein was synthesized and used to test for antibody reactivity in normal and CML patient sera. The purified GST-CML28 fusion protein has a molecular weight of \( M_r \ 58,000 \) corresponding to the combined size of GST (\( M_r \ 30,000 \)) and CML28. In the Western blots shown in Fig. 2, antibodies to CML28 were not detected in normal sera but were detected in sera obtained from CML patient A 6 months after DLI. Serum from patient A had been used to screen the CML expression library, and this result therefore confirmed that the CML28 protein was immunogenic in vivo. Antibodies to GST-CML28 were not detected by Western blot in serum from the same patient obtained before allogeneic BMT or before DLI (Fig. 2).

To additionally quantify the antibody response to CML28, serial serum samples obtained before transplant and at various times over a 2-year period after DLI were analyzed by ELISA using GST-CML28 as the coating antigen. In this assay, antibody reactivity with purified GST-CML28 was compared with antibody reactivity with purified GST. No reactivity was detected against CML28-GST or GST fusion protein in sera from 10 normal volunteers. Among the sera from 19 DLI responders tested, only patient A had reactivity to CML28-GST but not to GST. By using competition experiments, as described previously (5), in which addition of excess soluble CM28-GST fusion protein was found to abrogate evidence of serum reactivity on ELISA, the specificity of this interaction was established. As shown in Fig. 3, patient A did not have detectable antibodies to CML28 before BMT or before DLI. However, antibody titers to CML28 increased markedly 3 months post-DLI, peaked at 6 months post-DLI, and thereafter gradually declined. Specific antibody was no longer detectable 2 years after DLI. The isotype of anti-CML28 antibody was predominantly IgG1 (data not shown). The patient achieved a complete cytogenetic remission 3 months post-DLI, and, thus, the time course of antibody reactivity in this patient correlated well with the onset of cytogenetic remission. CML28-GST (range \( 0.083–0.92 \)) to CML28-GST (range \( 0.069–0.088 \)). The percentage of marrow metaphases containing the Philadelphia chromosome, and results of PCR analysis of patient blood and marrow samples for the presence of \( bcr-abl \) mRNA are also indicated.

CML28 Is Broadly Expressed in Tumor Cell Lines but Expression in Normal Tissues Is Limited. To examine gene expression of CML28, CML28 cDNA was used as a probe in a series of Northern blot hybridization experiments against total RNA obtained from a variety of normal and malignant tissues. As shown in Fig. 4, expression of a 1.1-kb transcript consistent with CML28 was present in only 1 of 28 normal human tissues (Fig. 4, B–D), human testis. Although background binding was noted in spleen, no specific hybridization band was noted in this tissue. In contrast, Northern blot hybridizations with this cDNA probe demonstrated high level expression in each of 8 human tumor cell lines that was examined. These included HL-60, K562, Molt-4, and Raji cell lines derived from myeloid or lymphoid tumors as well as 4 cell lines derived from a variety of epithelial malignancies and melanoma.

Protein expression of CML28 was also examined in primary normal and malignant hematopoietic tissue samples by Western blotting using a monoclonal antibody specific for CML28. A representative series of samples tested is shown in Fig. 5. CML28 was found to be highly expressed in a variety of cell lines including K562, BV173, and Jurkat, and prostate cell lines DU-145 and LNCAp. CML28 protein expression was consistently low or undetectable in lysates prepared from the normal bone marrow and 4 normal granulocyte colony-stimulating factor stimulated PBMC samples analyzed. In contrast, CML28 was highly expressed in 8 of 9 samples tested from patients with AML. CML28 was present at low levels or not detectable by Western blot in cell lysates from 2 myelodysplasia and 8 stable-phase CML samples tested. Of all of the primary leukemia samples tested, CML28 expression was highest in the 4 CML blast crisis samples. These results suggest that CML28 is highly expressed in highly proliferative tumors but is only expressed at low levels in more differentiated nonproliferating cells.

Quantitation of Specific IgG Response to CML28 in Normal Donors and Patients with Cancer. Because CML28 was highly expressed in a variety of epithelial tumor cell lines, we used ELISA to detect and quantitate levels of specific IgG antibody in sera obtained from patients with a variety of solid tumors. These results were compared with sera from patients with CML who had received vari-
ous therapies. As summarized in Fig. 6, of 19 CML DLI responders and 10 CML patients treated with hydroxyurea, only patient A had high level reactivity to CML28. Antibodies were not detected in normal donors \( n = 10 \), but specific CML28 reactivity was detected in sera from patients with lung cancer \( 2 \) of \( 15 \) patients), melanoma \( 5 \) of \( 17 \) patients), and prostate cancer \( 5 \) of \( 15 \) patients). In each instance where reactivity against GST-CML28 was greater than reactivity against GST, ELISA reactivity was blocked by prior incubation of sera with excess purified GST-CML28 (data not shown). These results confirm the specificity of the response to CML28 in these patients and suggest that CML28 was capable of eliciting humoral immune responses in patients with a variety of tumors.

**DISCUSSION**

CML28 is identical to hRpr46p, a constituent of the human exosome, of which the components have been found previously to elicit antibody responses in patients with autoimmune disease (13). The more completely characterized yeast exosome is composed of at least 10 proteins (including Rrp4p, Rrp40p, Rrp46p, Mtr3p, and Cds1p) and is a single complex of multiple 3’-5’ exonucleases (14). Exosome processing, which reduces some substrates to shorter forms and results in complete degradation of others, is required to produce the mature 3’ end of several stable RNAs. Gene deletion studies involving these components in yeast have demonstrated loss of viability, suggesting that most components, including hRpr46p, are essential for normal cellular function (15). The human counterpart of the yeast exosome is the PMScl particle (7, 15–17). Approximately 24% of patients with PMScl overlap syndrome have autoantibodies directed against the PMScl particle, with antibodies predominantly directed against the PMScl-100 antigen (17–21). Recently, auto-antibodies directed against other components of the human exosome have also been identified in sera from patients with a variety of autoimmune disorders (8). In one series, sera from >90% of patients tested were reactive to PMScl-100, and of those, 6–90% had additional auto-antibodies directed against other exosome components, including 13% who were reactive to hRpr46p.

In our studies, CML28 was identified as the target of a specific antibody response in a patient who developed an effective antitumor response after allogeneic DLI. Although the clinical and pathologic manifestations of GVHD bear many similarities with autoimmune disease, the patient who developed high titer antibodies to CML28 did not have clinically significant GVHD or manifestations of autoimmune disease. Thus, the humoral immune response to CML28, which we detected after DLI, appeared to be specifically associated with tumor rejection rather than with the development of autoimmune. In additional experiments, we also identified high titer autologous antibody responses to CML28 in patients with a variety of solid tumors. The demonstration of specific immune responses in patients with cancer indicates that the immunogenicity of CML28 is not dependent on its expression by allogeneic cells. Consistent with this
Expression in leukemogenesis.

CML28 is overexpressed in tumor cells

The expression profile of CML28 bears several striking similarities to that of another DLI-associated antigen termed CML66 (6), as well as CT antigens described previously (30, 31). All of these antigens have been shown to be expressed predominantly in tumors and a variety of different tumors. Although other CT antigens have a substantial degree of homology to each other (45–84%), CML28 has a much lower degree of homology (11–19%) to the other CT antigens. Nevertheless, the immunogenicity of CML28 is likely to be similar to CT antigens and be primarily related to aberrant tissue expression and overexpression rather than the presence of mutations within the protein. This mechanism of immunogenicity has been proposed previously for other CT antigens such as NY-ESO-1 (32–34) and the MAGE antigens (35).

In addition to high level expression in leukemia cells, our Northern and Western blotting experiments indicated that CML28 is also overexpressed in other cancers. These findings led us to examine whether patients bearing different solid tumors developed an antibody response against CML28. In fact, we found CML28 capable of eliciting specific humoral immunity in 10–33% of patients with melanoma, lung, and prostate cancer. Antibody responses to other overexpressed tumor-associated antigens such as prostate-specific antigen and HER-2/neu have been described previously in patients with prostate cancer (36, 37). However, in these studies, only a fraction of patients developed either antibody or T-cell responses to these antigens suggesting that host-specific factors also contribute to the generation of immune responses against self proteins that would otherwise be "ignored." Possibly, overexpression of antigens, such as CML28, lowers the threshold at which an immune response is initiated, as has been suggested by other investigators (38, 39).

Because the expression of this antigen in normal hematopoietic cells and other normal tissues is very limited, CML28 may be an excellent immunotherapy target. Recent efforts by other investigators have explored the potential of developing immunotherapy approaches against universal tumor antigen targets, such as survivin and telomerase (40–43). The shared features of these antigens include broad expression in most neoplasms, limited expression in normal tissues, and important roles in critical cellular functions. All of these criteria may be fulfilled by CML28.

Although our studies have not yet examined whether T-cell responses to CML28 are present, we anticipate that specific T-cell responses to CML28-derived peptide epitopes have been generated in those individuals with high titer-specific antibody to this antigen. T-cell interactions are known to be required for immunoglobulin isotype switching, and responses to CML28 detected in our patients are predominately IgG1 isotype. Previous studies with tumor-associated antigens such as NY-ESO-1, first identified as a target of an autologous antibody response, have demonstrated close correlations between antibody, and both CD4 and CD8 T-cell responses in vivo (32–34). Similarly, several MAGE antigens originally identified as tumor-associated antigens by T-cell clones have subsequently been shown to also elicit antibody responses in vivo (44, 45). Having identified individuals who have developed high titer antibody responses to CML28, it will now be necessary to determine whether T-cell responses have also been generated in vivo and to define the immunogenic epitopes within this protein. In conjunction with tumor-associated expression, the identification of T-cell responses to CML28 would additionally support the use of this antigen as a potential target for immunotherapy in patients with cancer.

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