Monoclonal Antibodies Specific to the Acute Lymphoblastic Leukemia t(1;19)-Associated E2A/pbx1 Chimeric Protein: Characterization and Diagnostic Utility

By Bi-Ching Sang, Liangru Shi, Peter Dias, Li Liu, Jia Wei, Zhi-Xue Wang, Craig R. Monell, Fred Behm, and Stefan Gruenwald

Nonrandom chromosomal abnormalities are found in most human malignancies, particularly leukemias and lymphomas. A characteristic t(1;19) (q23;p13.3) chromosomal translocation is detected in 5% of childhood acute lymphoblastic leukemia (ALL) cases. This translocation results in the formation of a fusion gene, which leads to the expression of an oncogenic E2A/pbx1 protein. Breakpoints in the E2A gene almost invariably occur within a single intron, and the identical portion of PBX1 is joined consistently to exon 13 of E2A in fusion mRNA. In this article, we report the development and characterization of monoclonal antibodies against E2A/pbx1 fusion protein using a specific peptide that corresponds to the junction region of the protein. The obtained antibodies recognize specifically the chimeric E2A/pbx1 fusion protein and lack cross-reactivities with E2A and pbx1. Immunohistochemical staining and flow cytometric studies show that these antibodies can distinguish t(1;19)-positive from t(1;19)-negative leukemic cells. These results indicate that the obtained E2A/pbx1-specific monoclonal antibodies might prove to be valuable diagnostic reagents and important tools for elucidating the mechanisms involved in oncogenesis and progression of t(1;19)-positive childhood ALL.

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was conjugated to maleimide-activated KLH (Pierce, Rockford, IL) according to the manufacturer’s instructions.

Recombinant pbx1 protein: pET21-pbx1 containing the entire coding region of PBX1 was transformed into BL21 (DE3) E. coli cells. The recombinant (His)_6-tagged pbx1 protein was expressed after isopropylthio-galactoside (IPTG) induction and purified using Ni-NTA beads (Qiagen, Chatsworth, CA) according to the manufacturer’s instructions.

Immunization and development of MoAbs. Balb/c mice were immunized with antigen on days 1, 7, 14, 21, and 28 and fused 3 days after the final immunization. The KLH-conjugated peptide 14 was used for the first three immunizations, and recombinant E2A/pbx1 junction protein together with conjugated-peptide 14 was used for the last two immunizations. Spleenocytes from immunized mice were then fused with F0 myeloma cells. Hybridoma supernatants were initially screened against KLH-conjugated peptide 14 by enzyme-linked immunosorbent assay (ELISA). Hybridomas secreting antibodies that recognize conjugated peptide 14 were further screened by dot blot analysis for specific reactivity against the peptide sequence corresponding to the junction of the chimeric protein. Selected hybridoma supernatants were purified via Sepharose G chromatography (Pharmacia) according to the manufacturer’s instructions.

Western blotting. The Namalwa Burkitt’s lymphoma cell line and the t(1;19)-carrying pre-B cell line 697 were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Cells in log phase were harvested and washed with phosphate-buffered saline (PBS). Cell lysates in sample loading buffer were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Immobilon-P membranes (Millipore, Bedford, MA). Western blotting was performed via the standard method using hybridoma supernatants and horseradish peroxidase (HRPO)-conjugated secondary antibodies and was developed by enhanced chemiluminescence (Pierce). For peptide blocking, 2 μg/mL of peptide 14 before using them in Western blot for reactivity with pbx1 and recombinant E2A/pbx1 junction analysis.

Immunohistochemical staining. Cells were attached to precoated slides (BioRad, Hercules, CA), fixed with 2% formaldehyde, washed with PBS, and permeabilized with 0.1% saponin plus 1% hydrogen peroxide in PBS. The cells were then blocked in 1% bovine serum albumin (BSA) plus 0.1% saponin and incubated overnight with the primary antibody. Biotinylated donkey antismouse immunoglobulin (Ig; Jackson ImmunoResearch Labs, West Grove, PA) was used as a secondary antibody. The staining was visualized using HRPO-conjugated streptavidin (Dako, Carpintera, CA) and 3,3’diaminobenzidine as chromogen.

Flow cytometric analysis. Approximately 5.5 × 10^6 cells were fixed with 4% paraformaldehyde, permeabilized with methanol, and blocked with 1% BSA. Cells were then incubated with 1 μg of E2A/pbx1 junction-specific MoAb for 30 minutes. An IgG1 isotype antibody was used as a negative control antibody. Cells were then stained with FITC-conjugated goat antirabbit Ig (2.5 μg/mL). For peptide 14 blocking experiments, 1 μg of junction-specific antibody was preincubated with 0.1 μg peptide 14 for 30 minutes before its incubation with cells. Cellular fluorescence was measured using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Clinical samples. Bone marrow samples of childhood leukemia were obtained from the tissue bank of St. Jude Children’s Research Hospital. The leukemic blast features of fourteen cases are summarized in Table 1.

RESULTS

Because initial attempts to raise MoAbs specific to the junction region using the conjugated peptide as the sole immunogen were not successful, we subsequently used purified recombinant GST fusion protein containing the E2A/pbx1 junction region as antigen for the last two immunizations to enhance the possibility of developing specific antibodies. This E2A/pbx1 junction fragment contained 20 amino acid residues from the E2A protein and 144 amino acid residues from pbx1. Of 1,000 hybridomas tested, 134 were positive with pbx1. Of these 134 clones was subcloned by limiting dilution and further tested with recombinant E2A/pbx1 junction protein by dot blotting. All of the 134 clones recognized the E2A/pbx1 junction protein. However, only antibodies from 22 clones failed to react with pbx1. These antibodies were further screened by Western blotting using cell lysate from

<table>
<thead>
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<th>Case</th>
<th>Sex</th>
<th>Leukocyte Count (×10^9/L)</th>
<th>Blast % *</th>
<th>FAB</th>
<th>Immunophenotype</th>
<th>Cytogenetics</th>
<th>Reactivity With G289-781</th>
<th>G265-061</th>
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<td>1</td>
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Abbreviation: FAB, French-American-British Cooperative Group Classification.

* Bone marrow blast percentage.
† Results of antibody reactivity by flow cytometry, immunohistochemistry, and Western blotting.
‡ Case of chronic myelocytic leukemia.
Fig 1. Western blot analyses of G289 MoAb clones. Total cell lysates from the E2A/pbx1 carrying line 697 (A) and the Burkitt’s lymphoma cell line Namalwa (B) were resolved on 4% to 20% SDS-PAGE and transferred to Immobilon-P membrane. Individual membrane strips were probed with various G289 clones, an E2A-specific G193-86 clone, and an isotype control MoAb using enhanced chemoluminescence.

Peptide 14 completely blocked the reactivity of those MoAbs to the E2A/pbx1 fusion protein in 697 cell lysate, as demonstrated in Fig 2. Peptide 14 could not block the E2A-specific reactivity of antibody G193-86.

To examine the specificity of these junction-specific MoAbs at a cellular level, the selected MoAbs were further characterized by flow cytometric analysis. Over 95% of 697 cells were stained by the G289-781 MoAb (Fig 3A) compared with fewer than 5% Namalwa cells (Fig 3B), a result similar to that obtained for the isotype control. The fluorescence of stained cells was intracellular because there was no staining observed by junction-specific antibody when cells were not fixed and permeabilized before the addition of anti-
result is consistent with published data that E2A/pbx1 is a nuclear protein.19,20

DISCUSSION

In childhood ALL, the t(1;19) (q23;p13.3) chromosomal translocation results in the fusion of the E2A and PBX1 genes, leading to expression of a fusion transcript. The translated chimeric protein is correlated with oncogenic properties, presumably due to its aberrant DNA binding and transactivation functions. However, results of transgenic mice studies indicate that the DNA binding domain of E2A/pbx1 is not essential for induction of malignant lymphomas, implying that the mechanism of action of the chimeric protein resulting from t(1;19) translocation is more complex. The availability of E2A/pbx1-specific MoAbs would therefore provide a valuable tool to elucidate the oncogenic mechanisms.

In the present study, we have developed MoAbs that are specific for the junction of the E2A/pbx1 chimeric protein. These antibodies do not interact with E2A or pbx1 alone. An especially important property of these antibodies is their ability to distinguish t(1;19)-positive from t(1;19)-negative body (Fig 3C). Furthermore, preincubation of 100 ng of peptide 14 with the MoAb completely blocked 697 cell staining (Fig 3A). These combined results indicate that the 697 cell staining was E2A/pbx1 specific. The significant difference in staining between E2A/pbx1-positive and -negative cells implies that these antibodies can be used for flow cytometric studies. At equal concentration, the G289-781 clone showed the strongest fluorescence staining in comparison with the other analyzed clones.

To test the diagnostic potential of the G289-781 MoAb, bone marrow specimens from 14 patients with leukemia were examined, without prior knowledge of the diagnosis based on cytogenetic analysis or immunophenotyping. Our data showed that only 6 of 14 bone marrow specimens were positive for the E2A/pbx1 chimeric protein by Western blotting, flow cytometry, and immunohistochemistry. These results were found to be in exact agreement with the diagnosis based on cytogenetic analysis (Table 1). Figure 4A and B shows results of the Western blotting and flow cytometric analysis of representative bone marrow specimens from four patients using the G289-781 MoAb. Figure 4C shows immunohistochemical staining of representative t(1;19)-positive and t(1;19)-negative bone marrow specimens. The staining
Fig 4. Detection of the E2A/pbx1 chimeric protein in patient bone marrow specimens using the G289-781 MoAb. (A) Western blot analysis of specimens from four representative patients (lanes 1 to 4), 697 cell lysate (lane 5), and Namalwa cell lysate (lane 6) using the G289-781 MoAb. (B) Flow cytometric analyses of the same patient specimens with an isotype control antibody (----) or G289-781 MoAb (-----). (C) Representative immunohistochemical staining of bone marrow specimens from patient 1 (top) and patient 3 (bottom) using an isotype-matched negative control antibody (left), an E2A-specific positive control antibody (G98-271.1, center), and the E2A/pbx1-specific G289-781 MoAb (right).
for the detection of minimal residual disease, and for monitoring early relapse and response to therapy.

In summary, we report the development and characterization of MoAbs that are highly specific for the common form of the E2A/pbx1 chimeric protein found in most t(1;19)-containing ALLs. These MoAbs do not react with either E2A or pbx1 protein alone. The use of a large number of clinical specimens from patients with leukemia will allow a detailed evaluation of their clinical application as well as their utility in understanding the pathogenic mechanisms of the E2A/pbx1 chimeric protein for this type of leukemia.

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