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# Chondroitin Sulfate Proteoglycan-4 (CSPG4)-Specific Monoclonal Antibody 225.28 in Detection of Acute Myeloid Leukemia Blasts

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Chondroitin sulfate proteoglycan-4 (CSPG4), a membrane-bound proteoglycan known to be expressed on the surface of malignant cells, has a restricted distribution in normal tissues. CSPG4 is a potential candidate tumor marker. We investigate CSPG4 expression on blasts in newly diagnosed acute myeloid leukemia (AML) patients and its relation with cytogenetic abnormalities and molecular markers known to have prognostic significance in this disease. Using hybridoma technology, we generated a specific monoclonal antibody (mAb), mAb 225.28, reactive with CSPG4. Blast samples obtained from the peripheral blood of newly diagnosed AML patients were analyzed for CSPG4 expression using the CSPG4-specific mAb and multiparameter flow cytometry. The results were correlated with cytogenetic and molecular characteristics of AML. CSPG4 was found to be expressed on a variable fraction of leukemic blasts in all AML patients with different leukemia morphology, including monoblastic cases. Reactivity of CSPG4-specific mAb with leukemic blasts was not limited to those with the rearranged MLL gene. CSPG4 was also expressed on AML blasts with a complex karyotype, FLT3 mutation, or NPM1 mutation. The results indicate that CSPG4 is expressed and detectable by flow cytometry using the mAb 225.28 on a proportion of blasts of all subtypes of AML irrespective of cytogenetic and molecular abnormalities. mAb 225.28 could be useful in detecting AML blasts by flow cytometry.

Key words: Chondroitin sulfate proteoglycan-4 (CSPG4); Acute myeloid leukemia (AML); Monoclonal antibody (mAb) 225.28; Cytogenetic and molecular abnormalities

## INTRODUCTION

Chondroitin sulfate proteoglycan-4 (CSPG4), a transmembrane proteoglycan, was originally identified as a highly immunogenic tumor antigen on the surface of melanoma cells. It consists of a 280-kDa N-linked glycoprotein and a proteoglycan component with the molecular weight of 450 kDa (1,2). CSPG4 is expressed on the surface of differentiated malignant cells, progenitor cells, and cancer-initiating cells in various types of solid tumors. CSPG4 has been shown to play an important role in the growth, migration, and metastatic dissemination of tumor cells (1,2). The complex mechanisms by which CSPG4 affects tumor progression are currently under investigation, and its association with other cell surface proteins and receptor tyrosine kinases as well as its potential role in modulating functions of these proteins are of special interest.

CSPG4 is highly expressed on malignant cells in various types of cancer and therefore is readily available to be targeted by monoclonal antibodies (mAbs). Therefore, investigating the expression of CSPG4 in patients with acute myeloid leukemia (AML) could lead to the development of effective CSPG4-targeted mAb-based therapies. Using a mAb known as 7.1 that recognized the human homolog of the rat NG2 chondroitin sulfate proteoglycan molecule, it was previously demonstrated that NG2 expression was variable in AML, correlated with 11q23 chromosomal abnormalities and was mostly detectable in monoblastic cases (3–9). Furthermore, its expression had a predictive value, as greater NG2 expression on leukemic blasts correlated with poor responses to chemotherapy and shorter progression-free survival. NG2 was not detected on the surface of normal hematopoietic precursor cells or leukemia stem cells (3–9).

Expression of CSPG4 on blasts in newly diagnosed AML patients using mAbs specific for the CSPG4 has not been extensively investigated. Also, CSPG4 expression on blasts and its relation to molecular markers known to

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have prognostic significance in AML merit further investigation. Using hybridoma technology, we generated a mAb 225.28 reactive with CSPG4 and used this mAb to characterize antigenic determinants expressed on AML blasts. Here we evaluate CSPG4 expression on myeloblasts isolated from peripheral blood of newly diagnosed adult AML patients and examine its relationship to different cytogenetic and molecular abnormalities and distinct morphologic subtypes of AML.

## MATERIALS AND METHODS

*Patients, Flow Cytometry, and Antibodies*

Blood samples were obtained from newly diagnosed AML patients prior to any treatment ($n = 18$). All subjects signed an informed consent approved by the Institutional Review Board at the University of Pittsburgh. Peripheral venous blood (20–50 ml) was collected into heparinized tubes. The samples were hand-carried to the laboratory and processed using Ficoll-Hypaque gradients. Peripheral blood mononuclear cells (PBMCs) were recovered, washed in AIM-V medium (Invitrogen, Carlsbad, CA, USA), counted in a trypan blue dye, and immediately used for experiments.

Mouse anti-human CD33-PE-Cy7 (IgG1) and mouse anti-human CD45-PerCP-Cy5.5 (IgG1) were purchased from eBioscience (San Diego, CA, USA). Mouse anti-human CD34-APC (IgG1), mouse anti-human CD117-PE (IgG1), and all mouse IgG1 κ isotype controls were purchased from Biolegend (San Diego, CA, USA). DAPI was purchased from Invitrogen (Grand Island, NY, USA).

The CSPG4-specific mouse mAb 225.28, an IgG2a, was developed and characterized in Dr. Soldano Ferrone's laboratory as previously described (9–11). Briefly, this mAb was generated from a BALB/c mouse immunized at weekly intervals with three injections of $1 \times 10^7$ melanoma cells (colo38) treated with recombinant IFN-γ (1,000 U/ml for 72 h). Splenocytes from immunized mice were hybridized with murine myeloma cells. Hybridization, subcloning, and growth of hybridoma in culture and in the peritoneal cavity of BALB/c mice were performed according to standard procedures (12). Ascitic fluids were obtained, clarified by centrifugation, diluted, and precipitated with 50% saturated ammonium sulfate. The precipitate was dialyzed against 5 mM Tris buffer, pH 7.5, and chromatographed on DEAE-Sepharose in the same buffer. Purity of collected fractions was determined by SDS-PAGE in 7.5% gel under nonreducing conditions; Ig fractions were combined and concentrated to 5–10 mg/ml on Amicon P-30 membrane. For use in flow cytometry, mAb 225.28 was conjugated with fluorescein isothiocyanate (FITC) at the Genomics and Proteomics Facility (University of Pittsburgh, Pittsburgh, PA, USA). Isotype control mouse IgG2a-FITC was purchased from BD Biosciences (San Jose, CA, USA). Human TruStain FcX was purchased from BioLegend.

For flow cytometry, cells were incubated for 10 min at 4°C with 5 µl of human TruStain FcX to block nonspecific binding. Cells were either stained individually or in combination with 5 µl of Abs specific for CD45, CD117, CD33, CD34, or CSPG-4 and corresponding isotype controls for 30 min at 4°C. After incubation, cells were washed twice with 2% BSA–PBS, resuspended in 400 µl 2% BSA–PBS, and 4 µl of DAPI was added to all samples prior to analysis. Approximately $6–8 \times 10^4$ live cells were acquired for analysis using a Cyan Beckman-Coulter flow cytometer (Brea, CA, USA). Prior to use, all mAbs were titrated to establish optimal staining dilutions using normal PBMC and AML blasts.

## RESULTS AND DISCUSSION

Evidence indicates that CSPG4 plays an important role in the proliferation, survival, and migration of malignant cells, as it is involved with the related signaling pathways. These properties of CSPG4 provide a mechanism for the ability of CSPG4-specific mAb to inhibit the in vitro growth and migration of cancer cells that express CSPG4 and their growth and metastatic spread in immunodeficient mice. As CSPG4 has restricted distribution in normal tissues, this may account for the lack of significant side effects when CSPG4-specific antibodies have been induced in patients by active specific immunotherapy (2,13–16).

In the current study, we evaluated CSPG4 expression on blasts in newly diagnosed AML patients and its relation with cytogenetic abnormalities or molecular markers known to have prognostic significance in this disease. Expression on the cell surface of markers that are not present on normal cells permits the identification of leukemia blasts. Since CSPG4 has highly restricted distribution in normal hematopoietic tissues, it could be considered as a potential AML blast marker and a potential therapy target. Using hybridoma technology, the mAb 225.28 reactive with CSPG4 was generated. It was labeled with the fluorochrome (FITC) and used for direct staining of peripheral blood cells by flow cytometry. Flow cytometry demonstrating expression of CSPG4 using the mAb 225.28 in acute leukemia cell lines and the gating strategy used for multicolor flow cytometry for the AML patients' blood samples is presented in Figure 1.

CSPG4 expression was detected in 18/18 peripheral blood samples containing AML blasts at levels ranging from 6% to 45% (Table 1). Three samples showed less than 10% positivity, three showed 10–19% positivity, four showed 20–29% positivity, and eight showed greater than 30% positivity. CSPG4 expression was not confined to

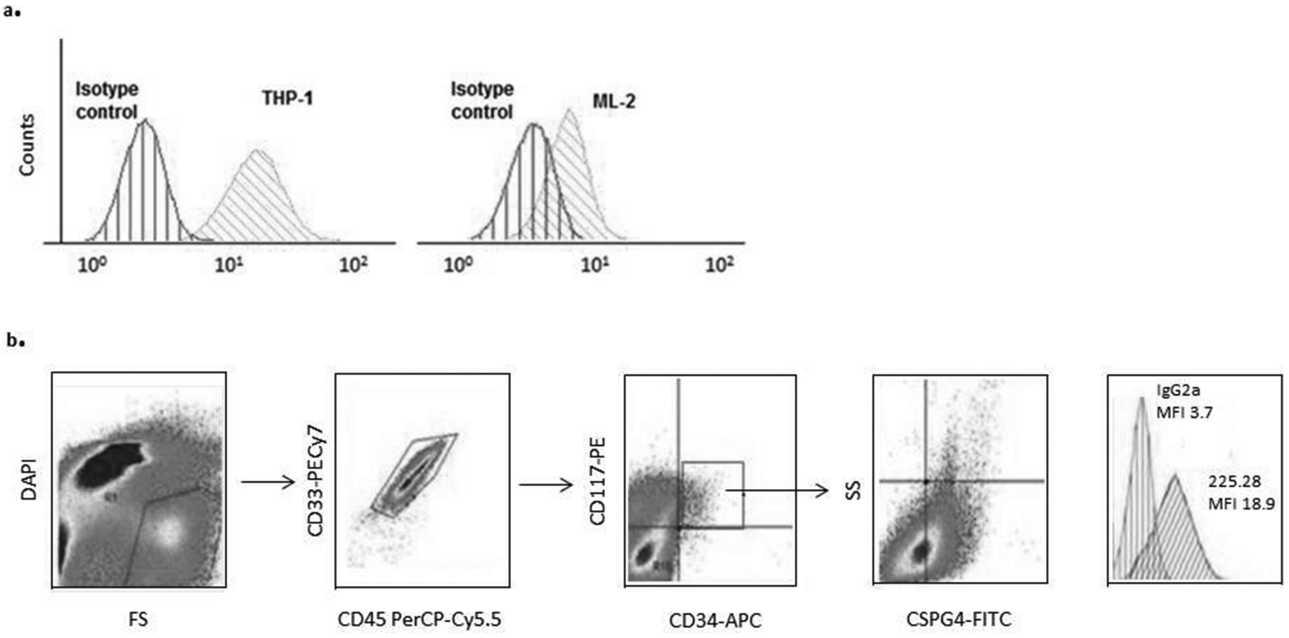


**Figure 1.** CSPG4 expression on acute leukemia cell lines and the gating strategy used for CSPG4 detection on myeloblasts in the peripheral blood of AML patients. (a) mAb 225.28 (or isotype control IgG2a Ab) was used to demonstrate expression of CSPG4 on the surface of THP-1 and ML-2 cells maintained in culture. (b) Abs specific for CD45, CD117, CD33, or CD34 were used to identify myeloblasts. Cells were individually stained with each antibody and used to set appropriate voltage and gain for each fluorochrome against its isotype. For analysis of patient samples, mAb 225.28 was labeled with FITC fluorochrome and used for direct staining of cells prior to flow cytometry.

any single blast phenotype as defined by costaining with CD45, CD34, CD117, and CD33, the markers that define leukemia blasts in this study. Though we found high levels (>30%) of mAb 225.28 reactivity in the two samples with t(9;11), other samples without 11q23 abnormality also had equally high CSPG4 expression. mAb 225.28 reactivity was also found in AML with other known prognostic factors: FLT3 mutation, NPM1 mutation, or complex cytogenetic abnormalities (Table 1). Furthermore, in the nine patients who had either myelomonocytic (M4) or monocytic (M5) leukemia, we found that all of these cases showed relatively high levels of CSPG4 expression irrespective of any cytogenetic or molecular abnormalities (Table 1).

Prior studies in AML using mAb7.1 recognized the chondroitin sulfate proteoglycan NG2 on blasts in some but not all leukemias (4,6,7). In contrast to prior studies, using mAb 225.28, we detected expression of CSPG4 in blasts of all AML evaluated; its expression was not restricted to 11q23/mixed-lineage leukemia (MLL) blasts. However, in all cases, only a proportion of blasts were positive, suggesting that the mAb 225.28 recognizes a unique CSPG4 epitope, which is expressed on AML blasts at a particular stage of leukemic differentiation (10,17). The fact that not all blasts were stained confirms the specificity of this mAb for CSPG4. We did not detect any correlation between the proportion of positive blasts and cytogenetic or molecular markers of poor prognosis in AML. This may be due to the fact that only a relatively small number of cases were investigated. This small number of cases, together with the variability of initial treatments in the AML cohort, likely contributed to our inability to draw any prognostic or predictive conclusions based on 225.28 mAb reactivity. Nevertheless, the study did include AML patients with a variety of cytogenetic and molecular abnormalities and demonstrated the presence of CSPG4+ blasts in all cases. In addition, we detected CSPG4 expression on acute leukemia cell lines with and without MLL gene rearrangement.

In aggregate, our results indicate that mAb 225.28 might be helpful in detecting AML blasts by flow cytometry in the peripheral blood of all AML patients regardless of the leukemia morphology or cytogenetic and molecular abnormalities. Further studies are necessary to investigate the differential CSPG4 expression in a proportion of the blasts and to determine if and how this differential expression correlates with that of established AML prognostic factors.

**Table 1.** Cytogenetic, Molecular Profile, and CSPG4 Expression Detected by mAb 225.28 in Patients With AML

| Patients | AML | WBC Count at Diagnosis, $10^9$/L | % Blasts in Peripheral Blood | Cytogenetic and Molecular Analysis | Percentage (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | CD45 | CD117 | CD33 | CD34 | CSPG4+Cells |
| 1 | Monocytic | 96.6 | 74 | XY; FLT3+, NPM1+ | 100 | 13 | 99 | 3 | 45 |
| 2 | Myelomonocytic | 9.9 | 7.5 | XY | 99 | 15 | 99 | 16 | 45 |
| 3 | Monocytic | 9.7 | 20 | t(9;11) | 99 | 0 | 95 | 0 | 41 |
| 4 | Monocytic | 101 | 16 | t(2;4), +8, monosomy 4q, trisomy 3q | 98 | 0 | 98 | 0 | 38 |
| 5 | Monocytic with mutated NPM1 | 7 | 19 | XY; NPM1+ | 100 | 11 | 97 | 1 | 38 |
| 6 | Monocytic | 20.9 | 27 | XX; FLT3+ | 99 | 32 | 97 | 4 | 36 |
| 7 | Myelomonocytic | 60 | 58 | t(9;11) | 100 | 1 | 99 | 0 | 36 |
| 8 | Monocytic | 72.5 | 78 | t(2;17), FLT3+, NPM1+ | 100 | 69 | 98 | 33 | 30 |
| 9 | Myelomonocytic | 48.8 | 25 | add 8 | 80 | 69 | 99 | 51 | 28 |
| 10 | AML, not otherwise specified | 14.3 | 61 | XX | 100 | 64 | 70 | 65 | 23 |
| 11 | AML with myelodysplasia-related changes | 7.6 | 62 | del x, del 5, +7, t(17;20), –16, –18 | 100 | 64 | 83 | 84 | 21 |
| 12 | AML with mutated NPM1 | 10.8 | 76 | XY; NPM1+ | 99 | 63 | 69 | 0 | 20 |
| 13 | AML with myelodysplasia-related changes | 15.7 | 77 | del 2, inv 4, –5, +8, del 8, del y, del 15, del 18, add 22, –21, +21 | 99 | 80 | 68 | 79 | 13 |
| 14 | AML with mutated NPM1 and FLT3 | 211 | 94 | XY; FLT3+, NPM1+ | 97 | 74 | 98 | 0 | 11 |
| 15 | AML, not otherwise specified | 62.4 | 87 | add 12 | 100 | 0 | 94 | 97 | 10 |
| 16 | AML with mutated FLT3 | 25 | 64 | XX; FLT3+ | 100 | 70 | 99 | 2 | 7 |
| 17 | AML with myelodysplasia-related changes | 6.9 | 9.5 | t(1;10), t(5;14), –12, –11, –14, inv 18, +21 | 94 | 53 | 94 | 36 | 7 |
| 18 | AML with mutated NMP1and FLT3 | 72.5 | 78 | XY; FLT3+, NPM1+ | 100 | 50 | 100 | 45 | 6 |

WBC, white blood cell; t, translocation; del, deletion; inv, inversion; FLT3, FMS-like tyrosine kinase-3; NPM1, nucleophosmin gene.

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