CD28, a Marker Associated with Tumoral Expansion in Multiple Myeloma

Nelly Robillard, Gaëtan Jego, Catherine Pellat-Deceunynck, Danielle Pineau, Denis Puthier, Marie-Paule Mellerin, Sophie Barillé, Marie-José Rapp, Jean-Luc Harousseau, Martine Amiot, and Régis Bataille

Laboratoire d’Hématologie [J. R., D. P., M-P. M., R. B.], Oncogénèse ImmunohématoLOGique, Institut National de la Santé et de la Recherche Médicale Unité 463 [G. J., C. P-D., D. P., S. B., M. A., R. B.], and Département d’Hématologie Clinique [M-J. R., J-L. H.], Institut de Biologie, 44 093 Nantes Cedex 01, France

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‡ The first two authors contributed equally to this work.

ABSTRACT

CD28 expression was thoroughly investigated on plasma cells of monoclonal gammopathy of undetermined significance, multiple myeloma (MM), and human myeloma cell lines. CD28+ plasma cells were detected in 19% of 31 monoclonal gammopathy of undetermined significance, 41% of 116 MM, and 100% of 13 human myeloma cell lines. CD28+ myeloma cells were detected in 21 of 79 (26%) MM cases at diagnosis, 13 of 22 (59%) at medullary relapse \((P < 0.009)\), and 14 of 15 (93%) at extramedullary relapse \((P = 0.05)\), including 10 of 10 (100%) secondary plasma cell leukemias \((P = 0.05)\). Serial studies in individual patients confirmed the emergence of CD28+ myeloma cells with tumoral expansion and treatment failure. This was significantly correlated with the expression of CD28 ligand, i.e., CD86 (but not CD80), and with an increase in the proliferative activity (labeling index) of myeloma cells in bone marrow. Whereas the expression of CD56 defines a particular subset of myeloma patients, CD28 is the only antigen for which expression correlates with tumor progression. Our data show that an aggressive compartment of CD28+ and CD86+ myeloma cells emerges during the course of MM in vivo, indicating that CD28 could be aberrantly expressed on highly malignant (possibly mutated) myeloma cells. Conversely, a subset of proliferative plasmablasts coexpressing CD28 and CD86 could be the normal counterpart of the clonogenic myeloma stem cell because a subset of CD28+ plasma cells was observed in 6 of 6 cases of reactive plasmocytosis.

INTRODUCTION

CD28, a well-documented antigen expressed on human T lymphocytes, plays an essential role in T-cell activation and T-cell-B-cell interactions through its specific ligands, especially CD86 (reviewed in Refs. 1–4). It is now well established that signaling through the T-cell receptor is necessary but not sufficient to induce antigen-specific T-cell activation and cytokine secretion. A costimulatory signal involving the T-cell surface molecule CD28 and its ligands is essential for T-cell clonal expansion, lymphokine secretion, and effector function. If this costimulatory signal is not delivered, T cells enter a state of long-term unresponsiveness to specific antigen (referred to as anergy; Refs. 1–4). In 1989, Kozbor et al. (5) reported the expression of CD28 on seven of seven HMCLs but not on resting B cells from peripheral blood or lymphoid organs, lymphoblastoid cell lines, or any other B-cell tumors. In 1990, Lee et al. (6) confirmed the expression of CD28 gene by malignant plasma cells from RPMI 8226 HMCL as a series of four distinct CD28 mRNA species: 1.3-, 1.5-, 3.5-, and 3.7-kb transcripts that were identical to those of T lymphocytes. More recently, we used flow cytometry and double immunofluorescence to reinvestigate the expression of CD28 on normal and malignant plasma cells and HMCLs (7–9) and found that normal plasma cells from tonsils and bone marrow did not express CD28 (7). However, this work showed for the first time that CD28 is expressed on fresh human myeloma cells from some but not all patients with MM (7). Finally, all HMCLs, unlike nonmalignant EBV+ lymphoblastoid cell lines, express CD28 (7–9). A major issue is to clarify the clinical significance of this antigen on human myeloma cells because our initial study showed a potential correlation between CD28 expression and disease severity (7). This study, which evaluated CD28 expression in a large number of patients with either benign or malignant plasma cell disorders, clearly shows that this antigen is a marker associated with disease progression and treatment failure.

MATERIALS AND METHODS

Patients. The expression of CD28 on plasma cells was investigated in 31 individuals with MGUS and 116 patients with MM, without \((n = 106)\) or with \((n = 10)\) a significant leukemic phase (secondary PCL). For these latter MM patients, the phenotype of myeloma cells was determined on bone marrow.
myeloma cells or both bone marrow and peripheral blood myeloma cells. Low- or high-risk MGUS was classified according to our previously reported criteria (10). The diagnostic criteria for MM were those of the Southwest Oncology Group (11). Seventy-nine MM patients (with or without a leukemic phase) were studied at diagnosis, 22 at medullary relapse, and 15 at extramedullary relapse (10 secondary PCLs, 3 pleural effusions, 1 skin and 1 ascites) and treatment failure. Six patients with peripheral blood reactive plasmacytosis during the course of viral infections (n = 4) or B- and T-cell lymphomas (n = 2) were included. In these patients, peripheral blood plasma cells were processed as myeloma cells.

**HMCLs.** The XGs and SBN1 HMCL were established by ourselves (8, 12). U266 and RPMI 8226 were purchased from American Type Culture Collection (Manassas, VA), and OPM2, LP1, L363, and NCI-H929 were from DSM (Germany). ANBL6 was a generous gift from D. Jelinek (Mayo Clinic, Rochester, MN).

**mAbs.** mAbs against the following antigens were used: Apo 2.7, CD19, CD28, CD38, CD45, CD56, CD80, CD86, and CD138 (i.e., syndecan-1). Apo 2.7-PE, anti-CD19-PE, anti-CD28-FITC, anti-CD38-FITC, anti-CD45-FITC, control IgG1-FITC, and control IgG1-PE were obtained from Immunotech (Marseille, France); anti-CD28-PE, anti-CD56-PE, and anti-CD80-PE were from Becton Dickinson (Heidelberg, Germany); anti-CD86-PE was from Pharmingen (San Diego, CA); rabbit anti-human k light-chain PE Ab was from DAKO (Glostrup, Denmark); and mouse anti-human Ig light-chain PE was from Caltag laboratories (San Francisco, CA). B-B4 mAb (CD138) is specific for normal and malignant plasma cells in bone marrow and peripheral blood and recognizes syndecan-1 (7, 13). Purified B-B4 mAb was biotinylated as described previously (7).

**Phenotypic Analysis.** For immunofluorescence staining, 5 × 10^5 cells were incubated with different FITC- or PE-conjugated or biotinylated mAbs for 30 min at 4°C in the presence of 20% human AB serum, followed by incubation (20 min at 20°C) with streptavidin-coupled to Quantum Red (Sigma Chemical Co., St. Louis, MO). Cells were fixed in 1% formaldehyde and analyzed on a FACSealibur flow cytometer with Cell Quest Software (Becton Dickinson). Data acquisition was always performed in two steps: first, 10,000 total cells were collected; and second, at least 5,000 plasma cells (CD138⁺) were acquired with an activated live gate on SSC versus CD138 dot plot. Apoptotic plasma cells were recognized by low expression of CD138 and Apo 2.7 staining and excluded from analysis.

CD138 plasma cell identification was controlled by coexpression of CD138, CD38, and intracytoplasmic k/λ immunofluorescence in a three-color assay. Briefly, cells were first surface-stained with CD138-biotin-streptavidin-Quantum Red and CD38-PE as described above, fixed, and permeabilized overnight in PBS with 1% formaldehyde and 0.01% Tween 20. For intracytoplasmic k/λ staining, permeabilized cells were washed and incubated for 30 min at 20°C with PE-conjugated Abs in PBS with 0.5% Tween 20. Cells were washed, and at least 5000 plasma cells (κ- or λ-positive cells) were collected. Careful analysis of the three-color staining revealed that >98% of either κ- or λ-positive cells coexpressed CD138 and bright CD38.

**Labeling Index of Myeloma Cells.** The labeling index of myeloma cells was measured according to a modification of Carayon and Bord (14) using anti-BrdUrd mAb (BU5-1; CytoBioscience, Euromedex, Soufflwegersheim, France) and analyzed by flow cytometry. The percentage of myeloma cells in S phase was determined by the number of positive cells for BrdUrd staining within the viable population of intracytoplasmic κ/λ-positive plasma cells. Briefly, 10⁶ cells were incubated with or without (control) 50 μM BrdUrd for 2 h at 37°C in 5% CO₂ incubator, washed, and permeabilized overnight in PBS containing 1% paraformaldehyde and 0.01% Tween 20 at 4°C. After two washes, cells were incubated for 30 min at 37°C with 50 Kunitz units of DNase I (Sigma). After DNase digestion, cells were washed in 0.5% Tween 20 PBS and labeled using PE-coupled anti-light chain Abs for 30 min at room temperature. Following two washes, both control cells and the incorporated BrdUrd cells were stained with 10 μl of FITC-BU5-1 mAb in PBS supplemented with 0.5% Tween 20 and 20% AB serum for 45 min at room temperature. After two washes, the cells were resuspended in PBS and analyzed immediately on a FACScalibur flow cytometer. For this purpose, data acquisition was performed on 50,000 cells and then on 10,000 live gated κ-/λ-positive cells when the plasmacytosis represented <20% of cells.

**Statistical Analyses.** The Wilcoxon rank sum test, the χ² test (with Yates correction, if necessary), and the Fisher exact test were used for statistical studies.

**RESULTS**

**CD28 Is Expressed on All HMCLs.** CD28 gene was expressed on all 13 HMCLs tested (Table 1) as a series of four distinct transcripts that were identical to those of T lymphocytes (data not shown). Although CD80, a CD28 ligand, was not expressed on HMCLs, a weak but significant expression of CD86, another CD28 ligand, was found on 4 of 13 (30%) HMCLs (Table 1).

**CD28 Myeloma Cells Emerge during Tumoral Expansion in Patients with MM.** To investigate the clinical and biological significance of CD28 expression in fresh human myeloma cells, we used the largest pool of patients with plasma
cell disorders ever studied. CD28 was clearly expressed on freshly explanted malignant plasma cells in 41% of patients with MM. The median percentage of CD28+ myeloma cells per patient was 100% (range = 10–100%). Few patients with <10% of CD28+ myeloma cells were classified as CD28− MM. A more detailed analysis of CD28 expression on malignant plasma cells showed a clear correlation with disease progression. As indicated in Fig. 1, CD28+ myeloma cells were only found in 26% of previously untreated patients with MM but in 72% of relapsing patients (P < 0.0001). When relapses were investigated more carefully, CD28+ myeloma cells were found in 59% of medullary relapses but in 93% of extramedullary relapses (P = 0.05). For the extramedullary relapses, 10 samples came from MM patients in leukemic phase (i.e., secondary PCL), and 5 came from ascitis, skin lesions, or pleural effusions. In the former cases, CD28 expression was found in 10 of 10 cases. Of note, in two of these secondary PCLs, CD28 was expressed on peripheral blood myeloma cells but not on bone marrow myeloma cells. In the latter cases, CD28 expression was found in four of five samples. Thus, tumor expansion was clearly associated with a growing CD28+ myeloma cell compartment. It is noteworthy that CD28 expression was observed in >90% of extramedullary tumors, which are usually associated with treatment failure. Serial evaluations of CD28 were performed in three patients to clearly show that CD28 expression emerged during tumor expansion in individual patients. In all three cases, medullary relapse was associated with the appearance of CD28. In one of these cases, CD28 evaluation was performed at diagnosis, medullary relapse, and extramedullary relapse (i.e., secondary PCL). As indicated in Fig. 2, an expanding CD28+ myeloma cell compartment was detected in conjunction with tumor progression (0, 53, and 94%, respectively, for the three evaluation times). Interestingly, CD28+ plasma cells were found in 19% of individuals with MGUS. The subset of CD28+ MGUS was too small (n = 6 of 31) to allow any conclusions to be drawn. However, the follow-up of these individuals, as compared to patients with CD28+ MM, will be of particular interest in terms of malignant conversion into overt MM.

**CD28 Myeloma Cells Also Emerge in the Special Subset of MM Lacking CD56.** CD19 and CD56 antigens were thoroughly investigated in conjunction with CD28. We and others have previously reported that malignant plasma cells lack CD19 but overexpress CD56, as compared to normal plasma cells (7, 15–17). As expected, most patients included in the current study did not express CD19 (98.8% of cases) but overexpressed CD56 (83% at diagnosis). Interestingly, as previously pointed out by Kawano and colleagues (17), no patient (of 116 total patients) presented the phenotype of normal plasma cells (CD19+, CD56−). Of note, CD56 was completely lost in malignant plasma cells of patients with secondary PCL, both in bone marrow and peripheral blood (n = 10). To determine whether this could have been due to late down-regulation of the antigen in secondary PCL, we re-investigated CD56 expression in the original samples at diagnosis from four CD56− secondary PCLs. In three of these four cases, CD56 was partially or totally lacking on bone marrow plasma cells at diagnosis. Thus, in contrast to CD28, CD56 did not correlate with disease progression but designated a specific subset of myeloma patients detectable at diagnosis. Interestingly, CD28 was more frequently observed at diagnosis in CD56− (57% of cases) than CD56+ patients (17%, P = 0.002; Table 2). Furthermore, as indicated in Table 2, CD28 expression increased significantly with disease progression in both CD56 subsets of patients.

**CD28 Expression Correlates.** The importance of ligands in the biology of CD28 led us to investigate the expression of CD80 and CD86 on fresh human myeloma cells. CD80 was not found in 20 consecutive patients with MM, regardless of disease activity, whereas significant CD86 expression was observed in 17 of 25 (68%) consecutive patients with MM. Interestingly, a significant association was found between CD28 and CD86 expression: all CD28+ MM patients (n = 10) expressed CD86 but only 46% of CD28− patients expressed CD86 (7 of 15; P = 0.018; Fig. 3A).
Given the significant association of a detectable compartment of CD28+ myeloma cells with disease progression, we searched for a correlation between the presence of this compartment and tumor kinetics in the bone marrow of newly diagnosed patients with MM. It was particularly noteworthy that the median labeling index of myeloma cells was 4% (range = 0.6–4.5%) in 7 CD28+ patients but only 1.1% (range = 0–4.5%) in 16 CD28− patients (P = 0.02; Fig. 3B). These data indicate that CD28+ MM were more proliferative (and thus more aggressive) than CD28− MM at diagnosis.

**CD28 Is Detected on Reactive Plasma Cells.** We previously found that nonproliferative and highly differentiated plasma cells within bone marrow did not express CD28 (7). In view of our current results in MM, it seemed to be of interest to investigate CD28 expression on reactive plasma cells, which are highly proliferative but less differentiated (18). Six cases of reactive plasmocytosis were studied using the same methodological approach, and the median percentage of reactive polyclonal plasma cells in peripheral blood was found to be 9% (range = 4–70%). In all cases, the phenotype was normal: CD138++, CD38++, k−λ−, CD19−, CD11a+, and CD56−. A significant population of CD28+ plasma cells was found in all cases (range = 1–12%, median = 6%).

**DISCUSSION**

CD28, a well-documented antigen expressed on human T lymphocytes, plays an essential role in T-cell activation and T-cell-B-cell interactions through its specific ligands: B7-1, i.e., CD80; and B7-2, i.e., CD86 (1–4). In 1989, Kozbor et al. (5) reported the expression of CD28 on 7 of 7 HMCLs, an observation confirmed by us and others (7–9, 19). Thus, it is clear that CD28 is a universal marker expressed on 100% of HMCLs but not on other cells of the B-cell lineage, including EBV− B-cell lines. At the transcriptional level, its expression on HMCLs is identical to that of T cells. Although all HMCLs express CD28, none express CD80, and only 30% express CD86 weakly.

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5 G. Jego et al., manuscript in preparation.
whereas EBV+ B-cell lines strongly express both CD80 and CD86.

More recently, we have found that (a) malignant plasma cells from some patients with MM express CD28, as do those of HMCLs (7), and that (b) normal well-differentiated and non-proliferative plasma cells (in bone marrow) do not express CD28 (7). Here, we carried out a more thorough assessment of this "T-cell antigen" on freshly explanted malignant plasma cells. Our current data clearly show that CD28 appears during disease progression. A continual increase in the percentages of MM patients expressing CD28 was found between the initial and terminal phases of the disease and at treatment failure, when almost all patients expressed CD28. This pattern was also observed in the important subset of MM lacking CD56 and is currently being investigated. Serial studies have clearly confirmed this concept in individual patients. The concept that CD28 expression is associated with tumoral expansion is also supported by the fact that the labeling index of newly diagnosed patients with CD28+ MM was 4 times greater than that of CD28− MM. This point strongly supports the concept that CD28+ MM is more aggressive (because it is more proliferative) than other forms. From a practical point of view, CD28 expression could be of prognostic value in MM and also in MGUS because CD28 was found to be expressed in some patients with these presenting features. Further investigation is needed to clarify these points. Basically, these in vivo data suggest that CD28 expression on malignant plasma cells could reflect intrinsic cell malignancy (in terms of oncogenic events accumulated in a clone) with higher proliferative capacity. It is of interest to emphasize that HMCLs generally emerge from samples obtained during extramedullary relapse or from the peripheral blood of secondary PCL, for which 100% of samples and cells express CD28 as HMCLs, with frequent Ras and p53 point mutations (20, 21). Further investigation will be necessary to clarify the mechanisms leading to the up-regulation of CD28 on these tumors. It would appear that these observations are not consistent with our finding of CD28 expression in 6 of 6 cases of reactive plasmocytosis in which plasma cells were not malignant. However, these cases of reactive plasmocytosis were highly proliferative. Thus, the intrinsic malignancy of myeloma cells related to the occurrence of Ras and/or p53 mutations (Refs. 20 and 21; or other unknown oncogenic mutations) during disease progression could make them proliferative enough to express CD28. Another possibility is that a normal but prolif-

Table 2 Expression of CD28 in both CD56+ and CD56− subsets of MM

<table>
<thead>
<tr>
<th>Disease status</th>
<th>CD56+ subset</th>
<th>CD56− subset</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnosis</td>
<td></td>
<td>P = 0.002</td>
</tr>
<tr>
<td>Medullary relapse</td>
<td>17% (10/56)</td>
<td>57% (12/21)</td>
</tr>
<tr>
<td>Extramedullary relapse</td>
<td>56% (9/16)</td>
<td>100% (5/5)</td>
</tr>
<tr>
<td>Extramedullary sites</td>
<td>75% (3/4)</td>
<td>100% (1/1)</td>
</tr>
<tr>
<td>Secondary PCL</td>
<td>No patient</td>
<td>100% (10/10)</td>
</tr>
</tbody>
</table>

Fig. 3 CD28 expression correlates. A, expression of CD86 on plasma cells (CD138+) according to CD28 expression. B, labeling index of plasma cells was defined as described in ”Materials and Methods.” Median values are indicated in the figure (—).
CD28 Expression on Human Myeloma Cells

Clear function for this antigen, not only on myeloma cells but also on normal (reactive) plasma cells. However, activation of this antigen through its CD86 ligand (although weakly expressed) could facilitate myeloma cell survival by up-regulation of antiapoptotic molecules such as Bcl-X<sub>L</sub>, as described for T lymphocytes. In this context, it is noteworthy that chemoresistance in MM, which is associated with overexpression of CD28 (this data), is also related to overexpression of Bcl-X<sub>L</sub>. Thus, further investigation is needed to delineate the role of CD28 on reactive plasma cells and human myeloma cells.

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