REVIEW

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Clinical and biological significance of surface molecules in myeloma

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Phenotypic analysis of tumor cells by flow cytometry is essential for various hematological malignancies. Surface antigen analysis of multiple myeloma cells is also widely used in clinical practice. However, the heterogeneity of surface molecule expression for malignant plasma cells often makes it difficult for hematologists and physicians to interpret the data. In this review, we focus on: 1) surface molecules for detection of myeloma cells; 2) surface molecules targeted by antibody-based therapy; and 3) surface molecules possibly expressed on myeloma-initiating cells. Understanding the significance and heterogeneity of surface molecule expression in multiple myeloma cells could lead to a better understanding of myeloma biology and myeloma treatment strategies.

Key words: multiple myeloma, surface molecules, anti-body therapy, myeloma-initiating cells

1. Introduction

Phenotypic analysis of tumor cells by flow cytometry is now essential for various hematological malignancies, such as acute leukemia and malignant lymphoma. Flow cytometric analysis is also widely used in the clinic for the diagnosis of multiple myeloma (MM) patients. The advantages of flow cytometry over other techniques, such as morphological analysis or immunohistochemistry, are that it is objective and results can be quantified. Considering these advantages, phenotypic analysis by flow cytometry is used for the differential diagnosis of MM, monoclonal gammopathy of undetermined significance and other related disorders, prediction of prognosis, quantification of minimal residual disease, and evaluation of treatment response.

The detection of plasma and MM cells by flow cytometry has previously been conducted using the CD38 gating method. However, although plasma cells are often a bright $CD38^{+}$ population, the heterogeneity of surface molecule expressions on malignant plasma cells often makes it difficult to interpret the data. It is widely debated whether antibodies against CD19, CD45, CD56, and CD138, should be combined with anti-CD38 antibodies to detect and distinguish malignant plasma cells from their normal counterparts.

Progress in flow cytometry techniques has led to the identification of cancer-initiating cells (cancer stem cells) in various tumors. The existence and phenotypes of myeloma-initiating cells has also been proposed by several groups, although these findings remain controversial. In this review, we discuss the current state of flow cytometry analysis related to MM.

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2. Surface molecules used to detect myeloma cells

2-1. Molecules expressed in normal and malignant plasma cells

CD38 is a type II transmembrane glycoprotein that is involved in cell adhesion, signal transduction, and regulation of intracellular calcium mobilization. Plasma cells and MM cells have been detected using CD38 as a primary gating antigen because they exist in $CD38^{++}$ (strongly positive) lesions [1]. Although hematopoietic stem cells, and T and B lymphocytes express CD38, plasma cells express CD38 at a much higher intensity. However, MM cells have been reported to express CD38 at lower levels than those in normal plasma cells [2–4].

CD138, also known as syndecan-1, is a heparan sulfate proteoglycan expressed by plasma cells but not by T or

B lymphocytes. CD138 mediates the adhesion of MM cells to extracellular matrix, especially type-1 collagen [5]. CD138 is also considered to be a differentiation antigen; during normal B-cell development, abundant CD138 expression is highly specific for terminally differentiated plasma cells in the bone marrow [6]. Because CD138 expression is also a hallmark of MM cells, it has been used for MM cell purification from bone marrow samples of patients [7]. CD138 expression should be evaluated carefully as it is known to be lost from the cell surface during apoptosis [8]. Heterogeneity of CD138 expression in bone marrow MM cells, and the existence of $CD138^-$ MM cells have been reported by several groups [9]. We reported a significant decrease in CD138 expression in patients with relapsed/progressive disease compared with that in untreated MM patients. Patients with low levels of CD138 had worse overall survival compared with those that had high levels of CD138 expression [10].

CS1 is a cell surface glycoprotein that belongs to the immunoglobulin (Ig) gene superfamily. CS1 is specifically expressed at high levels in normal and malignant plasma cells, and is expressed at low levels in natural killer (NK) cells and $CD8^+$ T cells [11]. Because CS1 is a downstream target of interferon regulatory factor 4 (IRF4) [12], a key transcription factor for plasma cell differentiation, it is considered a plasma cell differentiation molecule.

2-2. Differential expression of molecules between normal and malignant plasma cells

CD19, which is a pan-B-cell marker, is preserved in normal plasma cells but is absent in MM cells [1]. Expression of CD19 is absent in greater than 95% of MM patients [2]; the reasons for this absence are not clearly understood. However, alteration of PAX-5, a B-cell-specific transcription factor, which has been seen in MM cells, could be associated with the absence of CD19 expression [13]. CD20 is a transmembrane phosphoprotein specifically expressed in B cells, but not expressed in normal plasma cells. CD20 is known to be expressed in 15–20% of MM patients [2,14]; it is associated with small mature plasma cell morphology and t(11;14) [15]. A higher frequency of CD20 expression in plasma cell leukemia has been reported [16].

CD27 is a marker of memory B cells, and is also expressed in normal plasma cells. Interaction of CD27 with its ligand, CD70, is important for the differentiation of plasma cells. Although CD27 has been detected on normal plasma cells, its expression is lost as MM progresses. It is also known that CD27 expression is associated with better MM prognosis [17,18]. CD45, known as leukocyte common antigen, is a protein tyrosine phosphatase widely expressed in hematopoietic cells. CD45 is highly expressed in tonsil and peripheral blood plasma cells, while normal bone marrow plasma cells are a mixed population of $CD45^+$ and $CD45^-$ cells [19]. MM cells in the bone marrow are also a mixture of $CD45^+$ and $CD45^-$ cells, however the majority are $CD45^-$ [19,20]. Several reports have shown that patients lacking CD45 expression have relatively short survival [20,21]. CD45 is composed of several isoforms that vary in their extracellular region as a result of alternative splicing [22]. The major isoform expressed in MM cells is CD45RO, while normal plasma cells express CD45RA [23]. *In vitro* studies have shown that $CD45^+$ MM cells have a greater proliferative capacity [19] and are responsive to IL-6 stimulation [24].

Table 1. Aberrant surface antigen expression in MM cells compared with normal plasma cells

Antigen	Expression in normal plasma cells	MM cases with aberrant expression (%)
CD19	Positive	Negative (>95%)
CD20	Negative	Positive (15–20%)
CD27	Positive	Negative (40–50%)
CD45	Negative or Positive	Negative to Dim (70–80%)
CD56	Negative to Dim	Positive (70–80%)

CD56, also referred to as NCAM-1 (neural cell adhesion molecule 1), is a cell adhesion protein that is another member of the Ig superfamily, and is often considered an NK cell marker. CD56 is weakly expressed, or absent, in normal plasma cells; however it is over expressed in MM cells [25]. Lack of CD56 expression in MM cells is reportedly associated with a lambda isotype [25], t(11;14) [26], plasma cell leukemia [27], and fewer osteolytic lesions [28]. It was reported that $CD56^-$ MM patients have a poorer prognosis compared with $CD56^+$ patients [29]. Prognosis when combination therapies using novel agents are employed need to be re-evaluated. The aberrant expressions of surface molecules in MM cells are summarized in Table 1.

2-3. Combinations of surface molecules for gating MM cells

Traditionally, MM cells have been identified as $CD38^{++}$ $CD45^{-\text{ or dim}}$ cells; however there are some $CD45^+$ MM cells [14]. Harada et al. targeted three surface antigens (CD38, CD19, and CD56) to distinguish MM cells from normal plasma cells [1]. They reported that MM cells are $CD38^{++}CD19^-CD56^+$ or $CD38^{++}CD19^-CD56^-$, while normal plasma cells are $CD38^{++}CD19^+CD56^-$; as a consequence of these findings, several groups are now employing six-color screening panels. A British group used a combina-

tion of cytoplasm-λ, cytoplasm-κ, CD19, CD38, CD45, and CD138 to detect minimal residual disease with high efficacy [30]. The European Myeloma Network recommends using CD38, CD138, and CD45 for plasma cell identification, and CD19 and CD56 to detect abnormal plasma cells [3]. This network prefers to include CD20, CD117, CD28, and CD27 for additional immunophenotyping.

Many groups have used different gating patterns to identify MM cells. The fewer antibodies used for analysis, the higher the possibility of contamination by non-plasma cells, however using too many antibodies for the initial gating could underestimate the proportion of MM cells. To avoid this, researchers should gate with a combination of antigens that are expressed at equal levels on MM and normal plasma cells, in conjunction with one or two antigens differentially expressed between normal and malignant plasma cells. However, researchers should be aware that MM cells could be unexpectedly missed by gating analysis because they are highly heterogeneous with respect to expression of surface antigens.

3. Surface molecules as targets for antibody-based therapies

Daratumumab is a human CD38 antibody designed to kill MM cells *via* antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) [31]. Daratumumab stood out from other CD38 antibodies because of its strong ability to induce CDC in MM cells. Its cellular toxicity is significantly strengthened with Lenalidomide, through activation of effector cells showing ADCC activity [32]. CD138 monoclonal antibodies have also been considered for targeted therapy. Ikeda et al. investigated the antitumor effects of a murine/human chimeric CD138-specific monoclonal antibody, nBT062, conjugated with maytansinoid derivatives against MM cells [33]. The immunoconjugates significantly inhibited growth of MM cell lines and primary tumor cells from MM patients, and lacked cytotoxicity against peripheral blood mononuclear cells from healthy controls. They also inhibited MM tumor growth *in vivo* and prolonged the survival of xenograft mouse models of human MM, and a SCID-hu mouse model. Rousseau et al. reported preliminary biodistribution and dosimetry results in refractory MM patients in a phase 1/2 RAIT study using an iodine-131-labeled anti-CD138 monoclonal antibody [34]. Four patients with progressive disease were enrolled after three lines of therapy. Grade 3 thrombocytopenia was observed in two cases, with grade 4 hematologic toxicity not observed. One patient experienced partial response and total relief of pain, which lasted for one year.

Elotuzumab, a humanized anti-CS1 monoclonal antibody, inhibited MM cell binding to bone marrow stromal cells (BMSCs) and induced ADCC against MM cells [11]. Administration of elotuzumab significantly induced tumor regression in MM xenograft models [11]. A combination of elotuzumab with bortezomib significantly increased the *in vivo* therapeutic efficacy in the SCID-hu mouse model [35]. In a phase I study of elotuzumab involving relapsed/refractory MM patients, 26.5% of patients had stable disease [36]. The most common adverse events were cough, headache, back pain, fever, and chills; these were generally mild to moderate in severity. Clinical trials of elotuzumab in combination with novel agents have also been conducted. A phase I trial of elotuzumab and bortezomib in relapsed/refractory MM patients showed that 48% of patients had an objective response in terms of a partial response or better [37]. The most frequent grade 3–4 toxicities were lymphopenia (25%) and fatigue (14%). Elotuzumab in combination with lenalidomide, and a low dose of dexamethasone, resulted in an objective response in 82% of patients [38]. The most frequent grade 3–4 toxicities were neutropenia (36%) and thrombocytopenia (21%). Considering these findings, combining elotuzumab and novel agents is well tolerated by individuals and could be a potential new combination therapy.

Many other surface molecules are under consideration for use in antibody therapies against MM [39]. Although antibody therapy has the potential to improve the outcome of MM treatments, they might not be powerful enough to work as a single agent, and should be combined with other chemotherapeutic agents. It should be remembered surface molecules on MM cells are heterogeneous between patients, and could change according to disease progression.

4. Phenotype of myeloma-initiating cells

4-1. $CD19^+$ and myeloma-initiating cells

Cancer stem cells (CSCs) are often considered to contribute to relapse and drug resistance in various cancers [40]. Advances in flow cytometry technologies have led to the identification of CSCs in various tumors, including hematological malignancies, by searching for the progenitors of lineage-specific antigens. Previous reports regarding CSCs, such as acute myeloid leukemia

Table 2. Reported phenotypes of MM-initiating cells

Reports	Phenotype of MM initiating cells
Matsui W, et al. Blood. 2004; 103: 2332-6 Matsui W, et al. Cancer Res. 2008; 68: 190-7	CD19+/CD20+/CD27+/CD138–
Kim D, et al. Leukemia. 2012; 26: 2530-7	CD19–/CD45 (low or –)/CD38 high/CD138+
Hosen N, et al. Leukemia. 2012; 26: 2135-41	CD38 high/CD19–/CD138+ or –

stem cells [41], derived from undifferentiated progenitor cells have led researchers to focus on the B-cell compartment of MM. Previous reports have shown that $CD19^+$ B cells in MM patients express the same Ig gene sequence as that in MM cells [42,43]. These reports indicate the involvement of a less mature cell fraction compared with plasma cells during MM pathogenesis. Pilarski et al. showed that $CD19^+$ B cells from MM patients can be transplanted into NOD/SCID mice and generate MM [44,45]. Matsui et al. reported that clonogenic MM cells exist in $CD19^+CD20^+CD27^+CD138^-$ lesions and can be treated with rituximab, a CD20 monoclonal antibody [46,47]. However, Kim et al. showed that $CD19^-CD45^{low\ or\ -}CD38^{high}CD138^+$ cells are tumorigenic MM cells [48]. Hosen et al. reported that both $CD138^+$ and $CD138^-$ cells in $CD38^{high}CD19^-$ lesions can initiate MM [49]. These conflicting results (Table 2) are because of multiple issues, including the use of different xenograft models and the variation in the heterogenous phenotype of patients. The determination of antigen phenotypes for myeloma-initiating cells is still being widely debated.

4-2. Characteristics of $CD138^-$ MM cells

$CD138^-$ cells have been proposed as candidate myeloma-initiating cells by several groups, although whether $CD19^+$ or $CD20^+$ B cells are MM progenitors is yet to be fully confirmed. Because CD138 is highly expressed in mature plasma cells and is considered a differentiation molecule [6], it has been hypothesized that $CD138^-$ cells have a less mature phenotype. It has been reported that $CD138^-$ MM cells have high levels of B cell-specific transcription factor (BCL6) and low expression levels of plasma cell-specific transcription factors (IRF4, PRDM1, and XBP1) compared with $CD138^+$ cells in murine [50] and human MM cell lines [10]. Oct-4, SOX2, and NANOG, which are stem cell-related transcription factors, are reported to be up-regulated in $CD138^-$ MM cells [51,52]. We reported that patients with an increased number of $CD138^-$ MM cells had a poor prognosis [10]. Chaidos et al. showed that $CD19^-CD138^-$ cells are localized at extramedullar lesions, and are more resistant to chemotherapy compared with mature plasma cells [53]. Recently, it was reported that $CD138^-Xbp1s^-$ MM progenitors are resistant to proteasome inhibition [54], suggesting that $CD138^-$ cells are a source of drug-resistant or tumor-initiating cells.

The mechanisms regulating CD138 expression have also been studied. It has been reported that cytokines [55], osteoclasts [56], and bone marrow stromal cells [57] contribute to CD138 down-regulation in MM cells *in vitro*. We recently reported that hypoxia reduces CD138 expression levels and induces an immature phenotype in MM cells [58]. These data indicate that the bone marrow microenvironment has an important role for inducing an immature phenotype in MM cells (Figure 1). We

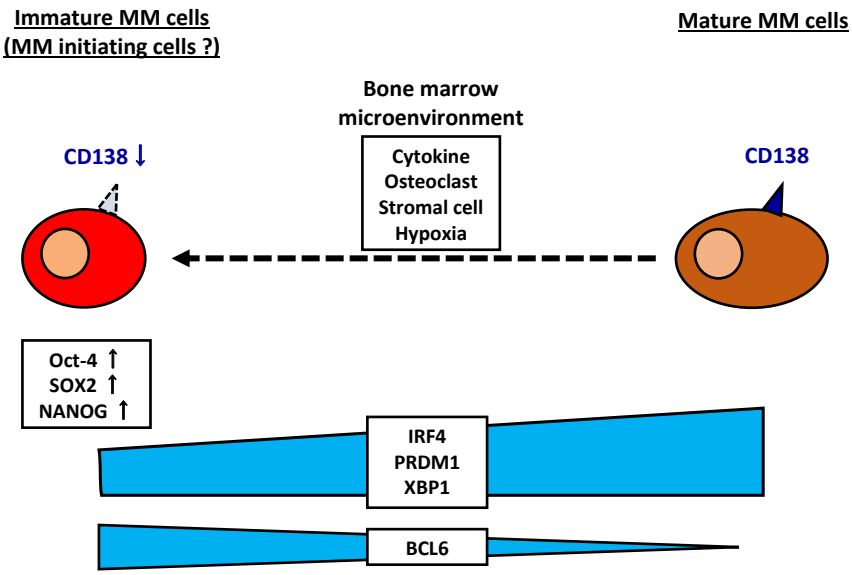

Figure 1. Characteristics of immature ($CD138^-$) MM cells. Immature MM cells are reported to have lower expression levels of plasma cell transcription factors (IRF4, PRDM1, XBP1), and higher expression levels of B-cell transcription factor 6 (BCL6) and stem cell transcription factors (Oct-4, SOX2, NANOG) compared with mature MM cells. The bone marrow microenvironment likely contributes to inducing mature MM cells toward the immature phenotype. To date, immature cells have not been confirmed as MM-initiating cells.

propose that these $CD138^{-}CD19^{-}$ cells are less mature and more resistant to chemotherapy compared with mature plasma cells. A similar model of plasticity between mature and immature MM cells has also been proposed [53]. However, immature $CD138^{-}$ cells are yet to be demonstrated as MM-initiating cells. New methods and further investigation are needed to identify MM stem cells.

5. Conclusion

Multi-color flow cytometry has enabled us to analyze the surface molecules of MM cells in a clinical setting. However, the heterogeneity of surface molecule expression for malignant plasma cells results in complications when attempting to detect minimal residual disease, and in the use of antibody therapies targeting surface molecules. Further information regarding the differences and similarities between normal and malignant plasma cells is essential for solving these problems. The development of novel agents has improved the survival of MM patients; however, it is still an incurable disease. Studying the expression of surface molecules in detail could lead to a better understanding of MM biology, in particular the phenotype of myeloma-initiating cells. A consequence of this, it is hoped, would be better molecular-targeting therapies against MM.

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