Review

CD44 and Hematologic Malignancies

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The expression of CD44 was upregulated in some hematological malignancies and is associated with metastasis and prognosis. The ligation of CD44 with specific monoclonal antibodies can trigger terminal differentiation of leukemic blasts in some subtypes, so it is probable to develop an anti-CD44 based differentiation therapy in leukemia. The effects of CD44 and its monoclonal antibodies are discussed in this review. *Cellular & Molecular Immunology*. 2006;3(5):359-365.

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Introduction

Adhesion molecule CD44 is a cell surface transmembrane glycoprotein encoded by single gene, involved in lymphocyte activation, recirculation and homing, adhesion of extracellular matrix, angiogenesis, cell proliferation, cell differentiation and cell migration, as a receptor for hyaluronic acid (1). All these biological properties are essential to the physiological activities of normal cells, but they are also associated with the pathologic activities of tumor cells. Elevated CD44 expression was correlated with poor prognosis in many malignancies, such as lung cancer (2), ovarian cancer (3), breast cancer (4), colorectal cancer (5), gastrointestinal neuroendocrine tumor (6), and so on. In recent years, scholars pay more attention to the association with CD44 and hematological malignancies. They presume that CD44 plays an important role in normal myelopoiesis because anti-CD44 antibodies profoundly alter *in vitro* myelopoiesis in long-term bone marrow cultures (7). In the context of leukemia, experiments have shown that it is possible to reverse differentiation blockage in some leukemic cells through CD44 ligation with specific antibodies (8), indicating new possibilities for the development of CD44-targeted differentiation therapy in leukemia.

The structure and function of CD44

Structure of CD44 gene and protein

Human CD44 gene is located at the short arm of 11 chromosome, containning at least 20 exons spanning some 50 kilobases of DNA. The gene is composed of two groups of exons, one group comprizing exons 1-5 and 16-20, are expressed together on all cell types as the standard form. The 10 variable exons (exons 6-15) can be alternatively spliced and included within the standard exons at an insertion site between exons 5 and 16 (9). Transcripts for this gene undergo complex alternative splicing that results in many functionally distinct isoforms. The variant isoforms differ in peptide units which are included in the extracellular region of the protein, theoretically alternative splicing would allow more than one thousand CD44 variants to be generated.

The smallest CD44 molecule, which lacks the entire variable region, is standard CD44 (CD44s). As it is expressed mainly on cells of lymphohematopoietic origin, CD44s is also known as hematopoietic CD44 (CD44H). It is composed of a distal extracellular domain (containing the ligand-binding sites), a membrane-proximal region, a transmembrane-spanning domain, and a cytoplasmic tail (Figure 1). Including several glycosylation sites and chondroitic acid-binding sites, the extracellular region can bind to different extracellular matrix. N-terminal is the region primarily responsible for the binding of hyaluronic acid. Transmembrane domain is fairly typical of most single-pass membrane glycoproteins, including a site that could link to hexadecanoic acid. The sequence of cytoplasmic end can be phosphorylated as the substrate of protein kinase C. As a GTP-binding protein, CD44 can bind to GDP substrate and has GTP enzymatic activity, so it can enhance the interaction of CD44 and ankyrin (10, 11).

CD44 variants (CD44v) are mainly expressed on epithelial cells, encoding amino acids with extensive glycosylation sites and chondroitic acid-binding sites. Splicing in continuous or septal way, different variable region exons combination encode different CD44 molecules. At present, there are more than 10 kinds of CD44v in many cell lines detected by polymerase chain reaction. Alternative splicing is

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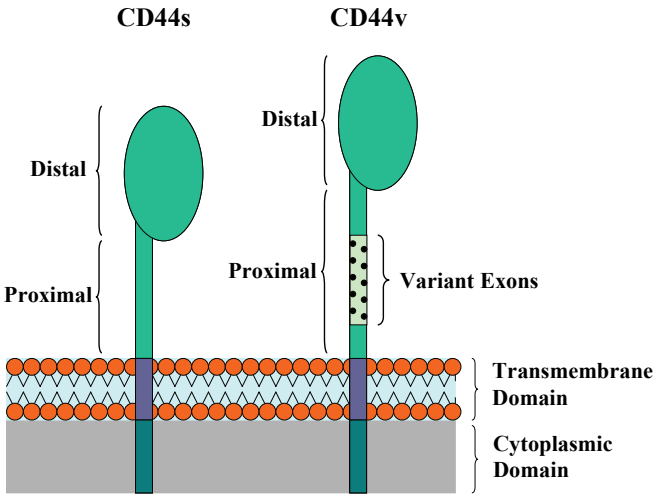

Figure 1. Model for the structure of CD44. The left diagram shows the four principal protein domains of the standard CD44 (CD44s), including the distal extracellular domain (link protein-homologous domain), the membrane proximal extracellular domain, the transmembrane domain, and the intracellular cytoplasmic domain. The right diagram shows that the alternatively spliced regions are inserted at the membrane proximal extracellular domain, giving rise to numerous variant isoforms of CD44 (CD44v).

the basis for the structural and functional diversity of this protein, and may be related to tumor metastasis (11). After immunological activation, the CD44v on T lymphocytes and other leukocytes was transiently upregulated. A CD44 isoform containing the last 3 exon products of the variable region (CD44v8-v10, also known as epithelial CD44 or CD44E), is preferentially expressed on epithelial cells. The longest CD44 isoform expressing in tandem eight exons of the variable region (CD44v3-v10) is detected in keratinocytes. The pMeta-1 (CD44v4-v7) and pMeta-2 (CD44v6, v7) are known as metastatic CD44 because their cDNA confers, upon transfection, metastatic potential on nonmetastatic rat tumor cells (Figure 2) (13).

Function of CD44

Cell adhesion molecules (CAMs) are essential for maintaining stable tissue structure. In dynamic situations, cells alter their cell-cell and cell-matrix interactions by virtue of altered expression and function of CAMs. The expression of CAMs is normally tightly regulated, thereby controlling cell proliferation, mobility, differentiation, and survival. As an adhesion molecule, the effects of CD44 are in many ways, for example, participating in lymphocyte homing, T-lymphocyte activation, promoting the adherence between fibroblast, lymphocyte and extracellular materia (ECM) such as hyaluronic acid, chondroitin sulfatase, fibronectin, laminin and collagen, participating in signal transmission, renewing the composition of interstitial tissue, accommodating drug absorption and drug sensitivity, and participating in pseudopod formation and cell migration (14).

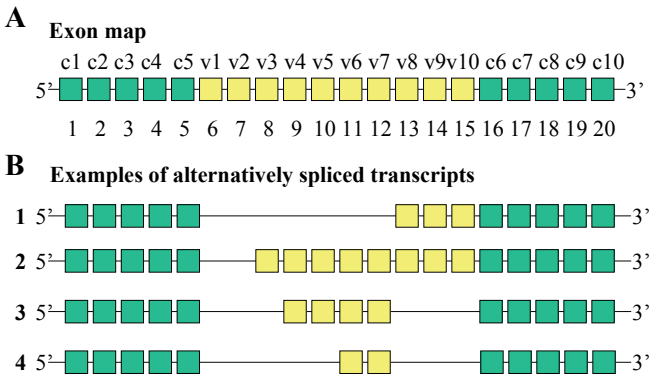

Figure 2. Exon organization of CD44. (A) Map of CD44 exons. The green squares represent the constant-region exons, while the yellow ones represent the variant exons. (B) Examples of alternatively spliced transcripts: 1) epithelial CD44 (CD44v8-v10); 2) keratinocyte CD44 (CD44v3-v10); 3) pMeta-1 (CD44v4-v7); 4) pMeta-2 (CD44v6, v7).

The role of CD44 in tumors

All the biological properties of CD44 are essential to the physiological activities of normal cells, but they are also associated with the pathologic activities of cancer cells. CD44s is expressed ubiquitously, but the expression of CD44v is far more restricted in normal tissues. In tumor tissue and cells, there is an increased level of CD44s and expression of CD44v.

The majority of epithelial, hematopoietic and other non-epithelial cells predominantly express the CD44s (15). The standard CD44 isoform is expressed on all types of mature blood cells, the majority of mononuclear bone marrow precursors, and all $CD34^+$ HPC. The level of its expression varies according to hematopoietic cell lineage and stage of differentiation. For example, it is high on monocytic cells, intermediate on polymorphonuclear cells (PMN) and $CD34^+$ HPC, and low on erythroid cells and platelets. The variant isoforms CD44-6v and CD44-9v have been detected on monocytes, macrophages, lymphocytes, and dendritic cells (16).

CD44 is highly expressed in many tumors, and correlated with the tumor biological behaviour including tumorigenesis, growth, metastasis and prognosis. It is a reliable indicator of tumor load and disease activity, and also called metastasis-associated protein. The fact that metastatic spread involves interaction between tumor cells and extracellular matrix as well as between tumor cells and endothelial cells has led to the hypothesis that especially the variant forms of CD44 may be involved in the process of metastatic spread. There are some commonness between activated lymphocyte and metastatic tumor cells, including the strong invasion, reversible adhesion to cell migration, accumulation and proliferation in draining lymph node, and eventually secreting to circulatory system and exosmosing to peripheral tissue. Seiter et al. presumed that these similarities probably on account of the common effects of CD44v6, indicating that

Table 1. The expression of CD44s and CD44v in different hematological malignancies

Disease	Expression of CD44	Ref
Acute myeloid leukemia	CD44s, CD44v3-v10	17,18
Acute lymphocytic leukaemia	CD44s, CD44v6	19-22
Chronic lymphocytic leukaemia	CD44s, CD44v6	23-25
Lymphoma	CD44s, CD44v6-v10	26-28
Multiple myeloma	CD44s, CD44v6	29-32

the mechanism of CD44v6 in tumor metastasis was the same as that in lymphocyte activation, i.e., the tumor cells possibly obtained lymphocytic disguise from overexpressed CD44v6, escaped from the recognition and killed by immune system, so they could invade lymph node and metastasized more easily (17).

Expression of CD44 in hematological malignancies

Recent studies have shown that CD44 overexpresses on hematopoietic cells and has been implicated in the interactions between bone marrow stromal layers and hematopoietic progenitors, and that its overexpression is associated with poor prognosis in a number of hematological malignancies (Table 1).

CD44 and acute myeloid leukemia

Bendall et al. compared the expression of CD44 variants on normal bone marrow, peripheral blood and $CD34^+$ hematopoietic progenitors with those expressed on blasts from 30 patients with acute myeloid leukemia (AML) (18). Normal bone marrow, peripheral blood and $CD34^+$ progenitor cells were negative for all variants tested by flow cytometry, while exons v3, v4, v5, v6 and v7 were expressed in AML cases. RT-PCR and Southern blotting revealed a more complex pattern of variant exon expression in leukemic samples in comparison to normal hematopoietic cells. The data demonstrated a striking increase in the complexity of CD44v expression in cells from patients with AML, along with surface expression of some variant CD44 proteins. They suggested that further studies should be made directly at how these altered the interaction of leukemic blasts with the bone marrow microenvironment and their diagnostic, prognostic and therapeutic potential. Florian et al. analysed the expression of target antigens on $CD34^+/CD38^-$ cells in patients with AML, myelodysplastic syndromes, chronic myeloid leukemia and systemic mastocytosis. Using multi-color flow cytometry, they reported that CD44 was expressed in all patients and neoplastic stem cells in various myeloid neoplasms expressed a similar phenotype including target antigens CD13, CD33 and CD44 (19).

CD44 and acute lymphocytic leukaemia

It has been reported that high levels of CD44 variants lead to poor clinical outcome in patients with acute lymphocytic leukaemia (ALL). Magyarosy et al. analyzed the expression of CD44v6 in bone marrow of sixteen pediatric ALL patients using immunocytochemistry (20). They detected that CD44v6 protein epitopes were expressed on leukemic cells in 6 ALL cases, primarily in the medium/high risk group (except one case). The feature was highly similar to the observations made in several adult solid cancers and indicated a possible association to an unfavorable outcome. The potential of CD44v6 expression on leukemic cells as prognosticator in pediatric ALL has to be evaluated in a larger clinical trial. Using oligonucleotide microarray analysis, Tsutsumi et al. analyzed the gene expression profiles of pediatric ALL samples according to their translocations (21). The results indicated that gene expression scores of FLT3, MeisI, and CD44 for samples with MLL rearrangements were particularly high compared with those for other ALL samples. Oh et al. reported that CD44 was associated with poor clinical outcome after analyzing tissue infiltration parameters in 86 patients with ALL (22).

Zittermann et al. analyzed CD44 expression in 38 samples of B cell precursors (BCP) from patients with ALL (23). They established five stages of BCP-ALL phenotypes that may represent different forms of interaction between BCP-ALL and bone marrow-adherent cells, according to the expression of CD10 and CD44. Then they analyzed the modulation of CD44 according to the expression of different BCP-ALL phenotypes by incubating the samples under different culture conditions, including addition of stromal cells and interleukin (IL)-7. In culture, the samples in stages 1 and 2 maintained high expression of CD44 and re-expressed this molecule when cultured after trypsin treatment, indicating ongoing synthesis of CD44. Similarly, the stage 3 samples cultured in the presence of stromal cells, IL-7, or both also upregulated CD44 expression in culture. In contrast, the low expression of CD44 on the presumably more mature stage 4 samples was not modified by the addition of stromal cells or IL-7 or when cultured after trypsin treatment, suggesting that those cells had arrested CD44 synthesis. They concluded that down-modulation of CD44 occurred in association with differentiation to phenotype stages 3 and 4 and they hypothesized that this down-modulation might be associated with the exit of BCP-ALL from the bone marrow.

CD44 and chronic lymphocytic leukaemia

Eisterer et al. evaluated the prognostic value of soluble CD44 in B-cell chronic lymphocytic leukaemia (B-CLL) and analyzed the source and regulation of CD44 secretion in B-CLL clones *in vitro* (24). Enzyme linked immunosorbent assay showed that serum levels of sCD44s and sCD44v6 were significantly elevated in B-CLL patients in comparison with normal persons. Elevated levels of sCD44s and sCD44v6 were associated with an advanced disease as reflected by an extended lymph node involvement, an advanced Binet and Rai stage and chemotherapy requirement. High levels of sCD44s were associated with high leukocyte counts and increased sCD44v6 was significantly associated

with splenomegaly. In B-CLL sCD44s as well as sCD44v6 was shed from leukemic cells as shown by *in vitro* cultures. Stimulation of B-CLL clones resulted in a proliferation-associated increased secretion of sCD44s and of sCD44v6. B-CLL clones from advanced stage patients were characterized by an increased capacity for proliferation and CD44 production in comparison with early stage patients. Bairey et al. studied the expression of CD44 in 42 patients with B-CLL (25). Using dual color flow cytometry, they detected an obvious higher level of CD44 and CD11c in patients with stage II(S) than that in patients with stages 0 and I. They considered that higher expression of CD44 and CD11c in cells of CLL patients with predominantly splenic manifestations may account for the tendency of their lymphocytes to home to the spleen. Molica et al. detected the sera from 94 previously untreated CD5-positive B-cell CLL patients taken at the time of diagnosis, and analyzed the presence of standard sCD44 using a commercial enzyme-linked-immunosorbent-assay (26). Patients with higher than median sCD44 levels (642 ng/ml) had a more advanced clinical disease stage, and had a median progression-free survival (PFS) of 36 months, whereas patients with an sCD44 level < 642 ng/ml experienced a longer PFS of the average of 8 years. In a stepwise multiple regression analysis, serum sCD44 levels > 642 ng/ml provided independent prognostic information regarding PFS. An increased serum level of sCD44 can be considered to be a promising parameter for predicting the risk of disease progression in patients with early CLL. All these results indicated that CD44 may represent a reliable prognostic marker in B-CLL and may be involved in the pathogenesis of B-CLL.

CD44 and lymphoma

It is reported that CD44 overexpression can cause genetoxic damage following the enhanced DNA repair and lead to poor prognosis in malignant lymphoma (27). Akisik et al. investigated expression of CD44v in chronic myeloid leukemia and lymphoma by reverse transcription-polymerase chain reaction (28). CD44v6 was detected in all patients and all individuals in the control group. CD44v6-v10 mRNA was observed in 25 patients but in none of the subjects in the control group. Moreover, CD44v6/v9-v10, CD44v6-v7, CD44 v6/v10 transcripts were detected in 11, 6, and 2 patients, respectively. They concluded that CD44v6-v10 expression may be associated with hematologic malignancies. Tacyildiz et al. detected that serum CD44 levels were significantly higher in patients with Hodgkin's disease (HD), non-Hodgkin's lymphoma (NHL) and Burkitt's lymphoma (BL) than those in the control group (29). Serum sCD44 levels significantly declined in HD and NHL patients who were in complete remission. Expression of CD44 was significantly high in patients with HD and NHL who were in advanced stages of disease. High serum CD44 level was also associated with high tumor tissue expression of CD44 in patients with HD and BL. In addition, patients with higher levels of serum sCD44, had a poorer outcome and survival than those with lower sCD44 levels in HD and NHL groups. They pointed out that a high serum sCD44 level and/or tumor tissue expression at diagnosis were associated with poor prognostic criteria and/or unfavorable outcome in lymphoma.

CD44 and multiple myeloma

Multiple myeloma (MM) is a malignancy characterized by the accumulation of monoclonal plasma cells in the bone marrow. Studies have indicated the expression of CD44 by MM cells. Danl et al. showed that CD44 is overexpressed on extramedullary plasma cells in patients with MM (30). Using flow cytometric analysis, Liebisch et al. examined CD44v6 expression in bone marrow samples from 57 patients (31). CD44v6 frequently expressed in advanced, high-risk MM. The expression was correlated with chromosomal band 13q14 deletions, a well-known risk factor in MM. The results suggested that this epitope was a potential new target for monoclonal antibodies such as bivatuzumab mertansine. Vincent et al. demonstrated that interleukin-6 (IL-6), the main survival and growth factor for myeloma cells, modulated CD44 RNA alternative splicing and induced the overexpression of all CD44 variant exons, and that IL-6-induced CD44 cell surface molecules have a functional polarized membrane distribution. Their findings suggested that a CD44/IL-6 amplification loop played a crucial role in myeloma cell survival (32). Caers et al. demonstrated that OPN affected 5T33 MM cell survival by increasing proliferation and inhibiting apoptosis (33). OPN also stimulated 5T33 MM cell migration, which was inhibited by anti-CD44v antibodies. In conclusion, OPN may act as a mediator of MM cell survival by engaging CD44v. They presumed that OPN involved in migration and invasion of MM cells through the activation of either $\alpha V\beta 3$ integrin or CD44v isoforms.

CD44 and leukemia therapy

The differentiation therapy, which consists in reversing the differentiation blockage of leukemic blasts, is the hot spot of current studies on hematonosis. All-trans-retinoic acid (ATRA) and arsenic differentiation therapy are succeeded in the clinical treatment of acute promyelocytic leukemia, but inefficient in the other subtypes. So it is our emphasis to detect new effective differentiation-inducing drugs and targets.

CD44 is highly expressed on human AML cells. Its monoclonal antibody or ligand hyaluronic acid is capable of triggering terminal differentiation of leukemic blasts in some subtypes. It has been reported that ligation of CD44 with some specific anti-CD44 monoclonal antibodies can reverse the differentiation blockage of leukemic cell lines. These results provide the perspective of developing a CD44-targeted differentiation therapy in most leukemia cases.

The specific monoclonal antibodies H90 and A3D8 directing to the CD44 cell surface antigen can trigger terminal differentiation of leukemic blasts in AML1 to AML5 subtypes, induce the differentiation of AML cell lines, inhibit their proliferation and, in some cases, induce their apoptotic death. Gadhoum et al. reported that H90 and/or A3D8 mAbs

may be able to inhibit the proliferation of leukemic progenitors, to promote the differentiation of the leukemic stem cells at the expense of their self-renewal, and, perhaps, to induce their apoptotic death, thereby contributing to decrease the size of the leukemic clone (34). Charrad et al. showed that A3D8 and/or H90 induced terminal differentiation of THP-1, HL60, and NB4 cell lines and strongly inhibited their proliferation (35). They also observed that incubation with A3D8 for 3 to 6 days induced an apoptotic cell death that was moderate in the case of THP-1 and HL60 cells, and massive in the case of NB4 cells. Besides, they demonstrated for the first time that it was possible to reverse the leukemic blockage of immature AML-M0 blasts KG1a cells by using both an anti-CD44 mAb and retinoic acid. The results provided a new experimental basis for a differentiation therapy in AML-M0 patients. The ligation of CD44 with A3D8 can trigger incomplete differentiation and apoptosis of the acute promyelocytic leukemia-derived NB4 cells. Maquarre et al. considered that both caspase-dependent and serine protease-dependent pathways contributed to A3D8-induced apoptosis (36). Artus et al. reported that exposure of human erythroleukemic HEL cells to the anti-CD44 mAb A3D8 resulted in cell growth inhibition followed by caspase-independent apoptosis-like cell death (37). Their data suggested that CD44 ligation triggered a novel caspase-independent cell death pathway *via* calpain-dependent apoptosis-inducing factor release in erythroleukemic HEL cells. IL-1R-associated kinase (IRAK)-M expression is enhanced in patients with chronic myeloid leukemia and is abolished by incubation with anti-CD44 Abs (38). Johnsson et al. analyzed gene expression profiling in several cell lines (K562 leukemia, MCF-7 breast cancer and S1 colon cancer) with acquired resistance against five cytostatic drugs (39). Using cDNA microarray, they reported that the expression of CD44 altered in the cell lines. These results are of great theoretical value in the development of CD44-targeted differentiation therapy.

The effects of A3D8 on myeloid cells were associated with specific disruption of cell cycle events and induction of G0/G1 arrest. Induction of G0/G1 arrest was accompanied by an increase in the expression of p21, attenuation of pRb phosphorylation and associated with decreased Cdk2 and Cdk4 kinase activities. Zada et al. observed that A3D8 treatment of AML patient blasts and HL60/U937 cells led to the down-regulation of c-Jun expression at mRNA and protein level (40). Transient transfection studies showed the inhibition of c-jun promoter activity by A3D8. Furthermore, A3D8 treatment caused a decrease in JNK protein expression and a decrease in the level of phosphorylated c-Jun. Ectopic overexpression of c-Jun in HL60 cells was able to induce proliferation and prevent the antiproliferative effects of A3D8. Their data identified an important functional role of c-Jun in the induction of cell cycle arrest and proliferation arrest of myeloid leukemia cells because of the ligation of the cell surface adhesion receptor CD44 by anti-CD44 antibody. Gadhoum et al. showed that CD44 ligation stabilized the cyclin-dependent kinase inhibitor p27(Kip1) protein, resulting in increased association with cyclin E/Cdk2 complexes and inhibition of their kinase activity (41). Moreover, using a p27 antisense vector, they provided direct evidence that p27 was the main mediator of cell growth arrested by CD44. CD44 ligation also led to p27 accumulation in THP-1, KG1a, and HL60 cell lines and in primary leukemic cells, suggesting that this process is general in AML. The results suggested that CD44 was an efficient means to increase the expression of p27 in AML cells. Considering that elevated expression of p27 is a factor of good prognosis in AML, these results provide a new basis for developing CD44-targeted therapy in AML.

Song et al. investigated the differentiation and apoptosis-inducing effects of another anti-CD44 monoclonal antibody HI44a on leukemic cells obtained from 31 patients with AML (42). The percentage of nitroblue tetrazolium $(NBT)^+$ cells and the expression of CD11b, CD14 and CD15 was increased significantly on the AML cells treated with HI44a, compared to that on control AML cells. HI44a was found to induce apoptosis of leukemic cells, as evidenced by Annexin-V assay. The mean percentage of apoptotic cells in HI44a-treated AML cells was significantly increased compared to that in control AML cells. The level of c-myc transcript expression on AML cells obviously decreased in all detected patients. These results indicated that HI44a effectively induced both differentiation and apoptosis of AML cells and this activity of the anti-CD44 antibody may be associated with its inhibitory effect on c-myc transcript expression.

As a new strategy of therapy, there are different opinions on the application of CD44 antibodies. Allouche et al. reported that prior incubation with A3D8 in HL60 and NB4 cells significantly decreased apoptosis induced by 3 drugs used in AML chemotherapy (43). In HL60 cells, CD44 ligation with A3D8 mAb fully abrogated the DNR-triggered generation of ceramide, a lipid second messenger involved in the DNR apoptotic signaling pathway. Moreover, results showed that the A3D8 mAb inhibited DNR-induced apoptosis in HL60 cells by overexpressing Bcl-2. These results suggested that, to eradicate AML blasts, the differentiation-inducing anti-CD44 mAb A3D8 should not be administered prior to apoptosis-inducing drugs.

Perspective

CD44 is expressed in many hematological malignancies, the level of which is associated with clinical condition and prognosis. The anti-CD44 monoclonal antibodies can effectively suppress the proliferation and induce differentiation or apoptosis of leukemic cells in some subtypes. Studies on CD44 antibodies therapy are just in the phage of primary experiments, and have not reached consensus, so further studies need to be made on this aspect. It is believed that the value of CD44 and its monoclonal antibodies in the diagnosis, metastasis, prognosis and therapy of hematological malignancies will be fully recognized and accompanied by the deep basic and clinical researches.

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