Bone Marrow Stromal Cells Protect Acute Myeloid Leukemia Cells From Anti-CD44 Therapy Partly Through Regulating PI3K/Akt–p27Kip1 Axis

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The anti-CD44 monoclonal antibody (mAb) A3D8 induces differentiation or apoptosis in vitro in various subtypes of acute myeloid leukemia (AML) via p27Kip1 upregulation. Bone marrow (BM) stromal cells play a vital role in the development of chemoresistance in AML cells attached to the stroma. To investigate the effect of BM stroma adhesion induced AML resistance to A3D8, we developed a co-culture system composed of an AML-derived cell line (NB4) cultured with either a human BM stroma cell line (HS-5) or mesenchymal stem cells (MSCs). We found that NB4 cells adhered to HS-5 cells or MSCs developed resistance against the anti-proliferative effects of A3D8, and this action is caused by the activation of PI3K/Akt signaling following p27Kip1 down-regulation and cytoplasmic re-localization. The stromal co-culture-induced resistance can be partially abolished by inhibiting the PI3K/Akt signaling pathway. Such findings were confirmed in two additional AML-derived cell lines as well as in primary AML cells. Our results suggest that BM stroma can induce A3D8 resistance in part via the PI3K/Akt–p27Kip1 axis, and blocking PI3K/Akt pathway may be necessary for anti-CD44 treatment on AML in BM microenvironment.

Key words: A3D8; bone marrow stroma; acute myeloid leukemia; PI3K/Akt; p27Kip1

INTRODUCTION

CD44 is a cell surface antigen that is highly expressed in acute myeloid leukemia (AML) cells, and it has been considered as a target to eradicate leukemia stem cells [1]. Notably, the anti-CD44 monoclonal antibody (mAb) A3D8 induces differentiation or apoptosis in myeloid cell lines and AML patient blasts in all AML subtypes [2–4]. In addition, A3D8 induces apoptosis in all trans retinoic acid (ATRA)-resistant NB4-derived cells and arsenic trioxide (As2O3)-resistant primary acute promyelocytic leukemia (APL) blasts [4].

Relapse of minimal residual disease (MRD) is a major problem following conventional chemotherapy in AML patients. Bone marrow (BM) is the major site for MRD, where the adhesion of AML cells to BM components such as BM stromal cells may provide protection from the therapeutic drugs [5]. The PI3K/Akt signaling pathway plays a critical role in the chemoresistance of BM-resident AML cells and determines the level of MRD in AML patients [6,7]. In the PI3K/Akt pathway, the intracellular signals are primarily transmitted through the activation of PI3K, leading to the production of the second messenger PIP3 on the plasma membrane. PIP3 binds with the intracellular signaling proteins Akt and PDK1. PDK1 promotes the phosphorylation of Akt at threonine 308 (Thr308) and serine 473 (Ser473), thereby activating or inhibiting its downstream target molecules, including p27Kip1 [8]. The recent data suggest that inhibition of the PI3K/Akt signaling pathway may facilitate the eradication of leukemia cells in the BM microenvironment [9]. In addition, the PI3K/Akt pathway was inhibited significantly in A3D8-induced differentiation and apoptosis in THP-1 cells [10]. However, the effect of A3D8 against AML cells bound to the BM stroma and the role of the PI3K/Akt–p27Kip1 axis remain unclear.

Here, we report that adhesion to HS-5 cells or mesenchymal stem cells (MSCs) enabled NB4 AML cells resistance to A3D8-induced proliferation inhibition, and that this resistance could be partially restored by the PI3K/Akt inhibitor LY294002. Such findings were also observed in human AML cells (the HL60 cell line, the THP-1 cell line, and primary AML cells) adhered to MSCs. Furthermore, our mechanistic

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results showed that A3D8 resistance is associated with the activation of PI3K/Akt signaling followed by p27Kip1 down-regulation and cytoplasmic re-localization in AML cells after BM adhesion. Thus, PI3K/Akt inhibition maybe considered when treating AML in BM microenvironment.

MATERIALS AND METHODS

Cell Culture

The cell lines were cultured in RPMI 1640 medium (Life Technologies, Carlsbad, CA) containing 10% FBS (Gibco, Carlsbad, CA) and 50 μg/mL of penicillin and streptomycin (Sigma-Aldrich, St.Louis, MO) in a humidified incubator with 5% CO2 at 37°C.

Isolation of Primary AML Cells and MSCs

The primary AML cells were derived from untreated AML patients at the Department of Hematology in the Union Hospital of Fujian Medical University. The samples were collected with the patients’ consent. Ficoll medium was used to separate the mononuclear cells, and the CD3+ cells were isolated using CD3+ magnetic beads. The AML patient data are presented in Table 1.

Co-Culture System and Magnetic Bead Sorting

The co-culture system has been described previously [11]. After 72 h of co-culture, AML cells were collected with nylon mesh (400 mesh, Becton and Dickinson Company, Franklin Lakes, NJ) to create a single cell suspension. CD45+ magnetic beads (Miltenyi Biotec Technology & Trading, Shanghai, China) were used to sort the adhered AML cells. The purity of the AML cells was >90% as determined by flow cytometry.

Reagents

The anti-CD44 mAb A3D8 (C7923) was purchased from Sigma. The same isotype mouse IgG1 (Life Technologies (AB & Invitrogen), Guangzhou, China) was used as a control. LY294002 (Cell Signaling Technology, Danvers, MA) was diluted to a concentration of 10 mM in DMSO and stored at −20°C.

Proliferation Assays

Cell proliferation was measured using an ELISA-based BrdU assay according to the manufacturer’s instructions (Roche, Indianapolis, IN).

Flow Cytometry Analysis

CD11b, CD44s, and CD44 cell surface expression was detected by staining with anti-CD11b, anti-CD44s, and A3D8, respectively, according to the manufacturer’s (Becton and Dickinson Company, Franklin Lakes, NJ) specifications, and the analysis was performed on a FACSscan (BD).

Akt Kinase Activity Analysis

Cells (5 × 10⁵ NB4) were washed twice with PBS, and a fixing agent was added for 30 min (ebioscience, San Diego, CA). Next, a cell permeabilization reagent was added and incubated for 30 min at room temperature. After washing with PBS, a PE-labeled activated Akt antibody was added and incubated for 30 min in the dark. The analysis was subsequently performed on a FACSscan (BD).

Cell Cycle Analysis

The cell cycle distribution was determined by performing flow cytometry analysis of propidium iodide-stained nuclei. Briefly, the NB4 cells were centrifuged at 1500 rpm for 3 min, washed with PBS, and fixed in ice-cold ethanol [70% (v/v) in water]. The DNA was stained with 100 μg/mL propidium iodide for 30 min at 4°C protected from light. The cells were then analyzed with the FACSscan (BD).

Western Blotting

Western blotting was performed as previously described. The following antibodies were used: anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-CD44s (Abcam, Cambridge, MA), anti-Akt, p-AktSer473, anti-p-AktThr308, anti-GSK3β, anti-pGSK3β, anti-p27Kip1, anti-Cyclin E, anti-Cdk2, anti-Cyclin D1, anti-Cdk4, anti-β-Tubulin, anti-lamin B1 (CST), and horseradish peroxidise-conjugated anti-mouse and anti-rabbit secondary antibodies (CST).

Protein Array

Protein was labeled with the following steps: (1) A simple centrifugation was performed for the biotin reagent; (2) Approximately 1 mg biotin reagent was added to 100 μL DMF (N,N-dimethylformamide) to achieve a final concentration of 10 μg/μL and was labeled as Biotin/DMF; (3) Approximately 50 μg protein samples were labeled by adding 3.0 μL Biotin/DMF to a final volume of 70 μL; and (4) The mixture

| Sample no. | Status at date of sampling | Gender/age | FAB | PB WBC count (×10⁹/L) | The percentage of primitive cells (%) |
|------------|---------------------------|------------|-----|----------------------|---------------------------------------|
|            |                           |            |     |                      | Before sorting | After sorting                    |
| 2          | Newly diagnosed           | Male/41    | M3  | 27.7                 | 81           | 94                                |
| 9          | Newly diagnosed           | Male/55    | M5  | 18.9                 | 97           | /                                 |

FAB, French-American-British classification; PB, peripheral blood; WBC, White blood cells.

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was vortexed, and the reaction was allowed to equilibrate for 2 h before adding 35 μL stop solution for 30 min. Protein hybridization was performed according to the manufacturer’s instructions (Full Moon, Sunnyvale, CA).

Statistical Analysis

All results are expressed as means ± standard error of the mean (SEM). Significant differences (P < 0.05) were identified using Student’s t-test.

RESULTS

Adhesion to HS-5 Cells Blocks the Inhibitory Effects of A3D8

In this study, we first determined if BM stromal adhesion confer protection of AML cells from anti-CD44 treatment. We used A3D8 anti-CD44 antibody to treat NB4 cells cultured alone (Al-NB4) or adhered to BM stroma (Ad-NB4). The HS-5 human BM fibroblast cells transformed with HPV-16 E6/E7 were used as an in vitro model of human BM stroma. The cell proliferation was analyzed using an ELISA-based BrdU assay, and the induction of differentiation and apoptosis was assessed by flow cytometry. As shown in Figure 1A,B, A3D8 inhibited Al-NB4 cell proliferation in a dose- and time-dependent manner, and induced the expression of the cell differentiation marker CD11b and cell apoptosis, but such inhibition was not observed on Ad-NB4 cells. As one of the most extensively studied CD44 monoclonal antibodies, A3D8 binds to the surface of CD44 standard molecules surface to perform its inhibitory function. To check if CD44 expression was altered by BM cell co-culture, we assessed the level of CD44s and CD44 in Al-NB4 and Ad-NB4 cells, respectively, by flow cytometry. The level of CD44s on the surface of Ad-NB4 cells was not altered compared to Al-NB4 cells, and CD44 expression was slightly increased in the Ad-NB4 cells (Fig. 1C). Such results suggest that although BM adherence did not alter CD44 expression on NB4 cells, it induced NB4 cells resistance to anti-CD44 treatment.

Adhesion Reduces p27Kip1 Levels

P27Kip1 is a major factor mediating CD44-induced inhibition of AML cell proliferation [12]. Mechanistically, p27Kip1 function as a CDK inhibitor to block cell cycle progression in the G1 phase [12]. Interestingly, it has been reported that adhesion to MSCs also transiently arrests tumor cells in the G1 phase of the cell cycle [13]. To determine if BM adhesion alters the level of p27Kip1 expression and cell cycle status of NB4 cells, we first examined the cell cycle of Al-NB4 and Ad-NB4 cells by flow cytometry. As shown in Figure 2A, we observed an increase in the percentage of Ad-NB4 cells in the G0/G1 phase and a decrease in the number of cells in the S phase compared with the Al-NB4 cells. We next investigated the expression of p27Kip1 and its related cell cycle regulators in the Ad-
NB4 and Al-NB4 cells. We measured the expression of p27Kip1, Cyclin E, CDK2, Cyclin D1, and CDK4 with a protein array followed by a Western blot. As shown in Figure 2B, adhesion did not affect CDK4 levels, and slightly increased the expression levels of CDK2, Cyclin E, and Cyclin D1 expression respectively. Strikingly, p27 level was markedly down-regulated by BM adhesion (Figure 2B).

Adhesion Reduces p27Kip1 Expression in Part Through PI3K/Akt Pathway Activation

Because p27Kip1 is localized in the cytoplasm and nucleus, we then examined the mechanism for reduced p27Kip1 expression in NB4 cells bound to HS-5 cells. As shown in Figure 2B, adhesion did not affect CDK4 levels, and slightly increased the expression levels of CDK2, Cyclin E, and Cyclin D1 expression respectively. Strikingly, p27 level was markedly down-regulated by BM adhesion (Figure 2B).

Adhesion Reduces p27Kip1 Expression in Part Through PI3K/Akt Pathway Activation

Because p27Kip1 is localized in the cytoplasm and nucleus, we then examined the mechanism for reduced p27Kip1 expression in NB4 cells bound to HS-5 cells. p27Kip1 is a direct target of the PI3K/Akt axis in HL60 cells and breast tumors [14]. Additionally, PI3K/Akt signaling is activated in AML cells attached to MSCs [11]. Thus, we checked if PI3K/Akt pathway was involved in adhesion-induced p27Kip1 down-regulation and A3D8 resistance. We first identified that the PI3K/Akt signaling (indicated by levels of p-Aktthr473, p-Aktthr388, and p-GSK3βser9) was dramatically inhibited in Al-NB4 cells treated with 4 μg/mL A3D8 (Figure 3A and Supplementary Figure S1). By contrast, adhesion to HS-5 cells significantly increased the phosphorylation of such proteins. Flow cytometry analysis further confirmed increased p-Akt activity in the Ad-NB4 cells (Figure 3B, right panel). Interestingly, such Akt activation requires cell-cell contact between NB4 cells and stroma cells (Supplementary Figure S2). Thus, A3D8 inhibited PI3K/Akt activity in NB4 cells, but adhesion to HS-5 cells activated PI3K/Akt pathway in NB4 cells.

As p27Kip1 is a direct target of the PI3K/Akt axis in HL60 cells and breast tumors [14], we next checked p27Kip1 protein level. A3D8 treatment increased p27Kip1 expression in the Al-NB4 cells (Figure 3A) and that adhesion to HS-5 cells decreased p27Kip1 expression in the Ad-NB4 cells (Figure 3B). As a cell cycle inhibitor, P27Kip1 translocates to the nucleus to bind with Cyclin E-CDK2 and Cyclin D-CDK4 to inhibit proliferation. As shown in Figure 3C (lower panel), p27Kip1 markedly accumulated in the nucleus of the Al-NB4 cells after treated with A3D8.

To confirm the functional role of the PI3K/Akt pathway, Ad-NB4 cells were treated with PI3K inhibitor LY294002 (10 nM) followed by A3D8 treatment. The cells displayed a dramatic decrease in the proliferation rate compared with cells treated with A3D8 alone (0% vs. 34.8 ± 4.2%). Furthermore, inhibition of PI3K/Akt pathway in Ad-NB4 cells by LY294002 increased p27Kip1 expression, and promoted the re-localization of p27Kip1 from the cytoplasm to the nucleus (Figure 3C).

The BM MSCs constitute a non-hematopoietic cell population that can differentiate into tissues of mesodermal origin (osteocytes, adipocytes, or chondrocytes). To determine whether the BM MSCs could also induce A3D8 resistance as the HS-5 cells, we established a co-culture system with NB4 cells and MSCs and analyzed the inhibitory effects of A3D8 on the NB4 cells attached to MSCs. Our data indicate that adhesion to normal BM MSCs induces A3D8 resistance (0% vs. 56.4 ± 3.1%). This resistance can be partially restored using treatment with LY294002 (31.7 ± 3.8% vs. 0%). Similar alterations in the PI3K/Akt pathway and p27Kip1 were observed in NB4 cells bound to the BM MSCs as the cells adhered to HS-5 cells (Figure 3D).

Adhesion to the BM MSCs Also Induces A3D8 Resistance in HL60, THP-1, and Primary AML Cells

To determine whether A3D8 resistance induced by adhesion to the BM MSCs also occurs in AML cells, we examined whether A3D8 induces differentiation or apoptosis in HL60 (myeloblastic), THP-1 (monoblastic), and primary AML cells bound to the BM MSCs. As shown in Figure 4A, the treatment of the Al-HL60 (HL60 cells cultured alone) and Al-THP-1 cells (THP-1 cells cultured alone) with different concentrations of A3D8 for 72h decreased cell proliferation and increased the expression of the cell differentiation marker CD11b. Of the nine primary AML cells from patients examined, A3D8 displayed the strongest inhibitory effect on the proliferation of AML2 (M3) and AML9 (M5) cells. Adhesion to BM MSCs also

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induced A3D8 resistance in HL60, THP-1, and primary AML cells (Table 2). Furthermore, our results show that the resistance induced by adhesion to the BM MSCs in the THP-1 (Figure 4B), HL60 (Figure 4C), and primary AML cells (Figure 4D,E) occurs in part through the activation of the PI3K/Akt-dependent down-regulation of p27Kip1. Strikingly, LY294002 pretreatment restored A3D8-mediated inhibition.

Figure 3. Stroma adhesion reversed A3D8-induced p27Kip1 accumulation partly through PI3K/Akt activation. (A) Western blot analysis of the expression of the indicated proteins from Al-NB4 cells treated with A3D8 for the indicated time points. (B) Left panel, analysis of protein expression in the Al-NB4 and Ad-NB4 cells at the indicated time points; Right panel, flow cytometry analysis of Akt kinase activity in the Al-NB4 and Ad-NB4 cells. (C) Western blot analysis of the indicated proteins (left panel) and p27Kip1 distribution in the nucleus and cytoplasm (right panel) in Al-NB4 or Ad-NB4 cells treated with LY294002 alone or LY294002 followed by A3D8. (D) Western blot analysis of the indicated proteins (left panel) and p27Kip1 distribution (right panel) in the nucleus and cytoplasm of Al-NB4 or NB4 cells cultivated with MSCs and treated with LY294002 alone or LY294002 followed by A3D8. t-, total. β-Actin showed equal loading of samples. The results are representative of three independent experiments.


discussion

The BM microenvironment plays a crucial role in cell growth and survival as well as in the drug resistance of AML [5,9]. The ligation of CD44 with A3D8 antibody efficiently inhibits the proliferation of AML cells, including ATRA-resistant NB4 cells and primary A53O3-resistant APL blasts [4]. However, the inhibitory effect of A3D8 in AML cells bound to the BM stroma has not been investigated to date. The results reported here are the first to demonstrate that the BM stroma protects AML cells from the inhibitory effects of the anti-CD44 mAb A3D8 in part via the PI3K/Akt-dependent down-regulation of p27Kip1.

Using NB4 cells that undergo massive apoptotic cell death in response to A3D8 treatment [12], we showed that NB4 cells adhered to BM stroma completely lost their sensitivity to A3D8. BM adhesion did not affect A3D8 binding to Ad-NB4. Instead, the adhesion activated PI3K/Akt signaling in NB4 cells. Furthermore, treatment with, LY294002 alone or LY294002 followed by A3D8. The results reported here are the first to demonstrate that the BM stroma protects AML cells from the inhibitory effects of the anti-CD44 mAb A3D8 in part via the PI3K/Akt-dependent down-regulation of p27Kip1.
In our study, we characterized the signaling cascades activated upon direct contact between AML cells and BM-derived stromal cells. Our findings demonstrate stromal-induced activation of the PI3K/Akt pathway by Akt and GSK3β phosphorylation. The PI3K/Akt pathway is involved in controlling p27Kip1 protein expression and re-localization from the cytoplasm to the nucleus. For example, Akt-mediated...
p27Kip1 plays a pivotal role in A3D8-induced differentiation and apoptosis of AML cells [12]. In AML cells treated with A3D8, the total levels of p27Kip1 increased and translocated into the nucleus, where p27Kip1 forms a complex with Cyclin E-CDK2 and Cyclin D-CDK4 and exerts anti-leukemia effects [12]. A3D8 was unable to inhibit cell proliferation of AML cells with p27Kip1 knockdown. Ramasamy et al. [13] reported that p27Kip1 expression was down-regulated in the B lymphocyte leukemia cell line BV173 cultivated with human BM MSCs for 72 h. Our protein array and Western blot results also demonstrated that p27Kip1 expression was reduced in the NB4 cells after BM adhesion. In addition, this reduction was also observed in a number of AML cell lines and primary AML cells adhered to BM MSCs. Furthermore, we found that p27Kip1 was sequestered in the cytoplasm in adhered AML cells, suggesting that adhesion inhibits the p27Kip1 nuclear localization through PI3K/Akt pathway activation. This finding is in agreement with previous findings from other laboratories that p27Kip1 is abundantly localized in the cytoplasm of HL60AR cells (HL60 cells with a constitutively activated PI3K/Akt axis), but primarily intranuclear in parental HL60 cells [14]. These results indicate that the resistance of adhered AML cells to A3D8 is associated with the down-regulation of p27Kip1 and the re-localization of p27Kip1 in the cytoplasm. However, the fact that A3D8 resistance is only partially restored even in the presence of high concentrations of LY294002 suggests that other signaling pathways may also regulate the growth and maintenance of leukemia cells. For example, Ravid et al. recently reported that the tumor microenvironment confers drug resistance through the stromal secretion of the hepatocyte growth factor (HGF), which activates the HGF receptor MET and the downstream MAPK and PI3K/Akt pathways in tumor cells [20].

Adhesion to the BM stroma induces a resting state (G0/G1) in the AML cells [13], and it is assumed that the negative cell regulator p27Kip1 is up-regulated. In addition, previous studies have also shown that the adhesion of myeloma cell lines to fibronectin (FN) induces cell-cycle arrest via increased p27Kip1 expression [21]. Unexpectedly, we observed a decrease in p27Kip1 expression in AML cell lines and primary AML cells after BM adhesion. Such discrepancy may be explained by the subtle difference in precise regulation of cell cycle phases by BM adhesion and p27Kip1. Accumulating evidence suggests that p27Kip1 is only negatively regulates the transition from the G1 phase to the S phase of the cell cycle [22,23], whereas BM MSCs regulate tumor cells at an early “quiescent” stage (G0/G1) of the cell cycle to preserve AML cell proliferation and self-renewal abilities [24,25] and support long-term survival [13]. Thus, stromal adhesion may alter the AML cell cycle via p27Kip1-independent mechanism.

Because BM MSCs can promote hematopoietic cell engraftment and reduce graft-versus-host disease, MSCs have been widely used in the treatment of hematological malignancies. However, emerging evidence indicates that MSCs form a niche that may preserve the self-renewal and proliferation of leukemia cells. Patients with hematological malignancies have increased relapse rates when MSCs and alloge- neic hematopoietic stem cells are used for transplantation compared with hematopoietic stem cells alone (60% vs. 20%) [26]. Our findings also demonstrate that the BM stroma induces resistance to chemotherapy as well as anti-CD44 therapy. In light of this result, caution should be taken when using MSCs for the treatment of hematological malignancies.

We also demonstrated that PI3K inhibitor can partly rescue the BM adhesion-induced CD44 resistance. Furthermore, the combination of arsenic and LY294002 has an even stronger inhibitory effect on the proliferation of adhered AML cells compared with the combination of LY294002 and A3D8 (data not shown), further supporting that pharmacological inhibitors of PI3K/Akt may sensitize leukemia cells.

Table 2. Inhibitory Rate of A3D8 Alone or in Combination With LY294002 in AML Cells Cultured With or Without MSCs

| Cells        | A3D8     | LY294002 + A3D8 |
|--------------|----------|-----------------|
| THP-1        | 49.59 ± 4.71% | 64.17 ± 5.9%    |
| HL-60        | 52.4 ± 4.37%  | 68.32 ± 4.06%   |
| AML2 (APL)   | 53.2 ± 9.57%  | 63.12 ± 7.33%   |
| AML9 (M5)    | 23.91 ± 8.74% | 28.64 ± 5.47%   |

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in the BM environment to traditional therapeutic agents.

In summary, BM stroma induces A3D8 resistance in AML cells in part by activating the PI3K/Akt pathway and sequestering the pathway’s downstream factor p27Kip1 in the cytoplasm. Therefore, A3D8 may not eradicate leukemia cells in the BM stroma and should not be used as a single-agent AML therapy in vivo. Our study may warrant future studies in preclinical and clinical investigations in developing combinational treatments of such tumors in BM.

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