Effects of decitabine on megakaryocyte maturation in patients with myelodysplastic syndromes

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Abstract. Thrombocytopenia is a common, often fatal complication experienced by patients with myelodysplastic syndromes (MDS). 5-aza-2'-deoxycytidine (decitabine) has been used to treat MDS patients with thrombocytopenia with a response rate of 45-50%. However, the mechanism of its effects on megakaryocytes remains unclear. In the present study, the effect of decitabine on megakaryocyte maturation was investigated. A total of 20 MDS patients diagnosed with thrombocytopenia were enrolled, including 16 refractory anemia with excess blasts (RAEB)-1 patients and 4 RAEB-2 patients], in addition to 20 leukemia patients that had achieved complete remission and 20 healthy donors. Overall, 65% of MDS patients exhibited a response to decitabine, with an increase in platelet count identified in 80% of patients. In the MDS group, the mean platelet count was significantly increased following one cycle of decitabine chemotherapy (36.85±24.54 vs. 84.90±61; P=0.001); however, no significant difference in megakaryocyte number was identified prior to and following treatment. Additionally, bone marrow mononuclear cells of the MDS patients were cultured *in vitro* with various concentrations of decitabine (0.0, 2.0, 2.5, 3.0 μM), and cluster of differentiation (CD)41 levels were examined via flow cytometry. The MDS and normal control groups exhibited the highest levels of CD41 expression following treatment with 2.0 μM decitabine (mean fluorescence intensity, 294.07±47.34 and 258.95±28.05, respectively). In conclusion, these results indicate that the DNA-hypomethylating agent, decitabine, may induce the differentiation and maturation of myelodysplastic megakaryocytes in MDS patients, even at low concentrations. Thus, the repeated administration of decitabine at lower doses in MDS patients may be useful in clinical practice, and may lead to the development of alternative treatments for other diseases of abnormal megakaryocyte differentiation, such as idiopathic thrombocytopenic purpura, however, future studies are required to investigate this.

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Introduction

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal disorders that originate from early hematopoietic progenitor cells. These syndromes are characterized by maturation defects that lead to ineffective hematopoiesis, resulting in anemia, leucopenia and thrombocytopenia (1). In the USA, the annual incidence rate for MDS is 2-4 cases per 100,000 individuals. Notably, MDS are most common in elderly individuals; the annual MDS incidence rate ranges between 15 and 50 cases per 100,000 individuals in patients aged >70 years (1). The majority of patients ultimately succumb to infection and/or hemorrhage. Thrombocytopenia (platelet count, $<100x10^9$/l) is common in MDS, occurring in 40-65% of patients (2). In severe cases of thrombocytopenia and/or hemorrhage, thrombocytopenia in MDS is treated via platelet transfusions. However, platelet transfusions often lead to a brief increase in platelet count and antibodies may be produced subsequent to multiple transfusions, causing human leukocyte antigen-alloimmunization. Furthermore, ≥30% of platelet transfusions result in complications, including bacteremia, graft-versus-host disease, acute pulmonary injury and exacerbated thrombocytopenia (3,4).

5-Aza-2'-deoxycytidine (decitabine) is a known inhibitor of DNA methylation and has been shown to exhibit a marked antileukaemic effect (5). Decitabine has been shown to induce response rates of 45-50% in elderly high-risk MDS patients, even inducing trilineage response and cytogenetic complete remission (CR) in ~30% of patients with cytogenetic abnormalities, by increasing platelet count (6). However, the mechanism by which decitabine increases the platelet count in patients with MDS remains unclear (6-9).

The association between decitabine and cellular differentiation was initially identified in the human erythroleukemia cell line, K562. Decitabine was found to induce irreversible hemoglobinization and morphological differentiation in a dose-dependent manner (7,8). A number of previous studies have suggested that the effects of decitabine treatment for thrombocytopenia in MDS patients may be due to enhanced megakaryocyte differentiation (6-12). Therefore, to elucidate

the mechanisms by which decitabine increases platelet count in patients with MDS, and to investigate the effects of decitabine on megakaryocyte differentiation and platelet release, the present study used cluster of differentiation (CD)41 (integrin, α IIb) as a cell surface marker for megakaryocyte maturation (13). Furthermore, the associated clinical data was collected prior to and after decitabine treatment, allowing analysis of the association between the clinical data and laboratory results.

Materials and methods

Patient characteristics. Patients with a hematological diagnosis of *de novo* primary MDS were considered eligible for the present study. A total of 20 MDS patients with thrombocytopenia (11 males, 9 females) with a median age of 55 years (range, 35-75 years) were enrolled at the General Hospital of Tianjin Medical University (Tianjin, China) between March 2013 and February 2014. According to the World Health Organization criteria (14), 16 patients exhibited refractory anemia with excess blasts (RAEB)-1 and 4 patients exhibited RAEB-2. Two control groups were also enrolled between March 2013 and February 2014. The first control group included 20 acute myeloid leukemia patients (12 males, 8 females) with a median age of 46 years (range, 20-69 years) who had achieved CR after induction chemotherapy [1 M2, 8 M4, 6 M3 and 5 M5 patients (15)]. The second control group included 20 healthy donors (10 males, 10 females) with a median age of 40 years (range, 22-57 years). Written informed consent was obtained from all patients and healthy donors, and the study was approved by the Ethics Committee of the General Hospital of Tianjin Medical University (Tianjin, China).

Treatment. All patients were administered with intravenous decitabine (20 mg/m^2/day; Xian Janssen Pharmaceutical Ltd., Beijing, China) for 5 consecutive days, according to Kantarjian's protocol proposed by the National Comprehensive Cancer Network (NCCN) (16). After one course of treatment, bone marrow aspiration was performed to assess the effects of decitabine.

Determination of treatment efficacy. MDS patient subtypes, blast cell count and treatment response was determined according to NCCN guidelines (16). After one cycle of decitabine treatment, all the patients underwent bone marrow aspiration to determine treatment efficacy. All bone marrow smears were evaluated by one hematologist. A bone marrow sorting counter (WZR-BM2; Ai Lin, Suzhou, China) was used by the hematologist to count nucleic cells in the bone marrow smears, and a total of 500 nucleic cells were counted in each smear. If the blast cell percentage increased, this was considered as disease progression, and if the blast cell percentage decreased by <50%, this was considered as a poor response. In all leukemia CR patients and donors, the blast cell count was <5% in the bone marrow.

Megakaryocyte count. The number of megakaryocytes in a 1.5x3.5-cm area of a standard bone marrow smear were determined under microscope (BX53; Olympus Corporation, Tokyo, Japan) using Wright's staining (Sigma-Aldrich, St. Louis, MO, USA) in all patients and donors, as described previously (17,18). Megakaryocyte morphology was also analyzed in all patients and donors (17,18).

Platelet count. Prior to and following one course of decitabine treatment, patients' peripheral blood platelet count was performed using a CytoFLEX blood cell counter (Beckman Coulter, Inc., Brea, CA, USA).

In vitro induction of primary bone marrow mononuclear cell (BMMNC) differentiation. A total of 5 ml bone marrow aspirate was obtained from each subject at the Department of Hematology (General Hospital of Tianjin Medical University). Overall, 80 bone marrow samples were collected; 40 samples were obtained from MDS patients (prior to and following one cycle of decitabine treatment), 20 samples from the leukemia CR patients and 20 samples from the healthy donors. EDTA (BD Biosciences, San Diego, CA, USA) was added to the aspirate at the time of collection. The samples were washed immediately with sterile phosphate-buffered saline (PBS; BD Biosciences). BMMNCs were separated with Facoil buffer (Sigma-Aldrich) and seeded in a 6-well culture plate. Cells (1.10-1.20x10^6 cells/well) were cultured at 37˚C in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) with 10 ng/ml recombinant human thrombopoietin (Thermo Fisher Scientific) and 5 ng/ml stem cell factor (Thermo Fisher Scientific) for 7 days. Cells were counted using a cell count plate (Eppendorf, Hamberg, Germany) prior to and following culture. To assess cell viability Trypan Blue (Sigma-Aldrich) was used to stain cells and only viable cells were counted. Decitabine was added to the wells in a concentration ladder (0.0, 2.0, 2.5 and 3.0 μM). Megakaryocyte differentiation was evaluated following 7 days of culture at 37˚C in at atmosphere containing 5% CO_2. At harvesting, 1 ml PBS was added to each well. Following gentle mixing with a plastic pipette (1 ml pipette; Thermo Fisher Scientific), cells were counted and harvested with the pipette. Megakaryocytes remained suspended in PBS and were incubated with 20 μl mouse anti-human CD41α-fluorescein isothiocyanate immunoglobulin G antibody (ready to use; cat. no. 340929; BD Biosciences) at 4˚C for 30 min in the dark. Following incubation, the cells were washed twice with PBS. Approximately 30,000 cells were acquired and analyzed for CD41 expression using fluorescence-activated cell sorting analysis (FACS; FACScalibur™; BD Biosciences) and CellQuest™ software version 6.0 (BD Biosciences).

Statistical analysis. All results are expressed as the mean or median ± standard deviation (SD). Data were analyzed using the independent samples t-test for comparisons between groups. All statistical analyses were performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). P<0.05 were considered to indicate a statistically significant difference.

Results

In vivo effects of decitabine. In the present study, all MDS patients enrolled were thrombocytopenic at the commencement of therapy. After one cycle of decitabine treatment, an increase in platelet count was identified in 16 patients

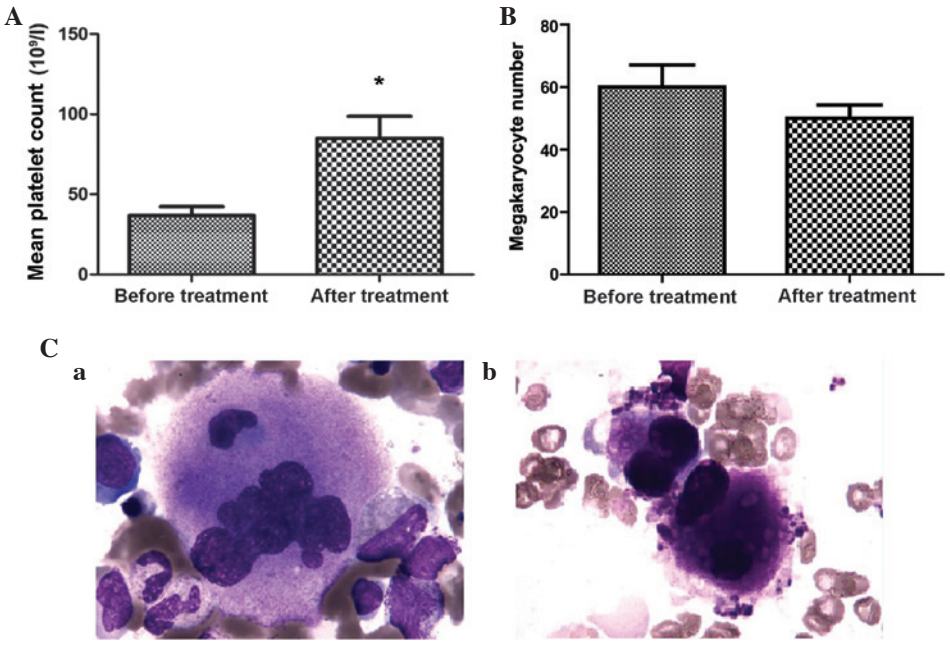

Figure 1. (A) Platelet counts of patients with MDS were significantly elevated after one cycle of 5-aza-2'-deoxycytidine (decitabine) treatment (20 mg/m^2/day). *P=0.001. Data are expressed as the mean ± standard deviation. (B) MDS patients' megakaryocyte number prior to and following one cycle of decitabine chemotherapy. Data are expressed as the mean ± standard deviation. (C) Megakaryocyte count and morphological analysis in a single patient with MDS (magnification, x100; Wright's staining). (Ca) Prior to treatment with decitabine (20 mg/m^2/day), the dysplastic megakaryocyte was immature and the release of platelets was dysfunctional. (Cb) In the same patient's bone marrow after decitabine treatment, a mature megakaryocyte producing platelets was observed. MDS, myelodysplastic syndrome.

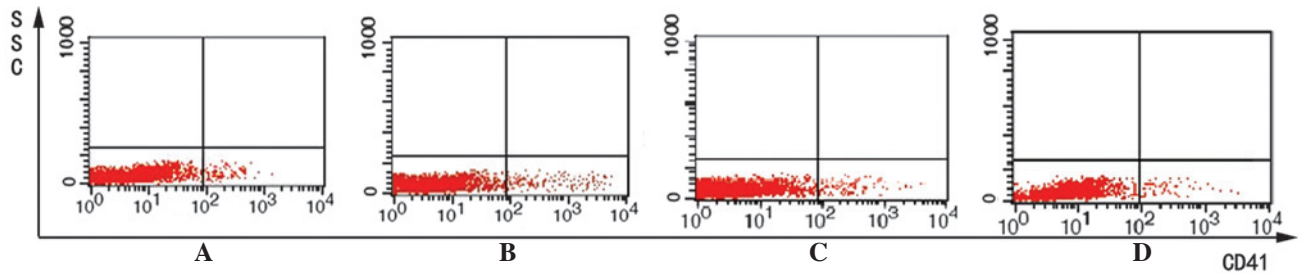

Figure 2. Megakaryocytes in bone marrow samples, detected by flow cytometry. (A) CD41 expression in the bone marrow mononuclear cells of a patient with myelodysplastic syndromes was extremely low prior to 5-aza-2'-deoxycytidine (decitabine) treatment [MFI, 206.73]. (B) CD41 expression increased after treatment with 2.0 μM decitabine (MFI, 308.83), and decreased progressively when cultured with (C) 2.5 μM (MFI, 288.10) and (D) 3.0 μM (MFI, 256.08) decitabine. CD, cluster of differentiation; MFI, mean fluorescence intensity.

(80%), however, platelet count remained stable (increased or decreased by <10x10^9/l) in 4 patients. The mean platelet counts were significantly increased in the MDS patient group, from 36.85±24.54x10^9/l before treatment to 84.90±61.85x10^9/l after treatment (P=0.001; Fig. 1A). However, no significant differences in megakaryocyte count were identified in the MDS patient group following one cycle of decitabine therapy (P>0.05). For the healthy donor group and leukemia CR patient group, the mean platelet counts were 198.55±54.25x10^9/l and 224.13±72.33x10^9/l, respectively, and these patients did not receive any treatment in the present study. Morphological analysis revealed megakaryocyte maturation and the production of platelets following treatment of MDS patients with 20 mg/m^2/day decitabine (Fig. 1B and C). Furthermore, in 13 patients, bone marrow blast cell count decreased by >50%. For 3/7 poorly responded patients, blast cell count increased after one cycle of decitabine chemotherapy. Notably, the platelet count increased to >30x10^9/l in all of the 3 progressed patients (data not shown). In the control group, the size and maturation of maturation of megakaryocytes was normal, and the blast cell percentage was <0.5%.

In vitro effects of decitabine. To investigate the effect of decitabine on megakaryocyte differentiation, megakaryocytes were cultured *in vitro* and exposed to various concentrations of decitabine (0.0, 2.0, 2.5 and 3.0 μM) to identify the optimal concentration required for megakaryocyte maturation. These results may provide a partial guide for the clinical use of decitabine in MDS patients with refractory thrombocytopenia. Briefly, BMMNC cells were incubated with various concentrations of decitabine (0.0, 2.0, 2.5 and 3.0 μM) for 7 days. The BMMNC cells were harvested, stained for the megakaryocyte marker CD41, and

Table I. Mean fluorescence intensity of membrane cluster of differentiation 41 in the bone marrow mononuclear cells of MDS patients, following treatment with decitabine at various concentrations.

	Mean fluorescence intensity (±SD)		
Decitabine (μM)	Control group	Leukemia CR	*de novo* MDS
0.0	284.53±38.12	318.91±24.70	226.19±17.61
2.0	294.07±47.34	307.42±55.40	258.95±28.05
2.5	273.25±34.26	273.05±47.54	242.89±24.11
3.0	272.93±38.36	232.43±33.90	224.23±16.05

MDS, myelodysplastic syndromes; decitabine, 5-aza-2'-deoxycytidine; CR, complete remission; SD, standard deviation.

evaluated by flow cytometry. The mean fluorescence intensity (MFI) of CD41 was compared between the four different treatment groups. In the MDS patients group, the results indicated that 2.0 μM decitabine induced the highest expression of CD41 following treatment (MFI, 258.95±28.05; P<0.05; Table I; Fig. 2), however, CD41 expression remained consistently and significantly lower than the healthy control group (P<0.05). In the leukemia CR patients group, the expression of CD41 was significantly higher than that of the healthy controls prior to decitabine treatment (MFI, 318.91±24.70 vs. 284.53±38.12; P<0.05; Table I); however, CD41 expression significantly decreased in the leukemia CR group with increasing decitabine concentration (P<0.05; Table I). In addition, the cell number of every well was calculated prior to and after cell culture. A total of 1.10-1.20x10^6 cells were seeded in every well. Following culture, no significant differences in cell number were identified in the 0.0, 2.0 or 2.5 μM treatment subgroups. However, following treatment with 3.0 μM decitabine, the cell numbers of all three groups decreased to 0.5-0.8x10^6/well and these differences were determined to be significant (P<0.05; Fig. 3).

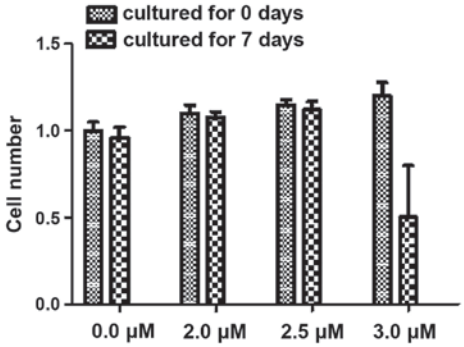

Figure 3. Cultured cell number (presented as the mean ± standard deviation) in different concentration groups (2.0, 2.5 and 3.0 μM) prior to and following 7 days culture.

Discussion

Previously, the treatment of elderly MDS patients with decitabine was found to result in a response rate of 45-50% (10,11). Increased platelet count is a major response observed in these patients. This is of particular importance, as supportive care involves platelet transfusions, which may be a burden for the patient, often leading to a refractory response to expensive platelet transfusions (1-3).

In the current study, after one cycle of decitabine treatment, a platelet response was observed in 16/20 (80%) MDS patients, with an mean increase of >30x10^9/l. In 2004, Van den Bosch *et al* (12) investigated 162 high-risk MDS patients. The authors reported that 58% of thrombocytopenic patients exhibited a platelet response following one cycle of decitabine therapy, and 69% of patients with a low platelet count exhibited a response during therapy. In addition, an increase in platelet count was found to be preceded by a positive trilineage response. The platelet responses observed in MDS patients following decitabine treatment in the present study and the study by Van den Bosch *et al* (12) (80 and 65%, respectively) were higher than the previously reported platelet response rates of 45-50% (6,10,11). Therefore, it is hypothesized that the megakaryocytic lineage pathways affected by decitabine may be independent from its effects on other lineages. Notably, in the current study, after one cycle of decitabine, the three progressed patients exhibited an evident platelet response, which enforces our hypothesis. Alternatively, dysplastic megakaryocytes may exhibit more sensitivity and thus respond to treatment earlier than other cell lineages (12). The maturation of megakaryocytes involves endomitosis (19), whereby the latter stages of mitosis are bypassed to allow an increase in DNA content and size of cells; therefore, megakaryocytes may be more sensitive to hypomethylation agents, such as decitabine, due to high levels of DNA replication (9).

In the present study, a concentration ladder of decitabine was used for *in vitro* cell culture. The results indicated that the MFI of CD41 was highest in the 2.0 μM decitabine subgroup (Fig. 2; Table I). Wang *et al* (9) performed a similar experiment *in vitro* using the mouse cell line, L8057, and demonstrated that decitabine induced the highest expression of CD41 at a concentration of 2.5 μM. Notably, these concentrations of decitabine are lower than the concentration used clinically, according to NCCN guidelines (16). CD41 is the surface marker of the megakaryocytic lineage which represents megakaryocyte maturation (20). In the current study, the higher concentration subgroup (3.0 μM) expressed a lower MFI and a decreased cell number after 7 days culture, which may be a result of severe cytotoxic side

effects of decitabine. However, the 3.0 μM subgroup, which approximates to the clinical dose used, resulted in higher CD41 expression than the 0.0 μM decitabine treatment group. These results indicate that a therapeutic regimen using a lower concentration of decitabine may be of clinical use for the induction chemotherapy of MDS patients. Furthermore, in the present study, although CD41 expression was significantly increased in the MDS group following treatment with 2.0 μM decitabine, the expression levels remained lower than those observed in the two control groups, which indicates that additional decitabine consolidation chemotherapy may be required for MDS patients following treatment with induction chemotherapy.

In the present study, the megakaryocyte number was calculated for the MDS patient group prior to and after one cycle of decitabine chemotherapy, however, no significant differences were identified. These results indicate that the increases in platelet counts and CD41 expression observed were due to the improvement of the quality of megakaryocytes, rather than megakaryocyte number. Thus, we hypothesize that decitabine affects the megakaryocytic lineage via the induction of differentiation and maturation of the lineage. Furthermore, maturation of megakaryocytes was observed directly via morphological comparison of patients' bone marrow smears prior to and after decitabine chemotherapy (Fig. 1B).

MDS is a malignant cloning disease. Typically, a small number of normal and dysplastic clones usually coexist in the bone marrow of MDS patients, (21,22). In the current study, megakaryocyte differentiation in healthy donors was also induced by decitabine, as indicated by increased expression of CD41, which peaked in the 2.0 μM subgroup (Table I). Similarly, Momparler *et al* (23) observed a 2-3-fold increase in the platelet count of patients treated for metastatic lung cancer using the same chemotherapeutic agent. In addition, in sickle cell anemia patients treated with even lower decitabine doses (0.15-0.30 mg/kg), an increase in hemoglobin F levels and concomitant increases in platelet counts were observed (24). These results indicate that the normal clones that exist in the bone marrow of MDS patients may also be induced by decitabine, which may explain the platelet response observed following decitabine treatment.

In conclusion, decitabine, as a DNA-hypomethylating agent, appears to induce the differentiation and maturation of myelodysplastic megakaryocytes in MDS patients, even at low concentrations. Therefore, the repeated administration of decitabine at lower doses in MDS patients may be useful in clinical practice, and may lead to the development of alternative treatments for other diseases of abnormal megakaryocyte differentiation, such as idiopathic thrombocytopenic purpura, however, future studies are required to investigate this.

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