Impaired differentiation and apoptosis of hematopoietic precursors in a mouse model of myelodysplastic syndrome

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ABSTRACT

Expression of a *NUP98-HOXD13 (NHD13)* fusion gene, initially identified in a patient with myelodysplastic syndrome, leads to a highly penetrant myelodysplastic syndrome in mice that recapitulates all of the key features of the human disease. Expansion of undifferentiated lineage negative (lin^{neg}) hematopoietic precursors that express *NHD13* was markedly inhibited (30-fold) *in vitro*. Decreased expansion was accompanied by decreased production of terminally differentiated cells, indicating impaired differentiation of *NHD13* precursors. Rather than differentiate, the majority (80%) of *NHD13* lin^{neg} precursors underwent apoptotic cell death when induced to differentiate. These findings demonstrate that *NHD13* lin^{neg} cells provide a tractable *in vitro* system for studies of myelodysplastic syndrome.

Key words: myelodysplastic syndromes, mouse model, *NUP98*, *HOXD13*, apoptosis.

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Introduction

Myelodysplastic syndrome (MDS) is a clonal stem-cell disorder characterized by ineffective hematopoiesis in one or more hematopoietic cell lineages. Despite peripheral blood cytopenias, the bone marrow (BM) of patients with MDS usually shows normal or increased cellularity.[1] This paradox can be explained by an increase in apoptosis, and several studies have demonstrated an increased rate of apoptosis in the BM of patients with early-stage MDS.[2-5] In addition to increased cell death or apoptosis, failure of differentiation contributes to the lack of terminally differentiated blood cells in patients with MDS.[6] *In vitro* colony assays demonstrate abnormal colony formation by granulocyte-macrophage (CFU-GM), erythroid (BFU-E, CFU-E) and megakaryocyte (CFU-Meg) progenitors in many patients with MDS.[7-9]

Important clues to the etiology of many hematologic malignancies, including MDS have come from cytogenetic studies. Although rare, translocations of the *NUP98* gene, especially those leading to fusions of *NUP98* with clustered homeobox (*HOX*) genes have been associated with both MDS and AML.[10] Recently, we developed a mouse model of MDS that recapitulates all of the critical features of the human disease by expressing a *NUP98-HOXD13* (hereafter *NHD13*) fusion gene in hematopoietic tissues under control of the *Vav* promoter.[11] In our initial studies, we showed that unmanipulated BM from *NHD13* and wild type (WT) mice gave rise to similar numbers of colony forming units (CFU) *in vitro*, and that BM from the *NHD13* mice showed increased replating potential.[11] However, those studies did not assess the growth, differentiation, and death of the hematopoietic stem cell (HSC) population. In this study, we demonstrate that *NHD13* lineage negative (lin^{neg}) murine bone marrow cells, which contain the hematopoietic stem and progenitor population, show increased apoptosis and differentiation failure, similar to the human disease.

Design and Methods

Mice

All of the *NHD13* transgenic mice were 7-9 months old on a C57Bl6 background. Bone marrow nucleated cells (BMNC) were obtained by flushing the femur and tibia. Diagnosis of MDS was confirmed by complete blood counts (CBCs) and cytospin examination of BM cells. CBCs were determined using a HEMAVET Multispecies Hematology Analyzer (CDC Technologies, Oxford, CT, USA). These studies were approved by the National Cancer Institute Animal Care and Use Committee.

Selection of lineage-negative (lin^{neg}) cells

We used the StemCep™ Mouse Progenitor Enrichment Kit (Stemcell Technologies, Canada) following the manufacturer's

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recommendations. The lineage cocktail contained the following antibodies: CD5 (Ly-1), Mac-1, B220, Gr-1, and Ter119. Purity of the purified lin^{neg} population was assessed by fluorescence activated cell sorting (FACS).

Liquid cultures and colony forming cell (CFC) assays

Lin^{neg} cells were cultured at 37°C/5% CO_2 in 6-well culture clusters (Corning Incorporated, NY, USA) in 3 mL of Iscove's Modified Dulbecco's Medium (Invitrogen, CA, USA) supplemented with 15% fetal bovine serum and the following cytokines: recombinant mouse stem cell factor (SCF, 100 ng/mL), recombinant mouse interleukin 3 (rmIL-3 , 6 ng/mL) and recombinant human interleukin 6 (rhIL-6, 10 ng/mL) (all from R&D Systems, MN, USA). On days 2, 4 and 7, cells were harvested, counted, and assayed for apoptosis. For CFC assays, 4×10^4 whole BM or 2×10^3 lin^{neg} cells were plated onto 35-mm Petri dishes in Methocult M3434 methylcellulose medium (Stemcell Technologies, Canada) respectively. Methocult M3434 is 2% methylcellulose supplemented with cytokines (SCF [50 ng/mL], rmIL-3 [10 ng/mL], rhIL-6 [10 ng/mL], and rhEpo [3 U/mL]). The CFC plates were incubated at 37°C in a 5% CO_2 incubator, and the number of colonies was counted 12 days after plating.

Apoptosis assay

Harvested cells from suspension culture were stained with FITC Annexin V (BD Biosciences, CA, USA) and propidium iodide (PI) (Sigma-Aldrich, MO, USA) using the manufacturer's recommended protocol. FACS analyses were performed using a dual laser FACScan (BD Biosciences, CA, USA).

Statistical analysis

Data are expressed as the mean ± standard errors of the mean (SEM). Differences between groups were analyzed by Student's t-test. *p*-values less than or equal to 0.05 were considered to be significant.

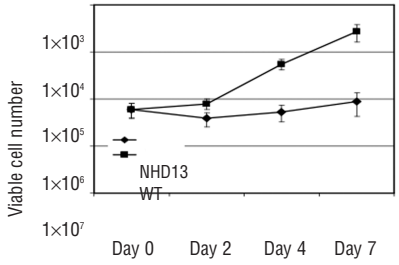
Results and Discussion

To determine whether the *NHD13* bone marrow nucleated cells (BMNC) were impaired in their ability to differentiate, we performed a standard colony forming cell (CFC) assay in methylcellulose. Although there was no dramatic difference in the numbers of colonies produced from whole BM, there was a clear difference in the type of colonies generated (Figure 1A). Whereas most of the colonies generated from WT BMNC were CFU-GM and CFU-GEMM, most of the *NHD13* colonies were abnormally small BFU-E and CFU-E colonies. To assess the differentiation potential of more primitive hematopoietic progenitors, we isolated lin^{neg} BMNC using magnetic beads as described above, and repeated the CFC assay with purified lin^{neg} BMNC. In these experiments there was an almost 10-fold decrease in the number of colonies produced by the *NHD13* lin^{neg} BMNC compared to WT lin^{neg} BMNC, suggesting a marked inhibition of growth and/or differentiation of the lin^{neg} progenitors. These results are similar to studies

A

	Whole BM (col/4x10^4 cells)		Lin^{neg} BM (col/2x10^3 cells)	
	WT	NHD13	WT	NHD13
CFU-E	9±2	42±45	5±2	2±2
BFU-E	22±9	28±7	35±2	5±1**
CFU-GM	84±44	24±21	93±21	4±5**
CFU-GEMM	6±2	0±0**	6±2	2±3
Total	121±49	94±29	139±21	13±6**

B



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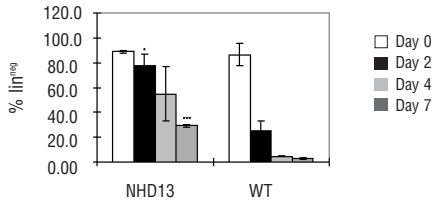

Figure 1. ***In vitro*** **expansion and differentiation of lin^{neg} cells. (A) CFC assay of whole BM and lin^{neg} BM cells from *NHD13* or WT mice. Colony counts represent total number from two plates for each sample, n=3 NHD13 and WT mice each. CFU-E, colony forming unit-erythroid; BFU-E, burst forming unit-erythroid; CFU-GM, colony forming unit-granulocyte, macrophage; CFU-GEMM, colony forming unit-granulocyte, erythroid, macrophage, megakaryocyte; **p<0.01. (B) Expansion of lin^{neg} cells from WT and *NHD13* mice grown in liquid culture. Cell counts represent pooled data from 3 independent experiments. (C) Percent lin^{neg} cells according to the culture day, pooled data from 3 independent experiments. (*p=0.051, ***p<0.01).**

of MDS patients comparing *in vitro* CFC results using CD34$^+$ purified cells instead of whole BM.[12,13] We, therefore, focused our subsequent studies on lin^{neg} BMNC.

On day 0, 6×10^4 lin^{neg} cells were placed in liquid culture with SCF, IL-3, and IL-6 as described above. The total number of viable cells was decreased in the *NHD13* cultures on day 2; thereafter, the total cell number slowly increased. By contrast, the WT cells accumulated briskly after a brief lag phase, such that by day 7 there was a 30-fold difference in cell number between the *NHD13* and WT cultures (Figure 1B).

As murine hematopoietic cells differentiate, they acquire cell surface markers such as Mac1 (monocyte/granulocyte), Gr1 (granulocyte), Ter119 (erythroid), B220 (B-lymphoid), and CD5 (T-lymphoid). We evaluated acquisition of these markers to determine whether the cells had differentiated after *in vitro* culture in the presence of SCF, IL-3, and IL-6. As early as day 2, 77.6±13.1% of the *NHD13* cells remained lin^{neg}, whereas only 25.2±10.7 % of the WT cells remained lin^{neg} (Figure1C). This trend continued, and on day 7 the fraction of *NHD13* lin^{neg} cells was almost 10-fold greater than the fraction of WT lin^{neg} cells (29.2±1.58% *vs.*

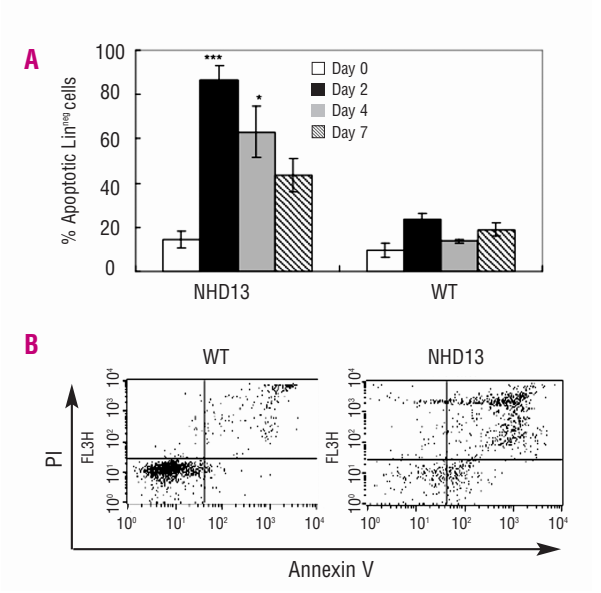

Figure 2. Apoptosis of lin^{neg} *NHD13* cells grown in liquid culture. (A) Apoptosis (%) of lin^{neg} cells in liquid culture (n=3, *p=0.051, ***p<0.01). (B) Representative FACS profiles of Annexin V and PI on culture day 2.

3.1±0.88%, p<0.01) (Figure 1C). These results demonstrate that *NHD13* BM cells are impaired in their ability to differentiate *in vitro*, consistent with the *in vivo* results demonstrating peripheral blood cytopenias and increased immature granulocytes in the BM of *NHD13* mice.[11] To determine whether increased apoptosis of *NHD13* lin^{neg} BMNC might contribute to the decreased number of NHD13 compared to WT cells, we used annexin V and PI staining to evaluate apoptotic cells. Lin^{neg} cells were again placed in liquid culture with SCF, IL-3, and IL-6. An increased proportion of apoptotic cells was evident in the *NHD13* culture as early as day 2 (Figure 2), and persisted for up to seven days. However, there was no clear increase in apoptosis of freshly isolated NHD13 lin^{neg} cells (Figure 2). These results suggest apoptosis of the *NHD13* cells is induced *in vitro* after the cells receive a differentiation stimulus. Alternatively, increased apoptosis of *NHD13* BM may be more difficult to detect *in vivo* as the apoptotic cells are quickly and efficiently cleared by phagocytes. In this context, it is important to note that increased apoptosis in BM from MDS patients is often difficult to appreciate, and has been attributed to rapid clearance of apoptotic hematopoietic cells *in vivo*, this hypothesis is supported by studies which show marked apoptosis of BMNC cells from MDS patients following culture *in vitro*.[14-16] The hypothesis that myeloid cells which are blocked in their ability to terminally differentiate undergo apoptosis in response to differentiation signals is supported by previous studies which showed that murine BMNC with enforced *Myc* expression had decreased terminal differentiation and increased apoptosis when treated with GM-CSF.[17]

In this report, we show that lin^{neg} hematopoietic precursors from *NHD13* mice are impaired in their ability to differentiate, and undergo apoptosis when induced to differentiate, similar to findings with human MDS. These findings are consistent with findings that embryonic stem (ES) cells with a *NHD13* "knock-in" allele were impaired in their ability to generate hematopoietic colonies *in vitro*.[18] Given that authenticated stable cell lines which recapitulate MDS characteristics *in vitro* and *in vivo* are not available,[2,6,19] we believe that BM obtained from an accurate mouse model of MDS can provide a useful *in vitro* platform for MDS studies. This *in vitro* approach is more amenable to high throughput studies than are *in vivo* models, and provide an accessible, *in vitro* culture system with which to study apoptosis and impaired differentiation associated with MDS.

Authorship and Disclosures

CWC: designed and conducted research and wrote the first draft of the manuscript; YJC: designed and conducted research; CS: designed and conducted research; PDA: designed research and wrote the final draft of the manuscript. The authors reported no potential conflicts of interest.

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