

# Dysregulated Human Myeloid Nuclear Differentiation Antigen Expression in Myelodysplastic Syndromes: Evidence for a Role in Apoptosis

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## Abstract

**Reduced levels of human myeloid nuclear differentiation antigen (MNDA) gene transcripts have been detected in both familial and sporadic cases of myelodysplastic syndromes (MDS). Numerous reports implicate elevated apoptosis/programmed cell death and death ligands and their receptors in the pathogenesis of MDS. MNDA and related proteins contain the pyrin domain that functions in signaling associated with programmed cell death and inflammation. We tested the hypothesis that MNDA is involved in the regulation of programmed cell death in human myeloid hematopoietic cells. Clones of K562 cells (MNDA-null) that expressed ectopic MNDA protein were established using retroviral transduction. MNDA-expressing K562 clones were resistant to tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-induced apoptosis, but were not protected from programmed cell death induced with genotoxic agents or H<sub>2</sub>O<sub>2</sub>. MNDA protein expression assessed in control and intermediate and high-grade MDS marrows showed several patterns of aberrant reduced MNDA. These variable patterns of dysregulated MNDA expression may relate to the variable pathophysiology of MDS. We propose that MNDA has a role regulating programmed cell death in myeloid progenitor cells, and that its down-regulation in MDS is related to granulocyte-macrophage progenitor cell sensitivity to TRAIL-induced programmed cell death.** (Cancer Res 2006; 66(9): 4645-51)

## Introduction

Myelodysplastic syndromes (MDS) are clonal disorders affecting hematopoiesis that lead to fatal cytopenias or acute myeloid leukemia (AML). The pathogenesis of MDS is unknown and most attempts to characterize MDS have concluded that excessive apoptosis/programmed cell death or sensitivity to the induction of programmed cell death contributes to the cytopenias (1). The classification of MDS into low-grade refractory anemia (relatively long survival), intermediate refractory cytopenia with multilineage dysplasia, and high-grade refractory anemia with excess blasts depends on percent blasts, karyotype, and peripheral cytopenias (2). Intermediate and high-grade cases manifest multiple abnor-

malities typically including neutropenia and granulocytic dysplasia that frequently progress to AML with an especially poor prognosis. Gene expression profiling analyses of MDS have documented differences in levels of transcripts in hematopoietic progenitor cells in MDS compared with normal cells (3, 4) or to AML (5). In the report by Hofmann et al. (4), the level of *human myeloid nuclear differentiation antigen (MNDA)* gene transcripts was lower in high-grade cases of MDS compared with low-grade cases. In another gene-profiling analysis, *MNDA* was the most significantly down-regulated gene in both familial and sporadic MDS cases (3).

*MNDA* is a member of the IFN-regulated 200 family of genes that contain one or two copies of a partially conserved 200-amino-acid domain that is thought to mediate protein-protein interactions specifically with other transcription regulatory proteins (6). All 200 genes are regulated by IFN and most members, including *MNDA*, also contain an 80 to 100 NH<sub>2</sub>-terminal amino acid pyrin domain that has been proposed to link a number of proteins through pyrin domain interactions to signaling pathways in programmed cell death and inflammation (7). The effects of the mouse *p202a* gene on programmed cell death has been examined in a number of experimental systems and found to both promote and inhibit programmed cell death (8–11). Forced expression of *p202a* increased the extent of tumor necrosis factor (TNF)-induced programmed cell death in breast cancer cells (10). The human 200 family gene *IFI16* was reported to promote programmed cell death in association with p53 activated by genotoxic agents (12). *MNDA* expression is limited to hematopoietic cells and this should be considered when investigating the possibility of its role in programmed cell death.

Our initial experiments assessed protein levels and changes in intracellular localization in HL60 cells undergoing programmed cell death and suggested that *MNDA* played an active role in this process. The effect of *MNDA* expression on sensitivity of cells to induced programmed cell death was examined by converting an *MNDA*-null cell line (K562) to positive through stable ectopic expression, followed by challenge with agents that induce programmed cell death. *MNDA* expression protected K562 cells from TNF-related apoptosis inducing ligand (TRAIL)-induced programmed cell death. We then used a multiparametric flow cytometric assay to analyze marrow samples from MDS patients to characterize the down-regulation of *MNDA* protein in MDS compared with control marrow in subsets of marrow hematopoietic cells. The results were consistent with the involvement of *MNDA* down-regulation in the pathogenesis of MDS by altering granulocyte-macrophage progenitor cell response to TRAIL-induced programmed cell death.

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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## Materials and Methods

**Cell lines and materials.** The human myeloid leukemia HL60 cell line was obtained from the American Type Culture Collection (Manassas, VA) and maintained using protocols recommended by the supplier. The origins of the nontransduced K562 parental cells, the vector control K562 cells, and the stable MNDA-expressing retroviral-transduced clones A2 and D2 were described previously (13). The reagents, H<sub>2</sub>O<sub>2</sub> (50-1,200 μmol/L), mechlorethamine (0.5-2.0 μmol/L), etoposide (25-100 μmol/L), and cisplatin (0.5-2 μmol/L) were obtained from Sigma-Aldrich (St. Louis, MO); cells were exposed to the range of concentrations as indicated. Leptomycin B (Sigma-Aldrich), recombinant human TRAIL (Calbiochem, San Diego, CA), and TNF-α (Sigma-Aldrich) were used at the concentrations specified.

Bone marrow aspirates were done for routine diagnostic purposes from sequential MDS patients receiving no current treatment, using a local institutional review board-approved protocol. Diagnosis of MDS was based on review by three observers (D.R. Head, S.A. McClintock-Treep, and F. Boulous) of peripheral blood and bone marrow morphology, clinical history, laboratory data, and cytogenetics. Patients lacking primary marrow disease and with normal hematologic variables in peripheral blood were used as controls.

**MNDA antibody conjugation.** The MNDA rat monoclonal antibody 3C1 was used in previous studies to document cell-specific expression (14-19) and was also used to immunoaffinity purify MNDA for sequence analysis (20). The monoclonal antibody 3C1 (Chemicon International, Inc. Temecula, CA) was used in all immunochemical assays of MNDA expression. The antibody was conjugated to Alexa 488 using a protein labeling kit (Molecular Probes, Eugene, OR). The conjugated product was tested for reactivity using a whole blood lysis/permeabilization technique using PermiFlow (InVirion, Frankfurt, MI) as the permeabilization reagent. Cell lines with known MNDA expression status (HL60, K562) were tested in a similar manner. The reactivity of the conjugated product was identical to prior testing using the purified monoclonal antibody in immunocytochemical and immunoblotting analyses that reveal reactivity restricted to monocytes and granulocytes and the cell line HL60 (data not shown).

**Flow cytometry.** Flow cytometry analyses were completed on a FC500 (Beckman Coulter, Fullerton, CA) using an established quality control program designed to ensure a consistent fluorescence readout (21). Cells from marrows were reacted with saturating amounts of CD45-PE and CD34-ECD (Beckman Coulter), washed twice in PBS + 2% FCS, and then permeabilized for 1 hour in PermiFlow. Following permeabilization, MNDA-Alexa 488 (at a concentration shown to react specifically with granulocyte-macrophage progenitors) and DRAQ5 (Apotech, San Diego, CA; for cell cycle analysis) were added. A separate aliquot of cells was not permeabilized and was used for Annexin V binding. Analysis of 250,000 total events was done using Winlist 5.0 software (Verity Software, Topsham, ME) with DDE links to ModFitLT 3.0 using modifications of published methods along with algorithms generated by Esoterix Center for Innovation (Brentwood, TN). Analyses were blinded to clinical results.

The mean fluorescence intensity (MFI) was determined by recording the linear mean channel of the MNDA histogram taken from either the CD45<sup>+</sup>, CD34<sup>+</sup> stem cells or the high side scatter/intermediate CD45/CD34-negative granulocyte-macrophage progenitors. A cell was considered MNDA negative if its fluorescence intensity matched the fluorescence intensity obtained in the lymphocyte population.

**Immunocytochemistry.** An immunohistochemical procedure for paraffin-embedded tissue, reported previously (16), was used to localize MNDA in bone marrow sections. The same procedure was used to localize MNDA in cultured cell lines attached to slides by cytocentrifugation (without deparaffinization).

Preparation of total cellular proteins for electrophoretic separation and blotting has been described previously (15). Blots were probed for MNDA with rat monoclonal antibody 3C1 (1:1,000; ref. 16) and a peroxidase-conjugated anti-rat IgG antibody (1:5,000; Jackson ImmunoResearch Laboratory, Inc., West Grove, PA). The same blot was probed with a goat anti-actin (1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and then a mouse anti-goat peroxidase conjugate (1:5,000; Santa Cruz Biotechnology).

A duplicate blot was probed with rabbit anti-human poly(ADP)ribose polymerase (PARP; 1:10,000; Santa Cruz Biotechnology), and antibody binding was detected with a peroxidase-conjugated anti-rabbit IgG (1:20,000; Santa Cruz Biotechnology Inc.). The antibody interactions were visualized using enhanced chemiluminescence detection (Amersham, Arlington Heights, IL).

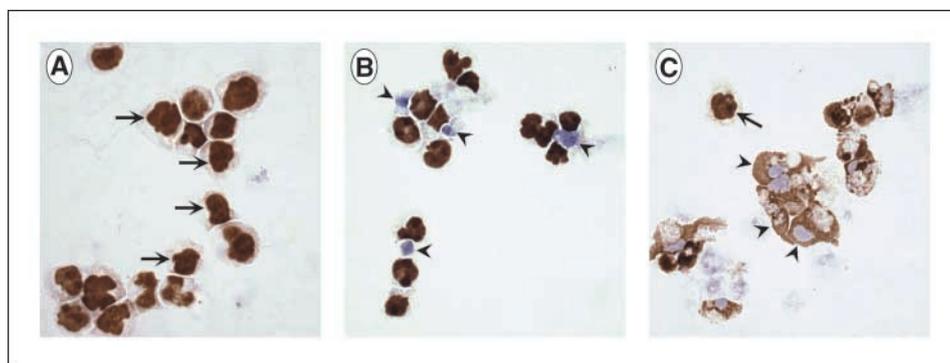
**Detection of programmed cell death.** The suspended cultured cells were induced to undergo programmed cell death. A 50 μL aliquot of the cell suspension was removed from the cultures at specified times and used to prepare slides by cytocentrifugation. Slides prepared at 24 hours were photographed using light microscopy without staining. Cells that had undergone programmed cell death appear as irregular, highly refractive clumps as confirmed by terminal deoxynucleotidyl transferase nick end labeling (TUNEL) reaction using a Vaso TACS kit (Trevigen, Inc., Gaithersburg, MD) as directed by the supplier. Loss of normal cell morphology was obvious and correlated with nuclear condensation, fragmentation, and reduced viable cell numbers determined by trypan blue dye exclusion. Slides prepared at 3 hours were used to detect histone H2B phosphorylation (Ser<sup>14</sup>), a specific late marker of programmed cell death (22, 23). A rabbit anti-human phospho-H2B (Ser<sup>14</sup>) serum (1:2,000; Upstate, USA, Inc., Charlottesville, VA) was used, and antibody binding was visualized using the universal vectastain ABC kit (Vector Laboratories, Inc. Burlingame, CA).

**Statistical analysis.** Statistical significance was accepted for a value of  $P < 0.05$  using Student's *t* test.

## Results

**MNDA expression in cell lines.** MNDA expression in untreated rapidly growing HL60 cells was investigated using immunocytochemistry. Nearly all HL60 cells contained uniform nuclear reactivity (Fig. 1A). When HL60 cultures were maintained without fresh medium for an extended time, the frequency of MNDA-negative cells in the culture increased noticeably and cellular morphology was consistent with programmed cell death (Fig. 1B). HL60 cells underwent programmed cell death following exposure to H<sub>2</sub>O<sub>2</sub> and most cells were MNDA negative 24 hours after exposure. MNDA-negative HL60 cells have morphology consistent with programmed cell death (data not shown). Exposing HL60 cells to H<sub>2</sub>O<sub>2</sub> also triggered early events. MNDA was relocated from nucleus to cytoplasm beginning at 3 hours and completed 4 hours after adding H<sub>2</sub>O<sub>2</sub> (Fig. 1C). A time course experiment examining levels of MNDA by immunoblot indicated that relocation of MNDA from the nucleus was followed by MNDA degradation that was preceded by cleavage of the 116 kDa death substrate PARP to an 85 kDa fragment that marks the execution phase of programmed cell death (Fig. 2). The cytotoxic agents (cisplatin, etoposide, and mechlorethamine) also induced an increase in the number of MNDA-negative cells in the cultures 24 hours after treatment (data not shown). An early relocation and subsequent degradation of MNDA was observed following exposure to each cytotoxic agent (data not shown). The early relocation of MNDA from the nucleus following the induction of programmed cell death with etoposide was not inhibited by leptomycin B (5-35 ng/mL; data not shown), indicating that active transport was not required.

**The effects of MNDA expression on programmed cell death.** K562 cells contain no detectable MNDA transcripts or protein (13). We previously generated stable K562 clones expressing ectopic MNDA (13). Both MNDA-null K562 parental cells and vector control transduced cells were compared with two ectopic MNDA-expressing K562 clones for responses to inducers of programmed cell death. K562 cells null for MNDA showed extensive degenerative changes in cell morphology when exposed to H<sub>2</sub>O<sub>2</sub> (Fig. 3A-D) and the same



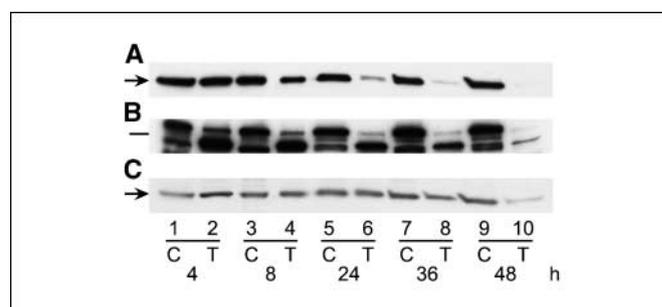
**Figure 1.** Immunocytochemical staining of HL-60 cells for MNDA (brown reaction product). Cells were counterstained with hematoxylin. *A*, control cells 2 days after feeding showing MNDA nuclear reactivity (arrows) in all cells. *B*, HL-60 cells maintained in culture for 11 days after feeding show a large number of cells without MNDA reactivity (arrowheads). *C*, HL-60 cells exposed to 500  $\mu\text{mol/L}$   $\text{H}_2\text{O}_2$  (4 hours) show a redistribution of the nuclear MNDA into the cytoplasm (arrowheads). Not all cells in the  $\text{H}_2\text{O}_2$ -treated cultures show relocation of MNDA (arrow), which is consistent with not all cells being induced to undergo programmed cell death. Original magnification,  $\times 400$ .

changes were observed in MNDA-expressing K562 cells (Fig. 3E-H). Forced MNDA expression in K562 cells did not influence the effectiveness of cisplatin, etoposide, or mechlorethamine to induce programmed cell death (data not shown). However, examination of programmed cell death induced through death receptors (extrinsic pathway) showed that clones of MNDA-expressing K562 cells were resistant to TRAIL-induced programmed cell death compared with nonexpressing K562 parental cells and vector control-transduced K562 cells (Fig. 4A-H). Use of an early marker of chromatin condensation [histone H2B phosphorylation (Ser<sup>14</sup>)] showed strong nuclear reactivity (dark nuclear reaction product in cells with hematoxylin counterstain) in most K562 parental or vector control MNDA-nonexpressing cells (Supplementary Fig. S1A and S1B), whereas the A2 and D2 clones of MNDA-expressing K562 cells showed only rare reactive nuclei (Supplementary Fig. S1C and S1D). An analysis of the sub-G<sub>1</sub> DNA content (flow cytometry) and TUNEL showed the same protection against TRAIL-induced programmed cell death in MNDA-expressing K562 cells compared with K562 null cells (data not shown). The MNDA-negative K562 parental and vector control cells were induced to undergo extensive programmed cell death with TRAIL (500 ng/mL) in all of our assays and the two stable clones of MNDA-expressing K562 cells were resistant to TRAIL up to 500 ng/mL. The same slides that were examined for morphologic changes at 24 hours in Fig. 4 (A, B, E, and F) were assessed for MNDA expression by immunocytochemical reaction. The results showed that the nontransduced K562 cells did not express MNDA both with and without exposure to 500 ng/mL TRAIL (Fig. 4I and J), whereas an MNDA-expressing clone of K562 cells did (Fig. 4K and L). That analysis also showed that MNDA remained in the nucleus of the expressing cells (Fig. 4K and L) that were protected from TRAIL (500 ng/mL)-induced programmed cell death. Higher levels of TRAIL (1,000-4,000 ng/mL) induced programmed cell death in the MNDA-expressing stable clones of transduced K562 cells and MNDA was relocated from the nucleus into the cytoplasm within 4 hours (data not shown) and was subsequently undetectable.

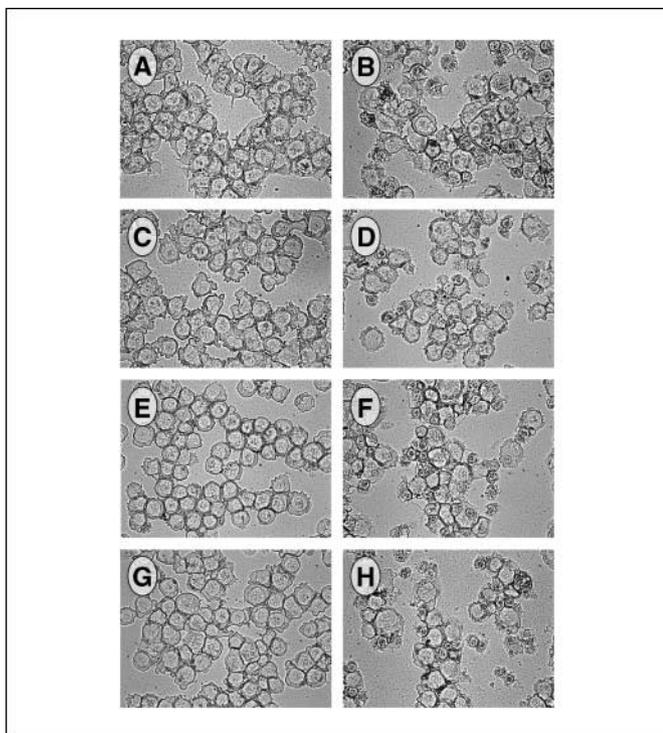
The extent of programmed cell death was similar in the MNDA-null K562 cells treated with 125 ng/mL TRAIL (Fig. 4M) as in the MNDA-expressing clones exposed to 1,000 ng/mL TRAIL (Fig. 4N). In addition, the viable cell counts in these cultures (MNDA-null at 125 ng/mL and MNDA-expressing 1,000 ng/mL TRAIL) treated for 72 hours were reduced to a similar extent (data not shown), indicating that MNDA expression was asso-

ciated with an 8-fold level of protection. TRAIL (500 ng/mL) has a long-term detrimental effect on MNDA-null K562 cell cultures, whereas the MNDA-expressing cells continued normal growth uninterrupted (Fig. 5). Additional experiments confirmed that parental K562 cells were inherently resistant to other agents (Fas-ligand, TNF- $\alpha$ ) that induce programmed cell death through the extrinsic pathway (24-27) precluding assessment of the ability of MNDA to protect against other effectors of the extrinsic pathway of programmed cell death.

**Expression of MNDA in MDS.** Considering the gene expression profiling results showing reduced *MNDA* transcript levels in MDS (3, 4), our results implicating MNDA in the regulation of TRAIL-induced programmed cell death, and reports of increased sensitivity of hematopoietic progenitors in MDS to TRAIL-induced programmed cell death, we then evaluated MNDA protein expression in MDS and control bone marrow samples to establish localization and relative levels of expression. Immunohistochemical analysis of MDS and control marrow sections showed nuclear localization of MNDA and no cytoplasmic reactivity in expressing cells, indicating that MNDA-expressing marrow hematopoietic cells were not in the late stages of programmed cell death (Supplemental Fig. S2, dark peroxidase-catalyzed nuclear



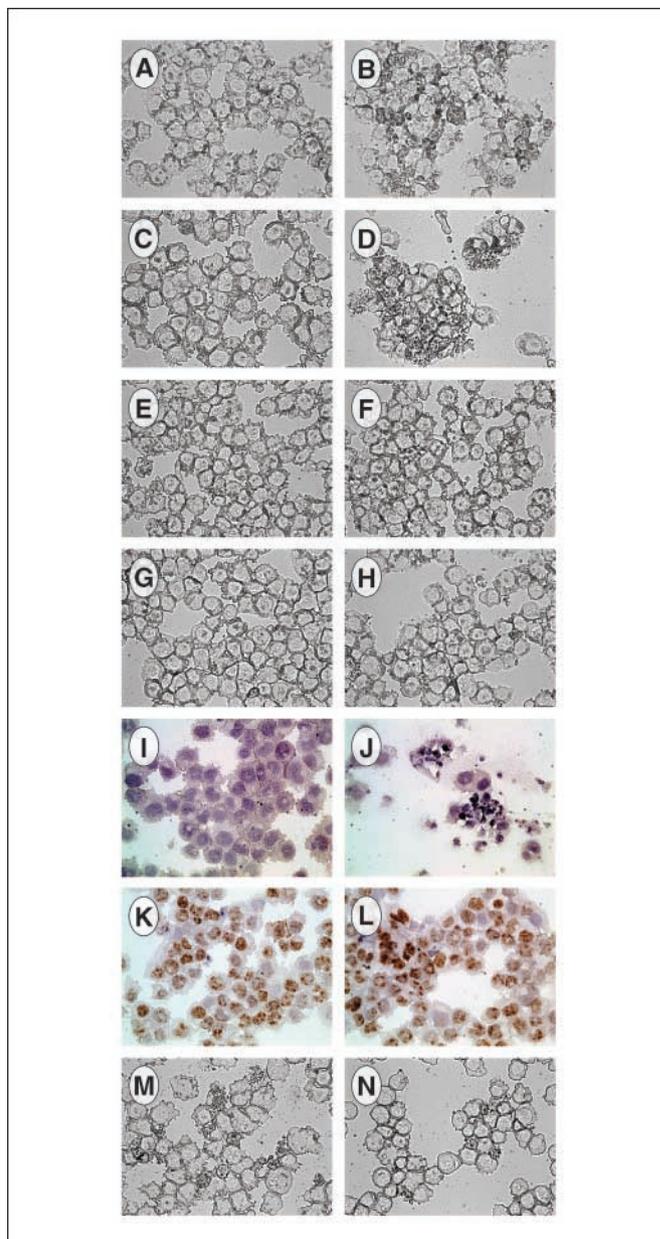
**Figure 2.** Immunoblot analysis of total cellular protein preparations from HL60 cells exposed to  $\text{H}_2\text{O}_2$  (7) from 4 to 48 hours with paired samples from untreated cultures (C). *A*, the MNDA antigen (arrow) migrates at 55 kDa and was progressively lost in treated samples with time. *B*, the cleavage of the intact death substrate PARP migrating at 116 kDa generates a 85 kDa band following  $\text{H}_2\text{O}_2$  exposure as evidenced by the progressive shift of the top band to the bottom in the treated samples with time (migration of 98 kDa marker indicated on left). *C*, the actin internal control band (arrow) is degraded in the treated sample at the latest time point consistent with nonspecific protein degradation. The degradation of MNDA was nearly complete after 24 hours of  $\text{H}_2\text{O}_2$  and the cleavage of PARP was nearly complete after 8 hours.



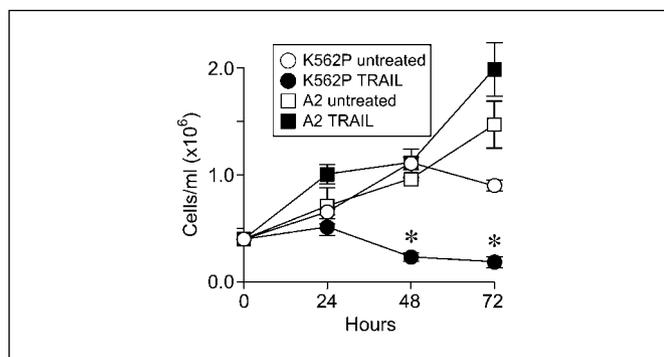
**Figure 3.** The effects of H<sub>2</sub>O<sub>2</sub> on K562 cells assessed by light microscopy of cells adhered to slides by cytocentrifugation. The MNDNA-negative, K562-nontransduced cells untreated (A) or exposed to H<sub>2</sub>O<sub>2</sub> (B). The MNDNA-negative K562 retroviral control cells (no insert) untreated (C) or exposed to H<sub>2</sub>O<sub>2</sub> (D). The stable MNDNA-expressing K562 cell clone A2 untreated (E) or exposed to H<sub>2</sub>O<sub>2</sub> (F). The stable MNDNA-expressing K562 cell clone D2 untreated (G) or exposed to H<sub>2</sub>O<sub>2</sub> (H). All cultures were exposed to 1.2 mmol/L H<sub>2</sub>O<sub>2</sub> for 24 hours. In all four lines, the increase in number of cells with abnormal morphology was accompanied by an increase in the number of irregular refractive clumps that marked for TUNEL (data not shown). The effects of H<sub>2</sub>O<sub>2</sub> were the same in the four cell cultures over concentrations of 50  $\mu$ mol/L to 1.2 mmol/L. Original magnification,  $\times$ 400.

immunochemical reactions). In contrast to the uniform strong reactivity in most cell nuclei in control marrow sections (Supplementary Fig. S2A and S2B), the MDS sections contained cells with low levels of reactivity, nonreactive late-stage granulocyte-macrophage progenitors, or a low percentage of MNDNA-reactive cells (Supplementary Fig. S2C-F).

The MNDNA reactivity in marrow sections detected by immunohistochemistry was consistent with lower MNDNA protein expression in MDS compared with normal bone marrow, but indicated that the basis for this varied with each case and might include combinations of hematopoietic cells with a normal level of MNDNA, low level expressing cells, or nonreactive cells. To resolve the variability in MNDNA reactivity detected by immunohistochemical staining, flow cytometric analysis was done in control and MDS marrow aspirates. Results of those analyses confirmed that MNDNA expression was specific for granulocyte-macrophage lineage cells in control marrow (Fig. 6, left). The majority of granulocyte-macrophage progenitors in control marrows express MNDNA at a uniformly very high level. We observed no difference in the level of MNDNA expression in the CD34<sup>+</sup> cell fraction in controls versus MDS marrows, with  $\sim$ 10% of the cells positive in each patient group (data not shown). A population of granulocyte-macrophage progenitor cells expressing a reduced level of MNDNA protein was present in most MDS samples (Figs. 6, right, and 7). It is also notable that some MDS marrow samples contain MNDNA-negative



**Figure 4.** The effects of TRAIL on K562 cells assessed by light microscopy of cells adhered to slides by cytocentrifugation. The MNDNA-negative K562 nontransduced cells untreated (A) or exposed to TRAIL (B). The MNDNA-negative K562 retroviral control cells (no insert) untreated (C) or exposed to TRAIL (D). The stable MNDNA-expressing K562 cell clone A2 untreated (E) or exposed to TRAIL (F). The stable MNDNA-expressing K562 cell clone D2 untreated (G) or exposed to TRAIL (H). Cultures were exposed to 500 ng/mL TRAIL for 24 hours. The MNDNA-negative nontransduced K562 cells and the MNDNA-negative vector control cultures showed a similar decrease in number of viable cells with normal morphology that was accompanied by an increase in the number of irregular clumps that were TUNEL positive (data not shown). The effects of TRAIL on the two MNDNA-null cultures were the same over concentrations of 100 to 500 ng/mL. The two MNDNA-expressing stable K562 cloned lines showed no effects of TRAIL from 100 to 500 ng/mL. The slides photographed in (A and B) were evaluated for MNDNA expression using immunocytochemical staining (absence of immunochemical reactivity) in (I and J). The slides photographed in (E and F) were evaluated for MNDNA expression using immunocytochemical staining (presence of nuclear immunochemical reactivity) in (K and L). The nontransduced MNDNA-null K562 cells were exposed to 125 ng/mL TRAIL (M) and the stable MNDNA-expressing K562 clone A2 was exposed to 1,000 ng/mL TRAIL (N) to achieve similar detrimental effects on cell morphology. Representative results from three independent experiments. Original magnification,  $\times$ 400.



**Figure 5.** The effects of TRAIL (500 ng/mL) on viable cell (trypan blue exclusion) counts of K562 cells nontransduced MNDA-negative untreated (K562P), or exposed to TRAIL for the times indicated. Viable cell counts of the clone of K562 cells transduced with MNDA-expressing retrovirus stably expressing MNDA (A2) untreated or exposed to TRAIL for the times indicated. Representative of three experiments. Points, mean; bars, SD. \*, significantly different from untreated cells at the 0.05 level.

granulocyte-macrophage progenitor cells, a situation not detected in control marrows (Fig. 7A). The MFI of MNDA in expressing granulocyte-macrophage progenitor cells in most MDS samples was lower than the MFI of MNDA in expressing granulocyte-macrophage progenitors in control marrow (Fig. 7A) with the exception of case M1 that also had a very high fraction of MNDA-negative granulocyte-macrophage progenitors (Fig. 7A). Overall, the average MNDA MFI for all granulocyte-macrophage progenitors in MDS cases was significantly less than that in control marrows (Fig. 7B).

## Discussion

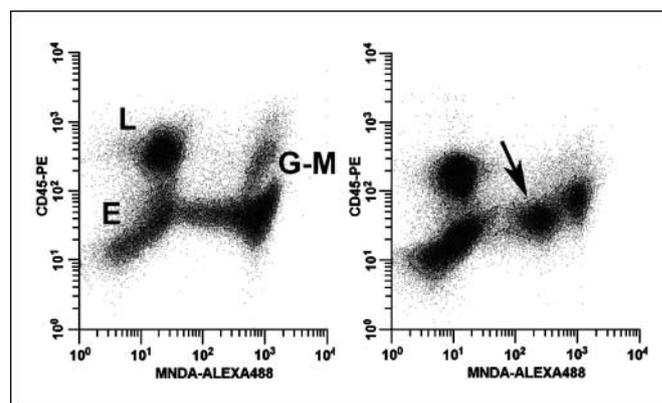
The reduced level of MNDA protein in granulocyte-macrophage progenitor cells in MDS cases detected in this study is consistent with the report of down-regulated *MNDA* message in the mononuclear cell fraction of marrows from familial and sporadic MDS cases (3). The down-regulation of *MNDA* expression in granulocyte-macrophage progenitors varied, being uniformly down-regulated in all granulocyte-macrophage progenitors in only one case and being composed of a mixture of MNDA-expressing and nonexpressing cells in four cases. Two cases had uniform cellular expression of MNDA slightly below the control levels and contained the lowest levels of Annexin V binding (Fig. 7A). All of our MDS cases, intermediate grade (refractory cytopenia with multilineage dysplasia) and high grade (refractory anemia with excess blasts), exhibited neutropenia and dysplasia of the granulocyte-macrophage progenitors. The possible significance of down-regulated MNDA expression in the pathogenesis of MDS, and the contribution of variability in this process to variability in the pathophysiology of individual cases, remains to be determined. The observations of defects in marrow cells in MDS patients vary to a large extent and may also include an admixture of nonclonal apparently normal cells.

Excessive programmed cell death and elevated sensitivity to induction of programmed cell death in MDS versus normal marrow have been reviewed recently (1). It was concluded that elevated programmed cell death contributes to the pathogenesis of MDS and accounts for the peripheral blood cytopenia associated with MDS. Most reports document elevated levels of programmed cell death in low-, intermediate-, and high-grade MDS cases (WHO

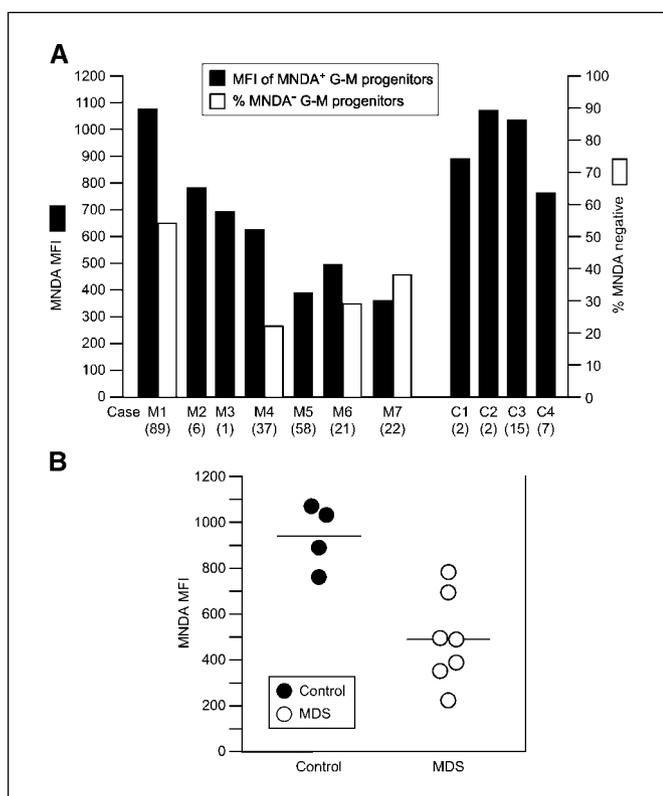
classification). Other results indicate that the programmed cell death levels within the maturing CD34<sup>+</sup> progenitor cells are consistently elevated, whereas programmed cell death levels in CD34<sup>+</sup> precursors vary with grade (28, 29) or are the same as normal regardless of grade (30). Our detection of subsets of granulocyte-macrophage progenitors (CD34<sup>+</sup> cells) with reduced levels or absence of MNDA in both intermediate- and high-grade MDS (Fig. 7) is consistent with the elevated levels of programmed cell death detected in MDS progenitor (CD34<sup>+</sup>) cells (28–30). Annexin V binding in our samples showed a 6-fold higher average level in the MDS cases versus the normal marrows (Fig. 7) and seemed to correlate inversely to some extent with MNDA expression in the granulocyte-macrophage progenitors.

A number of factors may contribute to programmed cell death in MDS, including defects in stromal cells, reduced levels of survival signals, as well as increased levels of death signals and receptors (1). Our detection of an MNDA-associated effect on TRAIL-induced programmed cell death might provide functional significance for the down-regulated MNDA expression in MDS. TRAIL has been found to suppress growth and differentiation of leukemia and MDS granulocyte-macrophage progenitors, whereas normal granulocyte-macrophage progenitors are resistant to TRAIL (31, 32). Exposing MDS marrows to exogenous TRAIL has also been shown to induce programmed cell death in granulocyte-macrophage progenitors. In addition, TRAIL and its receptors are found in MDS marrows (32).

Forced MNDA expression in K562 cells using retroviral transduction showed that programmed cell death induced by agents that act through the intrinsic pathway was not influenced by the presence of MNDA. However, we observed that TRAIL, which acts through the extrinsic pathway, was effective at inducing programmed cell death in K562 cells null for MNDA, as reported earlier (33), but was 8-fold less effective in stable MNDA-expressing K562 cells. The resistance to TRAIL observed in stable clones of MNDA-expressing K562 cells, considered with the reduced or absent MNDA protein expression in MDS, might relate to the previously described increased sensitivity of marrow progenitor cells in MDS to the programmed cell death-inducing effects of TRAIL (1).



**Figure 6.** Flow cytometric analysis of MNDA expression in a control marrow (left) and MDS case (right). In the control marrow, the MNDA-negative, nRBCs (E) and lymphoid (L) fractions are on the left side of the display and a continuum of increasingly positive granulocyte-macrophage progenitor cells (G-M) are in the middle and right side of the display. The majority of the granulocyte-macrophage progenitors express the highest level of MNDA and an intermediate level of CD45. Note that the MDS sample (right) contains a discrete subset of cells with a lower level of MNDA expression (arrow) relative to the highest MNDA-expressing granulocyte-macrophage progenitors.



**Figure 7.** The analysis of MNDA expression in MDS and control marrows using flow cytometry. *A*, the MFI of the MNDA-positive granulocyte-macrophage progenitors are plotted for each case. In addition, the percentage of MNDA-negative granulocyte-macrophage progenitors are included in cases where detected. *M1* to *M5*, intermediate-grade (refractory cytopenia with multilineage dysplasia) MDS cases; *M6* and *M7*, high-grade (refractory anemia with excess blasts) MDS cases. All seven cases had multilineage dysplasia that included the granulocyte-macrophage progenitors. *Numbers in parentheses*, percent Annexin V positivity. *B*, a plot of the MNDA MFI for total granulocyte-macrophage progenitors in control and MDS marrow aspirates. The control and MDS sample means are significantly different by a two tailed *t* test at the 0.05 level.

Our analyses of MNDA-expressing cell lines showed that degradation of MNDA during programmed cell death occurred after relocation from the nucleus to cytoplasm, yet our immunohistochemical analysis of MNDA in marrows of MDS patients showed only nuclear reactivity in granulocyte-macrophage progenitors, including those that exhibited reduced levels of MNDA. Thus, lower levels of MNDA in MDS do not seem to correlate with cells executing programmed cell death, and thereby degrading MNDA.

MNDA remained in the nucleus of the clones of MNDA-expressing K562 cells (transduced) exposed to 500 ng/mL TRAIL, indicating that protection against programmed cell death was apparently associated with MNDA function in the nucleus. MNDA and other genes in the 200 family are thought to influence gene expression through interactions with other proteins that regulate gene transcription. Possible specific gene targets for MNDA regulation include genes encoding functional TRAIL receptors (down-regulation) as well as decoy receptors (up-regulation; ref. 34). Other possible target genes include those encoding programmed cell death inhibitory factors, such as c-FLIP or survival proteins (35, 36), and proteins that can inhibit programmed cell death through interactions with receptor death domains or that associate with the death-inducing signaling complex (1). It should be noted that TRAIL acts exclusively

through the extrinsic pathway in K562 cells (37). A complete analysis of target genes influenced by MNDA expression is required to resolve this area.

Our initial results showed that the induction of programmed cell death in all MNDA-expressing (constitutive or ectopic) cells was followed by relocation of MNDA from nucleus to cytoplasm and then degradation. The significance of the relocation and degradation of MNDA remain unknown. However, these changes suggested MNDA involvement in programmed cell death and led to our discovery of its apparent specific role in restricting cellular response to TRAIL-induced programmed cell death. These early changes in MNDA localization might correlate with the ablation of MNDA effects on transcription regulation similar to the effect of translocating p53 out of the nucleus, which prevents continued p53 effects on gene transcription as well as allowing degradation of p53 in the cytoplasm (38, 39). In the case of p53, the relocation out of the nucleus is an active process inhibited by leptomycin B, whereas the translocation of MNDA was not. Because MNDA is a small protein (407 amino acids), it is unlikely that translocation from the nucleus depends on changes in the diffusion limits of nuclear pores that accompany programmed cell death (40). In addition, the time course over which these changes take place is inconsistent with being essential for the initiation or execution of programmed cell death. Relocation of MNDA from the nucleus to the cytoplasm is not complete until 4 hours after induction of programmed cell death and was then followed by degradation of MNDA. In the same cells, most PARP death substrate had been cleaved by 4 hours after induction of programmed cell death. Thus, our results indicate that relocation and degradation of MNDA are relatively late events in programmed cell death occurring after the execution phase has been initiated. The relocation of caspase-2 out of the nucleus and coinciding with nuclear fragmentation as in the case of MNDA (Fig. 1C) was considered a late event not associated with initiation of programmed cell death (41).

MNDA seems to act in the nucleus to alter the ability of K562 cells to respond to TRAIL. However, MNDA is relocated from the nucleus and degraded once programmed cell death was induced in the MNDA-expressing K562 or in other MNDA-expressing cells. The execution phase of programmed cell death progresses in the presence of MNDA, but seems to result in relocation of MNDA into the cytoplasm and ultimately to degradation. It is possible that the loss of MNDA late in programmed cell death might influence the ability of the cell to reverse the execution of programmed cell death (42), or it may simply represent nonspecific changes that accompany programmed cell death. The reduced levels of MNDA expression detected in MDS, the known sensitivity of MDS cells to TRAIL-induced programmed cell death, and the ability of MNDA to protect K562 cells from TRAIL-induced programmed cell death seems to implicate MNDA in the pathogenesis of granulocyte-macrophage progenitor cell programmed cell death in MDS. It is thus reasonable to suspect that the down-regulated MNDA expression in MDS might contribute to neutropenia in MDS by leading to excessive programmed cell death within the granulocyte-macrophage progenitors and therefore limit the production of mature effector cells. In addition, it has been proposed recently that reversing the early execution stage of programmed cell death generates proleukemogenic DNA damage in the genome of the recovered cell (43–45). In this scenario, excessive programmed cell death in MDS could not only compromise production of normal end-stage hematopoietic cells, but also provide a mechanism to produce genetic changes in cells when they recover from the

execution phase of programmed cell death rather than complete the process. The multistep model of MDS incorporates genetic instability that underlies disease progression. The role of down-regulating MNDA in MDS might be to enhance the level of programmed cell death and compromise hematopoiesis, while also contributing to disease progression. Our combined results implicate MNDA in the regulation of programmed cell death in granulocyte-macrophage progenitors and suggest that down-regulated expression in MDS might contribute to the complex

pathogenesis associated with fatal cytopenias or progression of MDS to leukemia.

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## Dysregulated Human Myeloid Nuclear Differentiation Antigen Expression in Myelodysplastic Syndromes: Evidence for a Role in Apoptosis

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