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A synthetic double-stranded RNA, poly I:C, induces a rapid apoptosis of human CD34$^+$ cells

Jiajia Liu[a,*], Yong-Mei Guo[a,*], Makoto Hirokawa[b], Keiko Iwamoto[a], Kumi Ubukawa[a], Yoshihiro Michishita[a], Naohito Fujishima[c], Hiroyuki Tagawa[a], Naoto Takahashi[a], Weiguo Xiao[d], Junsuke Yamashita[e], Toshiaki Ohteki[f,g], and Kenichi Sawada[a]

[a]*Department of Hematology, Nephrology, and Rheumatology, Akita University Graduate School of Medicine, Akita, Japan;* [b]*Clinical Oncology Center, Akita University Hospital, Akita, Japan;* [c]*Blood Center, Akita University Hospital, Akita, Japan;* [d]*Department of Rheumatology, The First Affiliated Hospital of China Medical University, Shenyang, China;* [e]*Bioscience Center, Radioisotope Division, Akita University Graduate School of Medicine, Akita, Japan;* [f]*Department of Biodefense Research, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan;* [g]*Japan Science and Technology Agency, Core Research for Evolutional Science and Technology (CREST), Tokyo, Japan*

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Toll-like receptor 3 (TLR3), retinoic acid-inducible gene I, and melanoma differentiation-associated antigen 5 (RIG-I/MDA-5) helicases are known to sense double-stranded RNA (dsRNA) virus and initiate antiviral responses, such as production of type-I interferons (IFNs). Recognition of dsRNA by TLR3 or RIG-I/MDA-5 is cell-type–dependent and recent studies have shown a direct link between TLRs and hematopoiesis. We hypothesized that viral dsRNA recognized by either TLR3 or RIG-I/MDA-5, affects the growth of human hematopoietic stem/progenitor cells. Here we show that polyinosinic polycytidylic acid (poly I:C)–mediated very rapid apoptosis occurs within 1 hour in CD34$^+$ cells in a dose-dependent manner. Polyadenylic-polyuridylic acid, another synthetic dsRNA that signals only through TLR3, had no effect. Poly I:C-LMW/LyoVec, a complex between low molecular-weight poly I:C and the transfection reagent LyoVec, which signals only through RIG-I/MDA-5, induces apoptosis of CD34$^+$ cells. A strong and sustained upregulation of messenger RNA and protein levels of Noxa, a proapoptotic BH3-only protein that can be induced by RIG-I/MDA-5 pathway, is found in CD34$^+$ cells treated by poly I:C. Although poly I:C upregulates type-I IFNs in CD34$^+$ cells, neither exogenous IFN-α nor IFN-β induces rapid apoptosis in CD34$^+$ cells and neutralization or blocking of type-I IFN receptor does not rescue CD34$^+$ cells, whereas Z-VAD, a pan-caspase inhibitor, rescues the cells from apoptosis. These results suggest that RIG-I/MDA-5, but not TRL3, signaling triggers poly I:C–induced rapid apoptosis of human CD34$^+$ cells, which will provide an insight into the mechanisms of dsRNA virus-mediated hematopoietic disorders. © 2012 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.

The initial sensing of infection is mediated by innate pattern recognition receptors, which include Toll-like receptors (TLRs), retinoic acid–inducible gene I (RIG-I)-like receptors, nucleotide binding oligomerization domain–like receptors, and C-type lectin receptors (for review see [1–3]). Each pattern recognition receptor, which recognizes microbe-specific pathogen-associated molecular patterns, activates specific signaling cascades to induce gene expression of targets such as proinflammatory cytokines and type-I interferons (IFNs) that coordinate the elimination of pathogens and infected cells [4]. A set of TLRs including TLR3, TLR7, TLR8, and TLR9 recognizes nucleic acids derived from viruses and bacteria, as well as endogenous nucleic acids in pathogens [4].

Recently, several lines of evidence have demonstrated a direct or indirect link between TLRs and hematopoiesis [5–7]. Nagai et al. [6] showed that TLRs and their coreceptors were expressed by multipotential hematopoietic stem cells, whose cell cycle entry was triggered by TLR ligation. They also showed that TLR signaling via the Myd88 adaptor protein drove differentiation of myeloid progenitors and also drove lymphoid progenitors to become dendritic cells.

*Drs. Liu and Guo contributed equally to this work.

Offprint requests to: Kenichi Sawada, M.D., Ph.D., Department of Hematology, Nephrology, and Rheumatology, Akita University Graduate School of Medicine, Hondo 1-1-1, Akita 010-8543, Japan; E-mail: ksawada@doc.med.akita-u.ac.jp

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Sioud et al. [7] showed that human BM $CD34^+$ progenitor cells constitutively express functional TLR7/8, a receptor that recognizes single-stranded RNAs from RNA viruses, and ligation of which can induce differentiation along the myeloid lineage without the addition of any exogenous cytokines. More recently, we demonstrated selective inhibition of erythroid growth and downregulation of the expression of erythropoietin receptor messenger RNA (mRNA) in human $CD34^+$ cells by CpG (cytosine linked to guanine by a phosphate bond) oligodeoxynucleotide-2006 (CpG-ODN2006), a TLR9 ligand that shares a consensus sequence with the parvovirus B19 genome [5]. Thus, nucleic acids derived from viruses and bacteria directly or indirectly affect hematopoiesis.

TLR3 detects viral double-stranded RNAs (dsRNAs) in the endolysosome and is involved in the recognition of polyinosinic polycytidylic acid (poly I:C), a synthetic dsRNA analog. A second family of pattern recognition receptors comprises the cytoplasmic sensors of viral nucleic acids, including RIG-I, melanoma differentiation-associated gene 5 (MDA-5), and laboratory of genetics and physiology 2. RIG-I and MDA-5 are DExD/H RNA helicases, possessing two caspase activation and recruitment domains at their amino terminus. Together with laboratory of genetics and physiology 2 they form the RIG-I-like receptors family. Laboratory of genetics and physiology 2 possesses only the helicase domain and lacks the caspase activation and recruitment domain [2,3]. RIG-I and MDA-5 share ~25% homology within the caspase activation and recruitment domain regions and 40% within the helicase domain. Poly I:C, which has been used frequently to study RNA sensing is at least 3 kb in length. By producing poly I:C of various lengths, it was determined that short segments of the polymer (~300 bp) do not activate MDA-5, but are potent ligands for RIG-I. In contrast, longer segments of poly I:C preferentially activate MDA-5 [8]. The mechanism underlying the discrimination between long and short dsRNA of MDA-5 and RIG-I remains to be elucidated.

We hypothesized that triggering TLR3, RIG-I, and/or MDA-5 with their cognate dsRNA ligands affects hematopoiesis through production of type-I IFNs and proinflammatory cytokines, and at the same time may trigger endogenous apoptosis as part of an antiviral host response. The results presented here partly support this hypothesis and even extend our understanding of dsRNAs in association with hematopoiesis. We identify a very rapid induction of apoptosis of human $CD34^+$ cells by poly I:C, and suggest that RIG-I and MDA-5 signaling triggers apoptosis of human $CD34^+$ cells.

Materials and methods

Reagents

Bovine serum albumin (BSA), Iscove's modified Dulbecco's medium (IMDM), and propidium iodide (PI) were purchased from Sigma (St Louis, MO, USA). Fetal bovine serum was from HyClone (Logan, UT, USA). Penicillin and streptomycin were from Invitrogen (Carlsbad, CA, USA). Insulin (porcine sodium, activity 28.9 U/mg) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Interleukin-3 (IL-3), stem cell factor, and thrombopoietin were kind gifts from the Kirin Brewery Co. Ltd. (Tokyo, Japan), and erythropoietin, and granulocyte colony-stimulating factor were from Chugai Pharmaceutical Co. (Tokyo, Japan). Vitamin B-12 was from Eisai Co. Ltd. (Tokyo, Japan) and folic acid was from Takeda Pharmaceutical Co. Ltd. (Osaka, Japan). RNase (Type III-A) was from Sigma. Carboxy-fluorescein diacetate succinimidyl ester was from Invitrogen. Poly I:C was from InvivoGen (San Diego, CA, USA) and Alexis Biochemicals (Lausen, Switzerland). Lipopolysaccharide (LPS) and bafilomycin A1 were from Sigma. R848 was purchased from Alexis Biochemicals.

CpG-oligodeoxynucleotide (ODN) 2006 with a modified nuclease-resistant backbone, phosphorothioate (PS) (ODN2006-PS), and CpG-ODN with the natural phosphodiester (PO) nuclease-sensitive backbone (ODN2006-PO) were commercially synthesized by Hokkaido System Science Co. Ltd. (Sapporo, Japan). Poly I:C/LMW/LeoVec, a complex between low molecular-weight poly I:C and the transfection reagent LyoVec, which signals only through RIG-I/MDA-5 [9], Poly I:C-rhodamine, BX795, and polyadenylic-polyuridylic acid, another synthetic dsRNA that signals only through TLR3 [10], were purchased from InvivoGen. Interferon (IFN)-α, IFN-β, mouse monoclonal antibody (Ab) against human IFN-α (anti–IFN-α Ab), and mouse monoclonal antibody against human IFN-α/β receptor (R) 1 (anti-IFNR Ab) were from PBL Biomedical Laboratories (Piscataway, NJ, USA). Goat monoclonal antibody against human IFN-β (anti–IFN-β Ab) and Z-VAD were from R&D Systems (Minneapolis, MN, USA).

Antibodies

Fluorescein isothiocyanate (FITC)–labeled monoclonal antibodies (mAb) specific for CD15 (H198) and phycoerythrin (PE)-labeled mAb specific for CD123 (IL-3Rα; 9F5) were purchased from Becton Dickinson (Mountain View, CA, USA). FITC or PE-labeled mAbs for glycophorin A (JC159), PE-CD34 (BIRMA-k3), and FITC-CD61 (Y2/51), FITC-CD11c (KB90) were purchased from Dako Japan Co. (Kyoto, Japan). Anti-Noxa mouse mAb (114C307) was purchased from Calbiochem (Tokyo, Japan). Anti-Puma (p53 upregulated modulator of apoptosis) rabbit polyclonal Ab, horse anti-mouse IgG/horseradish peroxidase–linked Ab and goat anti-rabbit IgG/horseradish peroxidase–linked Ab were purchased from Cell Signaling (Tokyo, Japan). FITC-Annexin V apoptosis detection kit was from Sigma. PE-Annexin V was from R&D Systems. PE-anti–IFN-α was from Miltenyi Biotec (Auburn, CA, USA).

Cell preparation

Granulocyte colony-stimulating factor–mobilized human peripheral blood $CD34^+$ cells were purified from healthy volunteers and stored in liquid nitrogen until use as described previously [11]. Informed consent was obtained from each subject before entry into this study, and the Akita University Graduate School of Medicine Committee preapproved the study for the Protection of Human Subjects. For the generation of progenitor cells toward various lineages, $CD34^+$ cells were thawed and prepared for the culture, as described previously. Cells were cultured in multilineage medium (IMDM containing 20% fetal bovine serum, 10% heat-inactivated pooled human AB serum, 1% BSA, 10 μg/mL insulin, 0.5 μg/mL vitamin B-12, 15 μg/mL folic acid, 50 nM β-mercaptoethanol, 50 U/mL penicillin,

Table 1. Primers used in this study

Primer	Direction	Sequence
TLR3	Forward	5′- AAC AGC ATC AAA AGA AGC AG -3′
	Reverse	5′- ACA GAG TGC ATG GTT CAG TT -3′
RIG-I	Forward	5′- GGG ACG AAG CAG TAT TTA G -3′
	Reverse	5′- CAT CTC CAA GCA CAG TGT A -3′
MDA-5	Forward	5′- CCA AAG CTG AAG AAC ACA T -3′
	Reverse	5′- ATC TTC TCT GGT TGC ATC T -3′
PKR	Forward	5′- TGG CTA TTC ATC ATG GCT GG-3′
	Reverse	5′- CTC AGC AGC ATT CCT TTT GG-3′
IFN-β	Forward	5′- TCA TGA GCA GTC TGC ACC T-3′
	Reverse	5′- AGA GGC ACA GGC TAG GAG AT-3′
p21	Forward	5′- ACT CTC AGG GTC GAA AAC G-3′
	Reverse	5′- CAC ACA AAC TGA GAC TAA GGC-3′
Bim	Forward	5′- TGG CAA AGC AAC CTT CTG-3′
	Reverse	5′- TGG CTC TGT CTG TAG GGA GGT A-3′
p53	Forward	5′- AGA TGT TCC GAG AGC TGA-3′
	Reverse	5′- CAG TGG GGA ACA AGA AGT-3′
Bcl-2	Forward	5′- TGT GGC CTT CTT TGA GTT C-3′
	Reverse	5′- CGG TTC AGG TAC TCA GTC ATC-3′
Bcl-xl	Forward	5′- GGT ATT GGT GAG TCG GAT CG -3′
	Reverse	5′- TCG GCT GCT GCA TTG TTC -3′
Noxa	Forward	5′- TGA TAT CCA AAC TCT TCT GC -3′
	Reverse	5′- ACC TTC ACA TTC CTC TCA A -3′
Puma	Forward	5′- GAC CTC AAC GCA CAG TA -3′
	Reverse	5′- CTA ATT GGG CTC CAT CT -3′
GAPDH	Forward	5′- GAA GGT GAA GGT CGG AGT C-3′
	Reverse	5′- GAA GAT GGT GAT GGG ATT TC-3′

GAPDH = Glyceraldehyde 3-phosphate dehydrogenase; Puma = p53 upregulated modulator of apoptosis.

and 50 μg/mL streptomycin, in the presence of 50 ng/mL IL-3, 50 ng/mL stem cell factor, 2 IU/mL erythropoietin, 100 ng/mL granulocyte colony-stimulating factor, and 100 ng/mL thrombopoietin) at a cell density of 2×10^4 cells/mL. For the short-term culture within 4 hours, $CD34^+$ cells were cultured at cell densities ranging from 5 to 10×10^4 cells/mL. Cells were maintained at 37°C in a 5% CO_2 incubator as described previously. The yield was measured by dye exclusion using 0.2% trypan blue dye and a hemocytometer.

Flow cytometry

The cells collected from culture were washed twice with IMDM containing 0.3% BSA. The cells were then incubated with FITC- and PE-labeled mAb, washed twice with MACS buffer (10 mM phosphate-buffered saline [pH 7.4], 0.5% BSA and 2 mM EDTA), and analyzed using a FACS Calibur (Becton Dickinson), as reported elsewhere [11].

Intracellular staining

Cells collected from culture were washed twice with IMDM containing 0.3% BSA. Cells were then incubated with 5 μg/mL ethidium monoazide bromide for 15 minutes in the dark, and washed twice with MACS buffer. After a 10-minute light exposure on ice, the cells were fixed and stained for intracellular PE-anti–IFN-α with BD Cytofix/Cytoperm kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions.

Apoptosis analysis

Annexin V-PE or Annexin V-FITC and PI (Sigma) were used to assess the incidence of apoptosis. A cell pellet was suspended in Annexin V binding buffer (10 mM HEPES/NaOH [pH 7.4] + 140 mM NaCl + 2.5 mM $CaCl_2$) with 0.2 ng/μL Annexin V-PE or FITC and 20 ng/μL PI. After incubation on ice in the dark for 15 minutes, cells were analyzed using a FACSCalibur (BD Biosciences).

Cell cycle distribution

Cells were harvested, washed with cold phosphate-buffered saline, and fixed in 70% ethanol. Cells were then stored at −20°C until analysis. Fixed cells were centrifuged at 1200 rpm, washed with cold phosphate-buffered saline twice and RNase A added at a final concentration of 0.5 mg/mL. Cells were then incubated for 10 minutes at 37°C. Next, 25 μg/mL PI was added and the cells were incubated for 30 minutes at room temperature in the dark. Cells were analyzed using a FACSCalibur. FlowJo software (Tree Star, Inc, Ashland, OR, USA) was used to determine the percentage of cells in the different cell cycle phases.

Confocal microscopy

Fluorescence staining was imaged using a Confocal Laser Scanning Microscope 510 (LSM510; Carl Zeiss Microscope Systems, Germany) equipped with a 100× objective lens and a 10× camera lens (Carl Zeiss Microscope Systems). Rhodamine was excited using a Hene laser at 543 nm. Detector slits were configured to minimize cross talk between channels and processed using a software package (LSM510, version 3.2) and Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from 1×10^5 cells per sample using TRizol reagent (Invitrogen). The extracted RNA was then reverse-transcribed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) in a 20-μL reaction volume. Complementary DNA was then subjected to real-time RT-PCR using LightCycler 480 SYBR Green I Master (Roche Applied Science, Basel, Switzerland). The relative gene expression levels were normalized with *glyceraldehyde 3-phosphate dehydrogenase*. Primer sequences are presented in Table 1 and were purchased from Nippon Gene Research Laboratories (Sendai, Japan).

Western blot analysis

$CD34^+$ cells were incubated in multilineage medium for 4 hours with or without 50 μg/mL poly I:C. Western blot analysis was carried out according to manufacturer's protocol (Invitrogen).

Statistical analysis

Significant differences between groups were calculated using the unpaired Student's *t*-test. Tests were undertaken using Stat View 4.0 and significant differences were defined as $p < 0.05$.

Results

Inhibition of multilineage differentiation from $CD34^+$ cells by poly I:C

To examine the effects of poly I:C on the growth of hematopoietic progenitors, human $CD34^+$ cells were cultured for 7 days in multilineage medium. Under these conditions, simultaneous differentiation of erythroid (glycophorin A^+), neutrophilic ($CD15^+$), and megakaryocytic ($CD61^+$)

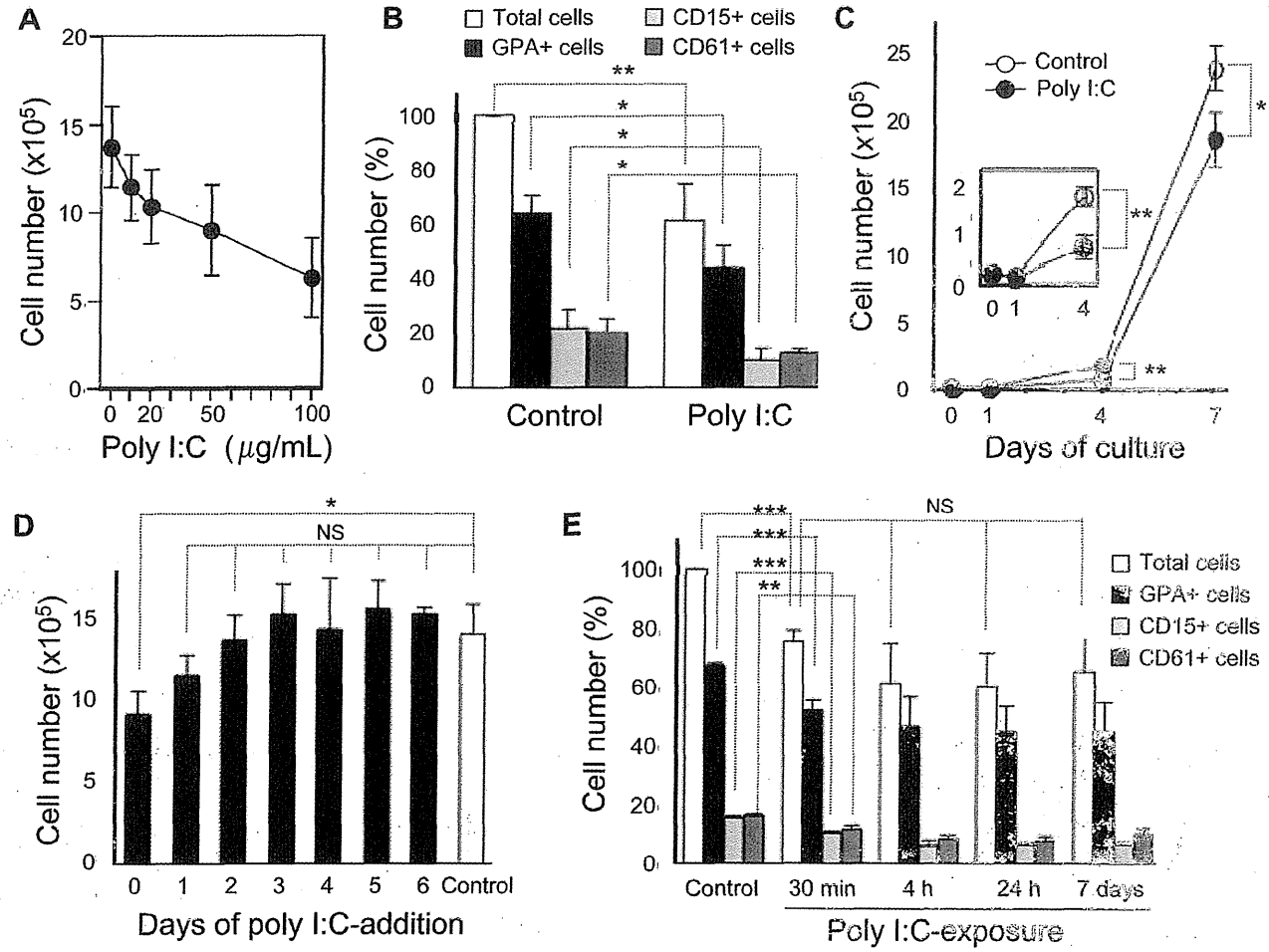

Figure 1. Inhibition of multilineage differentiation from $CD34^+$ cells by poly I:C. **(A)** Purified human $CD34^+$ cells at a density of 2×10^4 cells/mL were cultured with 1 mL multilineage medium containing IL-3, stem cell factor, erythropoietin, granulocyte colony-stimulating factor, and thrombopoietin with or without various concentrations of poly I:C. Seven days later, the cells were collected and the number of total cells was counted. Results presented are the mean ± standard deviation (SD) of five independent experiments. **(B)** Purified human $CD34^+$ cells at a density of 2×10^4 cells/mL were cultured with 1 mL multilineage medium. Seven days later, glycophorin A (GPA), CD15, and CD61 expressions of the generated cells with or without 50 μg/mL poly I:C were examined using fluorocytometry. Cell number is represented as a percentage relative to the total number of cells without poly I:C ($6.45 \pm 0.39 \times 10^5$ cells, n = 3). Results presented are the mean ± SD of three independent experiments. **(C)** Kinetics of cell growth. $CD34^+$ cells at a density of 2×10^4 cells/mL were cultured in 1 mL multilineage medium with (closed circle) or without (open circle) 50 μg/mL poly I:C. At the indicated days, cells were collected, washed, and counted. The inset represents amplification of the Y-axis. Results presented are the mean ± SD of three independent experiments. **(D)** $CD34^+$ cells at a density of 2×10^4 cells/mL were cultured in 1 mL multilineage medium. At the indicated days, 50 μg/mL poly I:C was added to the medium (closed bars). Seven days later from the beginning of the culture, the cells were collected, washed, and counted. Results presented are the mean ± SD of three independent experiments. **(E)** $CD34^+$ cells at a density of 2×10^4 cells/mL were cultured in 1 mL multilineage medium with 50 μg/mL poly I:C. At the indicated times, poly I:C was washed out from the medium. Seven days later, the cells were collected and glycophorin A (GPA), CD15, and CD61 expression were examined using fluorocytometry. Cell number is represented as a percentage relative to the total number of cells without poly I:C ($16.24 \pm 1.07 \times 10^5$ cells, n = 3). The results presented are the mean ± SD of three independent experiments. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. NS = no significance.

progenitors occurred [5]. During 7 days of culture, poly I:C significantly reduced the number of progenitor cells generated from $CD34^+$ cells in a dose-dependent manner (Fig. 1A). We also found that the decrease of the number of cells by poly I:C was due to decreases of erythroid, neutrophilic, and megakaryocytic progenitors (Fig. 1B). These data indicate that poly I:C inhibits growth of $CD34^+$ cells independent of the cell lineage and suggest that poly I:C inhibits multipotential progenitor cells.

To further understand the kinetics of the growth inhibition of $CD34^+$ cells by poly I:C, purified $CD34^+$ cells were cultured in multilineage medium in the presence or absence of poly I:C. The cell yield substantially increased in the cultures that did not contain poly I:C, and decreased in the cultures that did contain poly I:C after 4 days in culture (Fig. 1C), which indicated that poly I:C affected the growth of $CD34^+$ cells in the early stage of development. When purified $CD34^+$ cells were cultured for 7 days in multilineage

medium and poly I:C was added at the indicated time points (Fig. 1D), only the addition of poly I:C to the medium from the beginning of the culture resulted in significant inhibitory effects on the generation of progenitors (Fig. 1D). Additions of poly I:C to the medium later than 1 day after the initiation of culture resulted in no inhibitory effects on the generation of progenitors, which suggests that poly I:C inhibits growth of $CD34^+$ cells in the very early stage of development. To further investigate the developmental stage of $CD34^+$ cells susceptible to poly I:C, $CD34^+$ cells were transiently exposed to poly I:C and cultured in multilineage medium for 7 days. Surprisingly, only 30 minutes of exposure of $CD34^+$ cells to poly I:C significantly inhibited the growth of $CD34^+$ cells (Fig. 1E). These findings indicate that the inhibition of $CD34^+$ cells by poly I:C occurs within a very short time period.

Poly I:C induces apoptosis of $CD34^+$ cells

To investigate whether the inhibitory effects of poly I:C mediate the inhibition of cell division, $CD34^+$ cells labeled with carboxy-fluorescein diacetate succinimidyl ester were cultured in multilineage medium for 7 days with or without poly I:C. The progenitor cells generated with or without poly I:C exhibited the same carboxy-fluorescein diacetate succinimidyl ester intensity (Fig. 2A), which indicates that poly I:C-mediated inhibition of progenitor development does not depend on the decrease of cell division. On the other hand, after 4 hours of exposure to poly I:C, $CD34^+$ cells showed a marked heterogeneity in size and contained many shrunken cells (Fig. 2B), and an increase of Annexin V^+/PI^- apoptotic cells from 8.3% ± 1.8% to 46.4% ± 7.6% ($p < 0.01$, Fig. 2C), in a dose-dependent manner (Fig. 2E). These data indicate that poly I:C induces apoptosis of $CD34^+$ cells within a very short time period. In addition, when $CD34^+$ cells, cultured for 2 days in multilineage medium (day 2 cells), were treated with poly I:C for 4 hours and apoptosis was measured, an increase of Annexin V^+/PI^- apoptotic cells from 3.2% ± 0.2% to 7.0% ± 0.5% ($p < 0.01$, Fig. 2D) was observed, but to a lesser extent compared to that seen in day 0 $CD34^+$ cells (Fig. 2C). Consistent with the result shown in Figure 1D, this result also suggests that poly I:C inhibits the growth of $CD34^+$ cells in the very early stage of development.

Poly I:C-specific induction of apoptosis of $CD34^+$ cells

To examine the specificity of poly I:C in an immediate induction of apoptosis of $CD34^+$ cells, purified $CD34^+$ cells were cultured in multilineage medium for 4 hours in the presence or absence of various TLR ligands, such as LPS (a TLR4 ligand), R848 (a TLR7/8 ligand), nuclease-resistant 2006-PS, and nuclease-sensitive 2006-PO (TLR9 ligands). As illustrated in Figure 3A, poly I:C but not LPS, R848, 2006-PS, and 2006-PO, exhibited an increase of Annexin V^+/PI^- apoptotic cells. These data indicate that the effect of inducing immediate apoptosis of $CD34^+$ cells is specific in poly I:C among various TLR ligands.

In order to examine the intracellular localization of poly I:C, purified $CD34^+$ cells were cultured in multilineage medium for 4 hours with rhodamine-labeled poly I:C (Fig. 3B). Poly I:C-rhodamine was found in cells with a feature of flat and blurry cytoplasm (Fig. 3B, arrows in the middle panel), which suggests that the internalization of poly I:C in $CD34^+$ cells rapidly induces apoptosis of these cells. When bafilomycin A, a potent inhibitor of autophagosome-lysosome fusion [12], was tested against the effect of poly I:C, bafilomycin A completely blocked poly I:C–induced apoptosis (Fig. 3C), suggesting that $CD34^+$ cells internalize poly I:C through the autophagosome-lysosome pathway.

Cytosolic dsRNA receptors, RIG-I and MDA-5, and poly I:C–induced apoptosis of $CD34^+$ cells

Recognition of dsRNA by TLR3 or RIG-I/MDA-5 is cell-type–dependent. To identify the downstream molecules of poly I:C signaling, purified $CD34^+$ cells were cultured in multilineage medium in the presence or absence of poly I:C and expressions of TLR3, RIG-I, MDA-5, and dsRNA-dependent protein kinase R (PKR) mRNA were monitored at the indicated time points. PKR is a serine–threonine kinase that binds dsRNA [13]. PKR-deficient mouse embryonic fibroblasts have defective type-I IFN responses to poly I:C and some RNA viruses [14]. As illustrated in Figure 4A, TLR3, RIG-I, and MDA-5 mRNA, but not PKR mRNA, were found to be induced by poly I:C. Inductions of RIG-I and MDA-5 mRNA were evident as early as 2 hours into incubation with poly I:C. Polyadenylic-polyuridylic acid, another synthetic dsRNA, which signals only through TLR3 [10], had no effect on the proliferation (Fig. 4B) and apoptosis (Fig. 4C) of $CD34^+$ cells. On the other hand, poly I:C-LMW/LyoVec, a complex between low molecular-weight poly I:C and the transfection reagent LyoVec, which signals only through RIG-I/MDA-5 [9], induced apoptosis of $CD34^+$ cells accompanied with an increase of PI^+/Annexin V^- late apoptotic fraction (Fig. 4D). Collectively, RIG-I/MDA-5, but not TLR3, signaling induces poly I:C–mediated apoptosis of $CD34^+$ cells.

Induction of Noxa by poly I:C treatment

Apoptosis is regulated by various factors, such as transcription factors (e.g., c-Jun, p53, and c-Myc), cell cycle regulators (e.g., p21 and cyclin D1), Bcl-2 subfamily (e.g., Bcl-2 and Bcl-xl) and Bcl-2 homology 3 (BH3)-only proteins (e.g., Bim, Puma and Noxa) (for review see [15,16]). These alterations promote the sensitivity of $CD34^+$ cells toward cell death. Analyses of p53, p21, Bcl-2, and Bcl-xl during poly I:C–mediated apoptosis of $CD34^+$ cells showed strong transcriptional induction of p21 with RNA levels increasing 45.2- ± 10.1-fold within 2 hours of exposure to poly I:C. Among proapoptotic BH3-only proteins, critical initiators of mitochondrial apoptosis [17], a strong and sustained up-regulation (up to 22-fold) of Noxa mRNA and protein levels was found (Fig. 5A and B), and Bim mRNA to

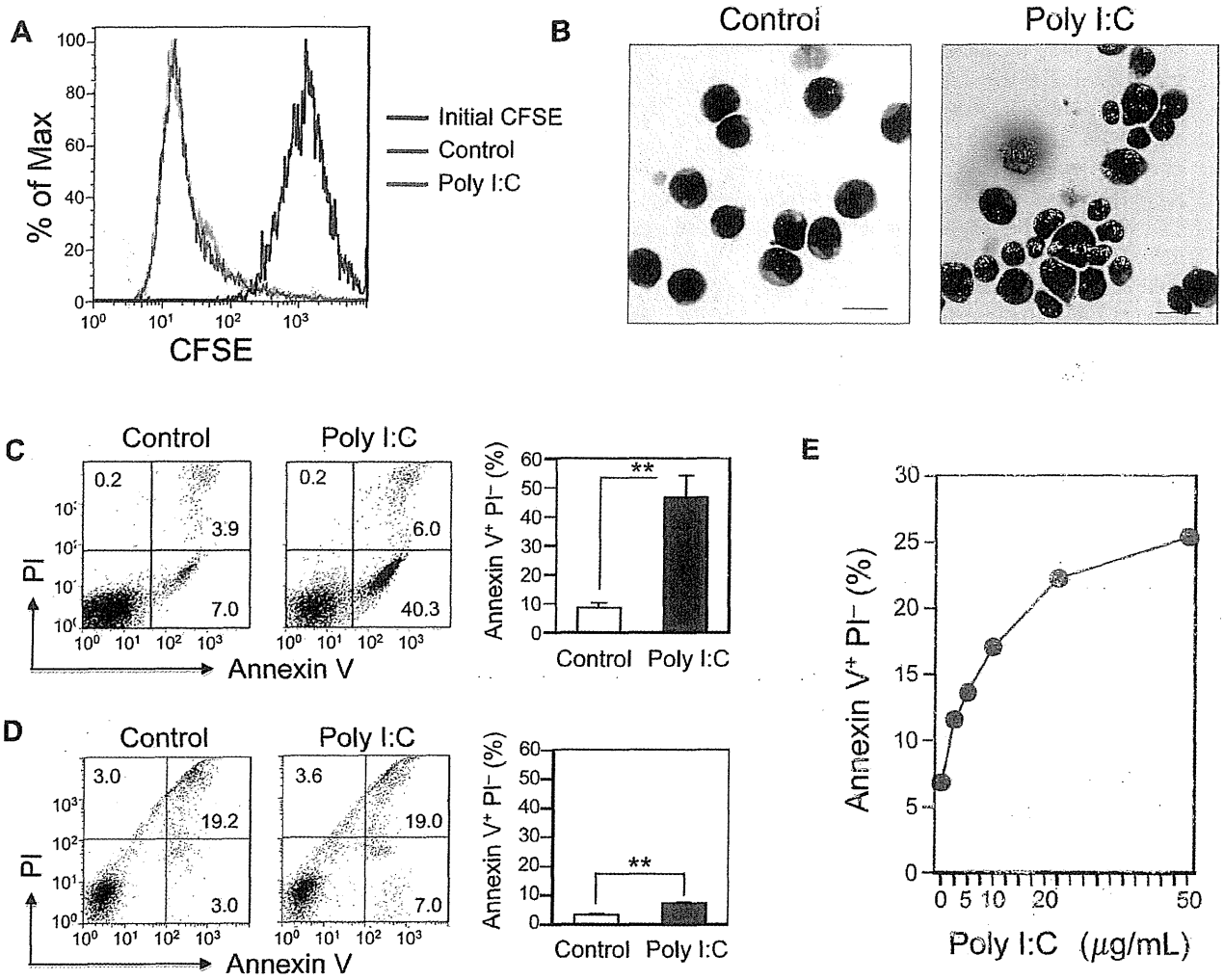

Figure 2. Poly I:C induces apoptosis of $CD34^+$ cells. (**A**) Carboxy-fluorescein diacetate succinimidyl ester (CFSE) dilution assay. Purified $CD34^+$ cells at a density of 5 to 10 × 10^4 cells/mL were stained with CFSE and cultured in 1 mL multilineage medium with or without 50 μg/mL poly I:C. Seven days later, cells were harvested and analyzed by fluorocytometry. Results are representative of two independent experiments. (**B**) Morphology of the generated cells. $CD34^+$ cells at a density of 1 × 10^5 cells/mL were cultured in 1 mL multilineage medium, with or without 50 μg/mL poly I:C for 4 hours and subjected to May-Grünwald-Giemsa staining (×600). Results are representative of two independent experiments. Scale bars: 20 μm. (**C, D**) Purified $CD34^+$ cells (**C**), and $CD34^+$ cells cultured for 2 days in multilineage medium (**D**), at a density of 5 to 10 × 10^4 cells/mL were cultured in 1 mL multilineage medium with or without 50 μg/mL poly I:C for 4 hours and were labeled with PI and Annexin V and analyzed using fluorocytometry. A representative of dot-plot analysis and the percentage of Annexin V^+ PI^- cells (mean ± standard deviation) of three independent experiments are shown. $^{**}p < 0.01$. (**E**) Dose-dependent induction of apoptotic cells (Annexin V^+ PI^- cells). $CD34^+$ cells at a density of 5 to 10 × 10^4 cells/mL were cultured in 1 mL multilineage medium with various concentrations of poly I:C for 4 hours and were labeled with PI and Annexin V and analyzed using fluorocytometry. A representative of two independent experiments is shown.

a lesser extent (Fig. 5A), whereas the changes of p53, Bcl-2, Bcl-xl, and Puma mRNA levels were minimal (Fig. 5A). There is evidence that RIG-I and MDA-5 trigger a p53-independent alternative pathway for the induction of Noxa [18]. Therefore, these results suggest functional links between dsRNA sensors, RIG-I and MDA-5, and the apoptosis program via Noxa.

Apoptosis induction by poly I:C involves caspases

The stress pathway initiated by BH3-only proteins can permeabilize the mitochondrial outer membrane, releasing cytochrome *c*, which provokes apoptotic protease-activating factor 1 to activate caspase-9 [15]. To analyze the relationships between poly I:C–mediated apoptosis and caspase activity, purified $CD34^+$ cells were cultured in the absence or in the presence of poly I:C with or without the broad-spectrum caspase inhibitor z-VAD-fmk [19]. Z-VAD-fmk strongly reduced the proportions of poly I:C–induced Annexin V^+/PI^- apoptotic cells (Fig. 6A, upper panel) as well as the poly I:C–mediated apoptotic fraction of DNA contents (Fig. 6A, lower panel). Quantitative analysis showed that z-VAD-fmk partially but significantly rescued poly I:C-mediated apoptosis of $CD34^+$ cells from 25.6% ± 6.2% to 14.7% ± 3.6% ($p < 0.05$, Fig. 6B), which indicates

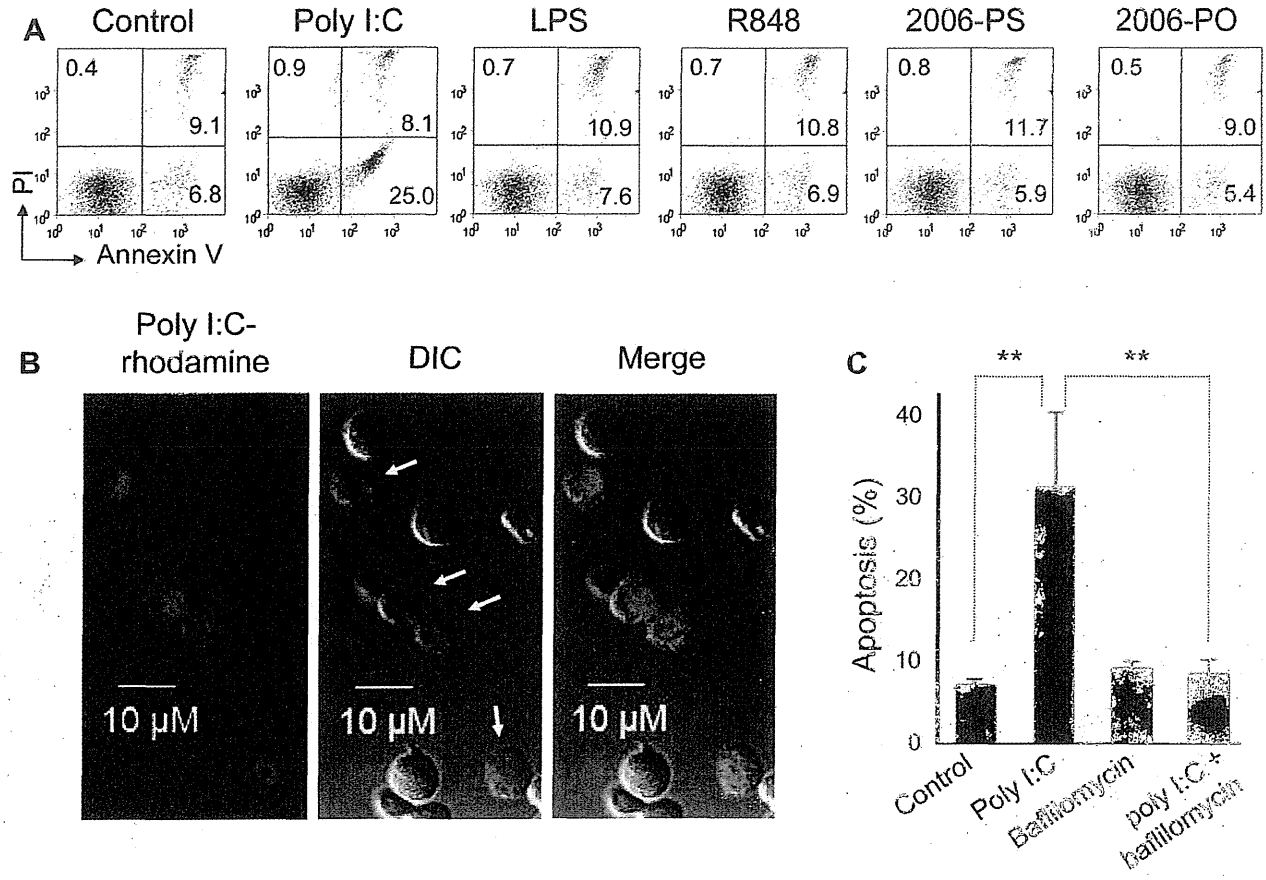

Figure 3. Poly I:C–specific induction of apoptosis of $CD34^+$ cells. (**A**) $CD34^+$ cells at a density of 5 to 10 × 10^4 cells/mL were cultured in 1 mL multilineage medium in the presence or absence of various TLR ligands (poly I:C 50 μg/mL, LPS 2 μg/mL, R848 10 μg/mL, 2006-PS 4 μM, and 2006-PO 4 μM). Four hours later, the cells were collected, washed twice, labeled with PI and Annexin V, and analyzed using fluorocytometry. Results are representative of two independent experiments. (**B**) $CD34^+$ cells at a density of 5 to 10 × 10^4 cells/mL were cultured in 1 mL multilineage medium for 4 hours in the presence of 50 μg/mL poly I:C-rhodamine (red) and were observed by confocal microscopy in a differential interference contrast (DIC). Arrows indicate the cells with a feature of flat and blurry cytoplasm. Results are representative of two independent experiments. (**C**) $CD34^+$ cells at a density of 5 to 10 × 10^4 cells/mL were cultured in 1 mL multilineage medium for 4 hours in the presence or absence of 50 μg/mL poly I:C and 100 nM bafilomycin A and were subjected to apoptosis and cell cycle analysis. Results presented are the mean ± standard deviation of three independent experiments. $^{**}p < 0.01$.

that apoptosis induction by poly I:C involves caspase signaling.

Apoptosis induction by poly I:C and IFN signaling

Type I IFNs, especially IFN-α and IFN-β, are transcriptionally activated by viral infection, and the secreted IFNs are recognized by a cognate IFN receptor complex, which is composed of IFN-α/β receptor IFNR1 and IFNR2 [20]. Exposure of $CD34^+$ cells to poly I:C increased IFN-α–producing cells within 4 hours and significantly induced IFN-β mRNA expression within 2 hours (Fig. 7A). In addition, when $CD34^+$ cells, cultured for 2 days in multilineage medium, were exposed to poly I:C for 4 hours, no increase of IFN-α–producing cells was observed, while a strong but transient upregulation (up to 142-fold) of IFN-β mRNA was found (Fig. 7B). These results suggest that poly I:C–treated $CD34^+$ cells change the cytokine production pattern depending on the stage of development. To analyze the role of IFN signaling in apoptosis induction, $CD34^+$ cells were cultured in multilineage medium with or without IFN for 4 hours and subjected to apoptosis analysis. Surprisingly, neither IFN-α nor IFN-β at a pharmacological dose increased Annexin V^+/PI^- apoptotic cells (Fig. 7C), suggesting that type-I IFNs are not associated with poly I:C–mediated rapid apoptosis of $CD34^+$ cells.

To analyze the role of IFNs secreted from the $CD34^+$ cells and IFNR signaling in apoptosis induction, $CD34^+$ cells were cultured in multilineage medium for 4 hours in the presence or absence of poly I:C and with or without anti-IFNR Ab, anti–IFN-α Ab, or anti–IFN-β Ab, and subjected to apoptosis analysis. None of these antibodies affected poly I:C–mediated apoptosis of $CD34^+$ cells (Fig. 7D). On the other hand, these antibodies were biologically active in blocking type-I IFN-mediated inhibition of the growth of hematopoietic progenitors (Fig. 7E). These observations suggest that poly I:C–mediated

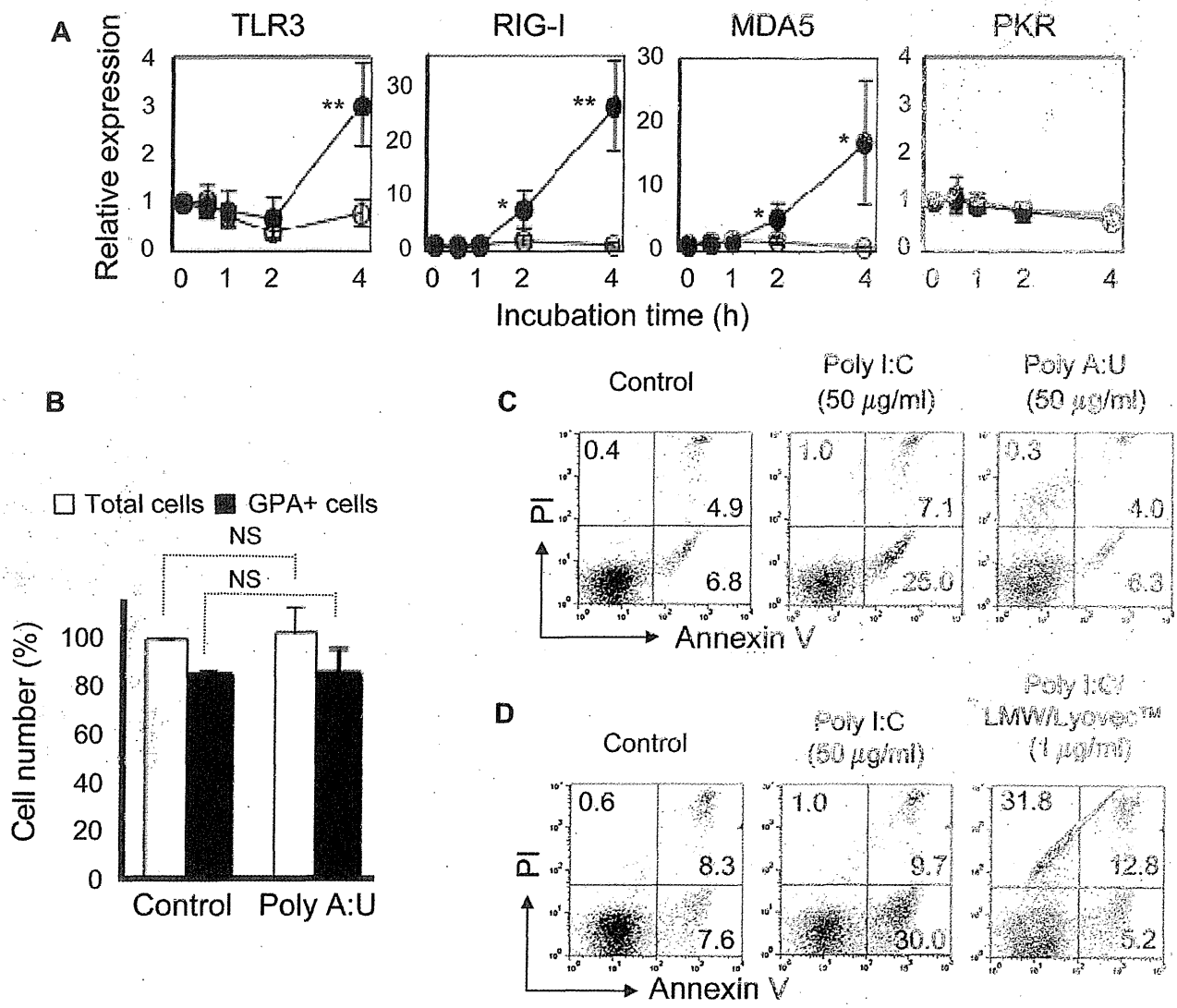

Figure 4. Cytosolic RNA receptors, RIG-I and MDA-5, and poly I:C–induced apoptosis of $CD34^+$ cells. (**A**) Quantitative analysis of poly I:C receptors using real-time RT-PCR. Purified $CD34^+$ cells at a density of 5 to 10 × 10^4 cells/mL were cultured in 1 mL multilineage medium with (closed circles) or without (open circles) 50 μg/mL poly I:C. At the indicated time, the cells were harvested and mRNA extracted from the cells was subjected to real-time RT-PCR. The relative gene expression levels were normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts and represented as a unit relative to $CD34^+$ cells. Results presented are the mean ± standard deviation (SD) of three independent experiments. $*p < 0.05$ and $**p < 0.01$ compared to the control. (**B, C**) Effects of polyadenylic-polyuridylic acid (poly A:U), a ligand of TLR3 on $CD34^+$ cells. (**B**) $CD34^+$ cells at a density of 2 × 10^4 cells/mL were cultured in 1 mL erythroid medium with or without poly A:U. Seven days later, the cells were collected, washed, and counted, and examined for glycophorin A (GPA) expression using fluorocytometry. Cell number is represented as a percentage relative to the total number of cells without poly A:U (10.59 ± 1.69 × 10^5 cells, n = 3). Results presented are the mean ± SD of three independent experiments. NS = no significance. (**C**) $CD34^+$ cells at a density of 5 to 10 × 10^4 cells/mL were cultured in 1 mL multilineage medium with or without 50 μg/mL of poly A:U for 4 hours and subjected to apoptosis analysis. Results are representative of three independent experiments. (**D**) Effects of Poly I:C/LMW/Lyovec, ligand of RIG-I and MDA-5 on $CD34^+$ cells. $CD34^+$ cells at a density of 5 to 10 × 10^4 cells/mL were cultured in 1 mL multilineage medium with or without poly I:C (50 μg/mL) or poly I:C/LMW/Lyovec (1 μg/mL) for 4 hours and subjected to apoptosis analysis. Results are representative of two independent experiments.

rapid induction of $CD34^+$ cells is independent of IFN signaling.

Discussion

In this study, we demonstrated the inhibitory effect of poly I:C on the growth of progenitor cells derived from human $CD34^+$ cells. The decrease of progenitor cells by poly I:C mediates immediate apoptosis of $CD34^+$ cells that occurs within 4 hours. Polyadenylic-polyuridylic acid, another synthetic dsRNA that signals only through TLR3 [10], had no effect. Poly I:C-LMW/LyoVec, a complex between low molecular-weight poly I:C and the transfection reagent LyoVec, which signals only through RIG-I/MDA-5 [9], induces apoptosis of $CD34^+$ cells. Among

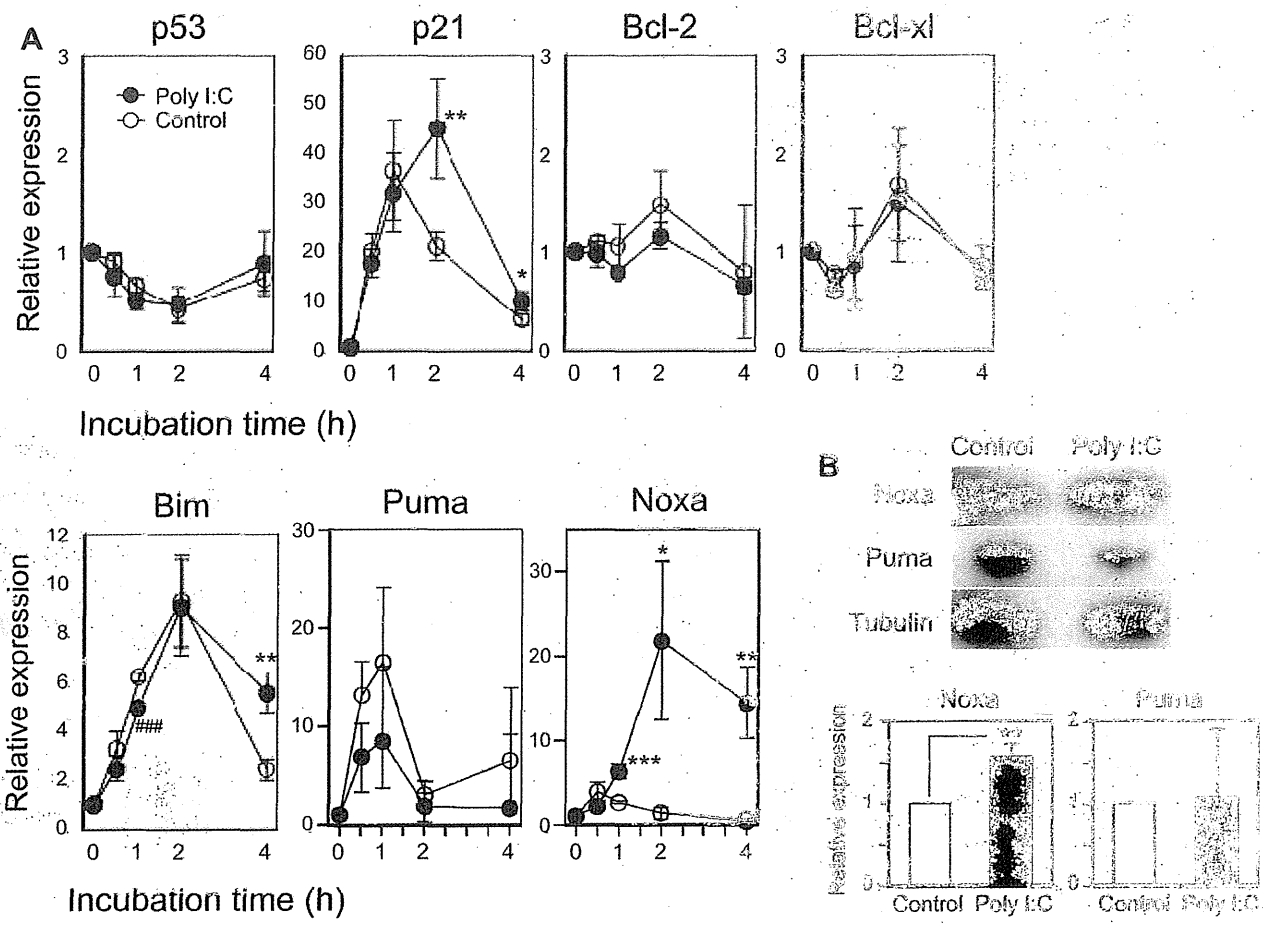

Figure 5. Induction of Noxa by poly I:C treatment. **(A)** $CD34^+$ cells at a density of 5 to 10 × 10^4 cells/mL were cultured in 1 mL multilineage medium with (closed circles) or without (open circles) 50 μg/mL poly IC for the indicated periods, and levels of p53, p21, Bcl-2, Bcl-xl, Bim, Puma and Noxa mRNA were measured by quantitative RT-PCR. The relative expression levels were normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts and represented as a unit relative to $CD34^+$ cells. **(B)** Western blot analysis of Noxa and Puma. Human $CD34^+$ cells were cultured in 1 mL multilineage medium with or without 50 μg/mL poly I:C. After 4 hours, the cells were harvested and the protein obtained from 2 × 10^6 cells was applied to each lane. The relative expression levels of proteins were normalized with tubulin expression and are the mean ± standard deviation of three independent experiments. $*p < 0.05$ and $*p < 0.01$.

proapoptotic BH3-only proteins, a strong and sustained up-regulation of Noxa mRNA and protein levels was found, whereas the changes of p53, Bcl-2, Bcl-xl, and Puma mRNA levels were minimal. There is evidence that RIG-I and MDA-5 trigger a p53-independent alternative pathway for the induction of Noxa and that induction of Puma by RIG-I and MDA-5 depended on p53 [18,21]. Therefore, these results for the first time suggest functional links between dsRNA sensors, RIG-I and MDA-5, and the apoptosis program via Noxa in human $CD34^+$ cells.

We showed that the broad-spectrum caspase inhibitor z-VAD-fmk significantly reduced the proportion of poly I:C–induced Annexin V^+/PI^- apoptotic cells, which indicates that apoptosis induction by poly I:C involves caspase signaling. The previous studies demonstrate that human MDA-5 augments FasL-mediated DNA degradation [22]. Caspase-mediated cleavage of human MDA-5 has been observed in poliovirus-infected cells [23]. Similar to MDA-5, RIG-I also displays apoptosis-inducing properties [24,25] and cytosolic poly I:C or influenza A virus infection activates caspases 1 and 3 resulting in pro–IL-18 processing [25]. The relationship between RIG-I and/or MDA-5 with hematopoiesis remains largely unknown. Recent evidence has suggested that RIG-I can act, in the absence of viral infection, to regulate myeloid cell differentiation [26]. Zhang et al. reported that a significant RIG-I induction occurs during normal myelopoiesis and that disruption of the RIG-I gene in mice leads to the development of a progressive myeloproliferative disorder [26]. Using a mouse model in which a different region of the RIG-I gene was deleted, they also showed that these animals develop granulocytosis, indicative of defects in myeloid development. In the same study, it was noted that the granulocytosis in the rig-$I^{-/-}$ mice is attributable to a reduction in expression of IFN consensus sequence-binding protein, which plays a central role in regulating myeloid differentiation [26]. A potential role for MDA-5 in differentiation was not investigated and is an area worth further exploration.

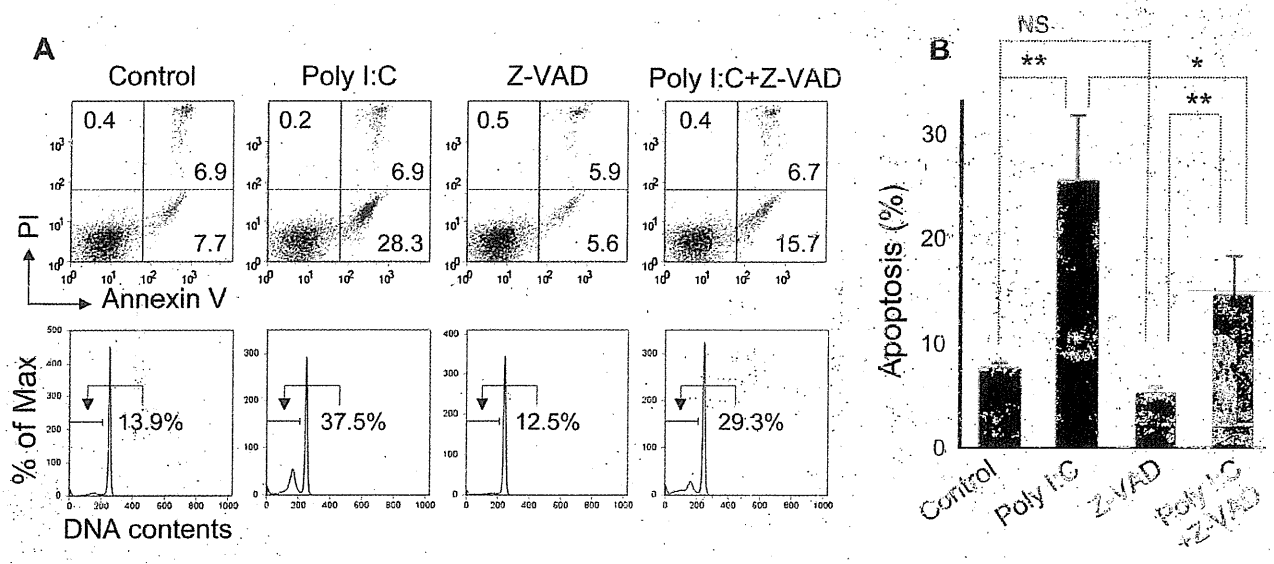

Figure 6. Apoptosis induction of poly I:C involves caspase signaling. (**A**) $CD34^+$ cells at a density of 5 to 10 × 10^4 cells/mL were cultured for 4 hours in 1 mL multilineage medium in the presence or absence of poly I:C and with or without 50 μM Z-VAD and were subjected to fluorocytometry. Apoptotic cells were identified as Annexin V^+ PI^- cells. (**B**) Data shown in this figure are the mean ± standard deviation of three independent experiments. $*p < 0.05$ and $**p < 0.01$. NS = no significance.

TLR3, RIG-I, and MDA-5 helicases are known to sense dsRNA viruses and initiate antiviral responses such as production of type-I IFNs. Several previous studies [27–32] have established that type-I IFNs are potent regulators of normal hematopoiesis in vitro and in vivo. The negative effects of IFNs are exerted on progenitor cells of all hematopoietic lineages, including early and late erythroid (burst-forming unit erythroid and colony-forming unit erythroid), myeloid (colony-forming unit granulocyte-macrophage), megakaryocytic progenitors and $CD34^+CD38^-$ cells, a primitive cell population [27–33]. The mechanisms by which such effects occur remain largely unknown. It has been reported that activation of the p38 mitogen-activated protein kinase mediates the suppressive effects of type-I IFNs on normal hematopoiesis [34]. We have demonstrated that the apoptosis of erythroid progenitors was induced by IFN-α [32] and showed here that poly I:C upregulates type-I IFNs in $CD34^+$ cells. In this connection, upregulation of type-I IFNs could explain the apoptosis and growth inhibition of $CD34^+$ cells by poly I:C, nevertheless, the biological effects of type-I IFNs on human $CD34^+$ cells are definitely different from those seen in poly I:C. Firstly, delayed addition experiments of poly I:C indicate that poly I:C affects only the early stage of hematopoietic $CD34^+$ progenitor cells, although type-I IFNs affects progenitor cells in any stage of differentiation. Secondly, neither IFN-α nor IFN-β causes an immediate induction of apoptosis of $CD34^+$ cells. In addition, the neutralization or blocking of type-I IFN receptor does not rescue $CD34^+$ cells, whereas Z-VAD, a pan-caspase inhibitor, rescues the cells from apoptosis. These results strongly suggest that the apoptosis induction in $CD34^+$ cells by poly I:C is independent of IFN signaling.

Infection of RNA viruses to $CD34^+$ cells or endocytosis of dsRNA by human $CD34^+$ cells leading to immediate apoptosis of these cells is of particular interest. Human $CD34^+$ cells have been shown to be susceptible to infection with a number of viruses, including human immunodeficiency virus type 1 [35], hepatitis C virus [36], JC virus [37], human cytomegalovirus [38], human herpes virus (HHV) type 6 [39], HHV-7 [40], HHV-8 [41], and human T-cell lymphotropic virus type 1 [42]. Suppression of hematopoiesis has been documented to occur following infection of $CD34^+$ cells with human cytomegalovirus [38,43], HHV-6 [39], human immunodeficiency virus type 1 [44], and measles virus [45], either by direct infection of $CD34^+$ cells or by indirect mechanisms, such as disruption of cytokine expression or stromal cells. This study provides a possibility that viral or cellular dsRNA, in itself, causes a rapid apoptosis of human $CD34^+$ cells.

In conclusion, the present study for the first time suggests that poly I:C–mediated RIG-I and MDA-5 signaling triggers apoptosis of human $CD34^+$ cells in a type-I IFN-independent pathway, which may represent an antiviral host response to eliminate infected cells, here, hematopoietic stem/progenitor cells. Alternatively, the induction of immediate apoptosis of $CD34^+$ cells may represent a viral strategy to evade host defense because the reduction of progenitors comprises dendritic cell progenitors [11], specialized antigen-processing and antigen-presenting cells, as well as erythroid, neutrophilic, and megakaryocytic progenitors. In either case, the induction of apoptosis of $CD34^+$ hematopoietic stem/progenitor cells by viral or cellular dsRNA could be a cause of hematopoietic disorders, such as bone marrow failure syndrome characterized by pancytopenia, which should be an area for further exploration.

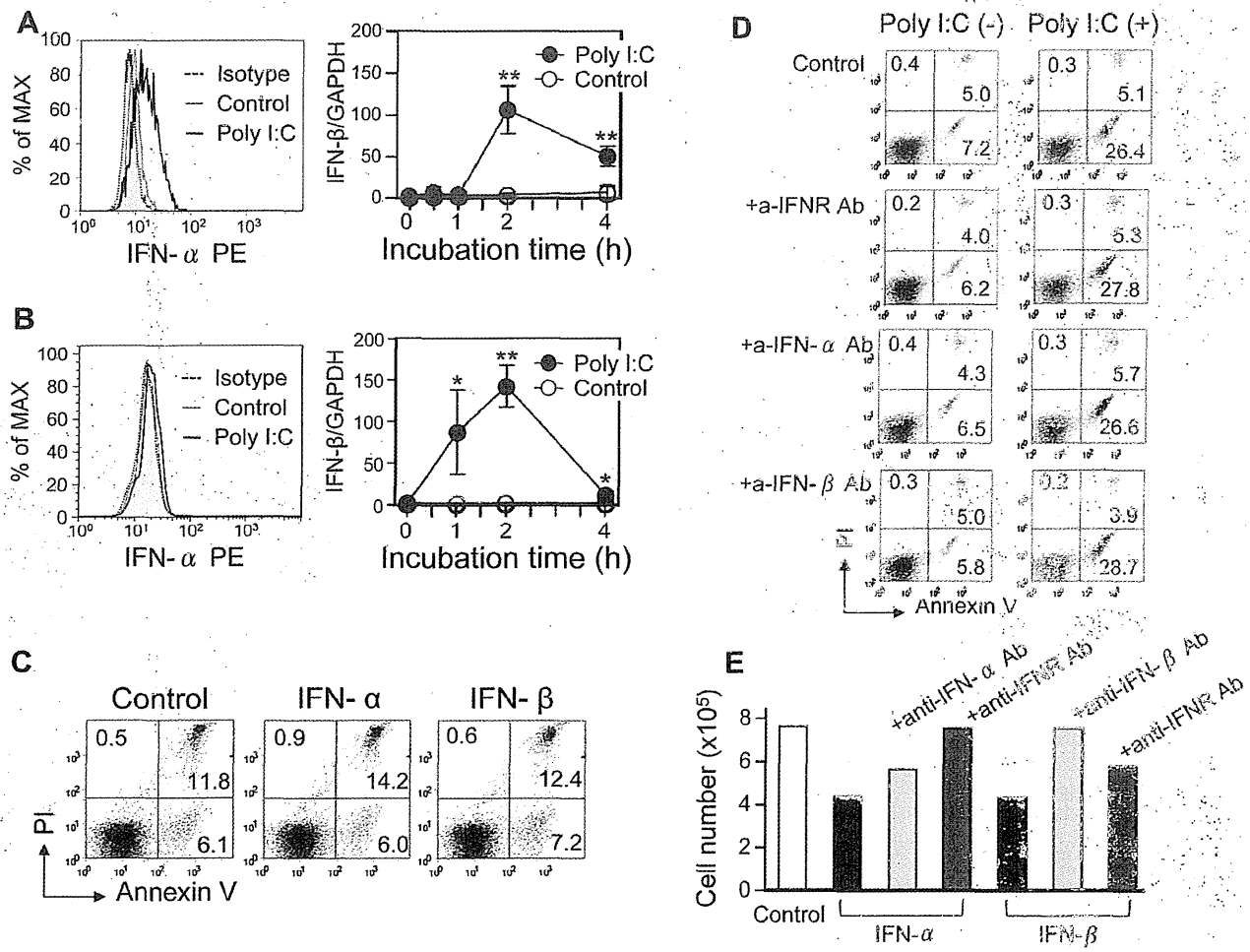

Figure 7. Apoptosis induction by poly I:C and IFN signaling. (**A**, **B**) IFN-α and IFN-β expression of the cells treated with or without poly I:C. Purified $CD34^+$ cells (**A**), and $CD34^+$ cells cultured for 2 days in multilineage medium (**B**), at a density of 5 to 10×10^4 cells/mL were cultured in 1 mL multilineage medium with or without 50 μg/mL poly I:C. IFN-α expression was analyzed by fluorocytometry after 4 hours of incubation. A representative of two independent experiment is shown. IFN-β expression was analyzed at the indicated period by quantitative RT-PCR. The relative gene expression levels were normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts and represented as a unit relative to $CD34^+$ cells. Results presented are the mean ± standard deviation (SD) of three independent experiments. (**C**) Effects of IFN-α (10,000 U/mL) and IFN-β (10,000 U/mL) on $CD34^+$ cells. $CD34^+$ cells at a density of 5 to 10×10^4 cells/mL were cultured in 1 mL multilineage medium with or without IFN for 4 hours and subjected to apoptosis analysis. (**D**) $CD34^+$ cells at a density of 5 to 10×10^4 cells/mL were cultured in 1 mL multilineage medium for 4 hours in the presence or absence of poly I:C and with or without the type I-IFN inhibitors (anti-IFNR Ab 25 μg/mL, anti–IFN-α Ab 1000 U/mL, and anti–IFN-β Ab 1 μg/mL) and subjected to apoptosis analysis. (**E**) $CD34^+$ cells at a density of 5 to 10×10^4 cells/mL were cultured in 1 mL multilineage medium in the presence or absence of IFN-α or IFN-β and with or without the type I-IFN inhibitors (anti-IFNR Ab 25 μg/mL, anti–IFN-α Ab 1000 U/mL, and anti–IFN-β Ab 1 μg/mL). Seven days later, the total cell yield was counted. Data are representative of two independent experiments or the mean ± SD of three independent experiments. $^{**}p < 0.01$. NS = no significance.

Acknowledgments

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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TIM-3 as a therapeutic target for malignant stem cells in acute myelogenous leukemia

Yoshikane Kikushige and Koichi Akashi

Department of Medicine and Biosystemic Sciences, Kyushu University Graduate School of Medicine, Fukuoka, Japan

Address for correspondence: Koichi Akashi, Department of Medicine and Biosystemic Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-Ku, Fukuoka 812-8582, Japan. akashi@med.kyushu-u.ac.jp

Acute myeloid leukemia (AML) originates from self-renewing leukemic stem cells (LSCs), an ultimate therapeutic target for AML. Recent studies have shown that many AML LSC–specific surface antigens could be such candidates. T cell immunoglobulin mucin-3 (TIM-3) is expressed on LSCs in most types of AML, except for acute promyelocytic leukemia, but not on normal hematopoietic stem cells (HSCs). In mouse models reconstituted with human AML LSCs or human hematopoietic stem cells, a human TIM-3 mouse IgG2a antibody with complement-dependent and antibody-dependent cellular cytotoxic activities eradicates AML LSCs *in vivo* but does not affect normal human hematopoiesis. Thus, TIM-3 is one of the promising targets to eradicate AML LSCs.

Keywords: leukemic stem cell; AML; TIM-3

Introduction

Acute myeloid leukemia (AML) is a clonal malignant disorder derived from a small number of leukemic stem cells (LSCs). The concept of LSCs has been proposed based on the finding in the 1980s that only a small fraction of AML blasts can form colonies *in vitro*. In 1996, Dick *et al.* showed that the $CD34^+CD38^-$, but not other fractions of bone marrow cells from AML patients, can reconstitute human AML in immunodeficient mice, demonstrating direct evidence for the presence of LSCs.[1] LSCs can self-renew and generate a large number of clonogenic leukemic blasts.[1–3] Although recent studies have suggested that LSCs are present in either in the $CD34^+CD38^+$ fraction[4] or the $CD34^-$ blastic fraction, at least in some types of AML[5,6] the $CD34^+CD38^-$ population represents highly-enriched LSCs in the vast majority of cases.[7] The $CD34^+CD38^-$ phenotype is, however, shared with normal human hematopoietic stem cells (HSCs) that have long-term reconstitution activity.[8,9]

In the clinic, conventional chemotherapies can currently achieve complete remission in ~90% of AML cases. However, a considerable fraction (~60%) of AML patients still relapse after intensive chemotherapy. The recurrence of AML in these patients could be caused by regrowth of the remaining LSCs. Therefore, LSCs could be the ultimate therapeutic target to achieve cure in AML patients. Thus, to selectively kill AML LSCs, sparing normal HSCs, one of the most practical approaches is to target the AML LSC–specific surface or functionally indispensable molecules. To achieve specificity for LSCs, the target molecule should be expressed on LSCs at a high level and not on normal HSCs.[10] It should not matter whether the molecule is expressed in normal blood cells or normal progenitor cells, because normal HSCs that are spared would replenish all mature blood cells after treatment.

Recently, two papers have reported the T cell immunoglobulin mucin-3 (TIM-3) as a surface molecule expressed in LSCs of most AML types.[11,12] Here, we discuss the potential usefulness of TIM-3 in eradicating AML LSCs, leaving normal HSCs intact.

Expression and functions of TIM-3 in normal hematopoiesis

TIM-3 was originally identified as a surface molecule expressed in interferon (IFN)-γ–producing $CD4^+$ Th1 cells and in $CD8^+$ T cytotoxic type 1 (Tc1) cells[13] in mouse hematopoiesis. TIM-3 plays an important role in limiting and controlling

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Th1-dependent immune responses and in inducing immune tolerance.[13–15] The ligand of TIM-3 in lymphocytes is galectin-9, an S-type lectin with two distinct carbohydrate recognition domains that can bind to carbohydrate chains on the TIM-3 IgV domain. Engagement of TIM-3 by galectin-9 induces Th1 cells to undergo apoptosis and inhibits their production of IFN-γ.[16] Thus, TIM-3 is a negative regulator of Th1- and Tc1-driven immune responses.

TIM-3 is also expressed in myeloid cells, including $CD11b^+$ macrophages, $CD11c^+$ dendritic cells (DCs), and mast cells, and it recognizes apoptotic cell–expressed phosphatidylserine (PS) through the TIM-3 IgV domain.[17–20] The binding of PS to TIM-3 does not interfere with binding of galectin-9 to TIM-3, as the binding sites of these molecules are located at opposite sides of the IgV domain. In TIM-3–expressing DCs, recognition of PS by TIM-3 enhanced phagocytosis of apoptotic cells and cross-presentation of apoptotic cell–associated antigens to $CD8^+$ T cells.[18]

In human steady-state hematopoiesis, TIM-3 is expressed mainly in monocytes and a fraction of NK

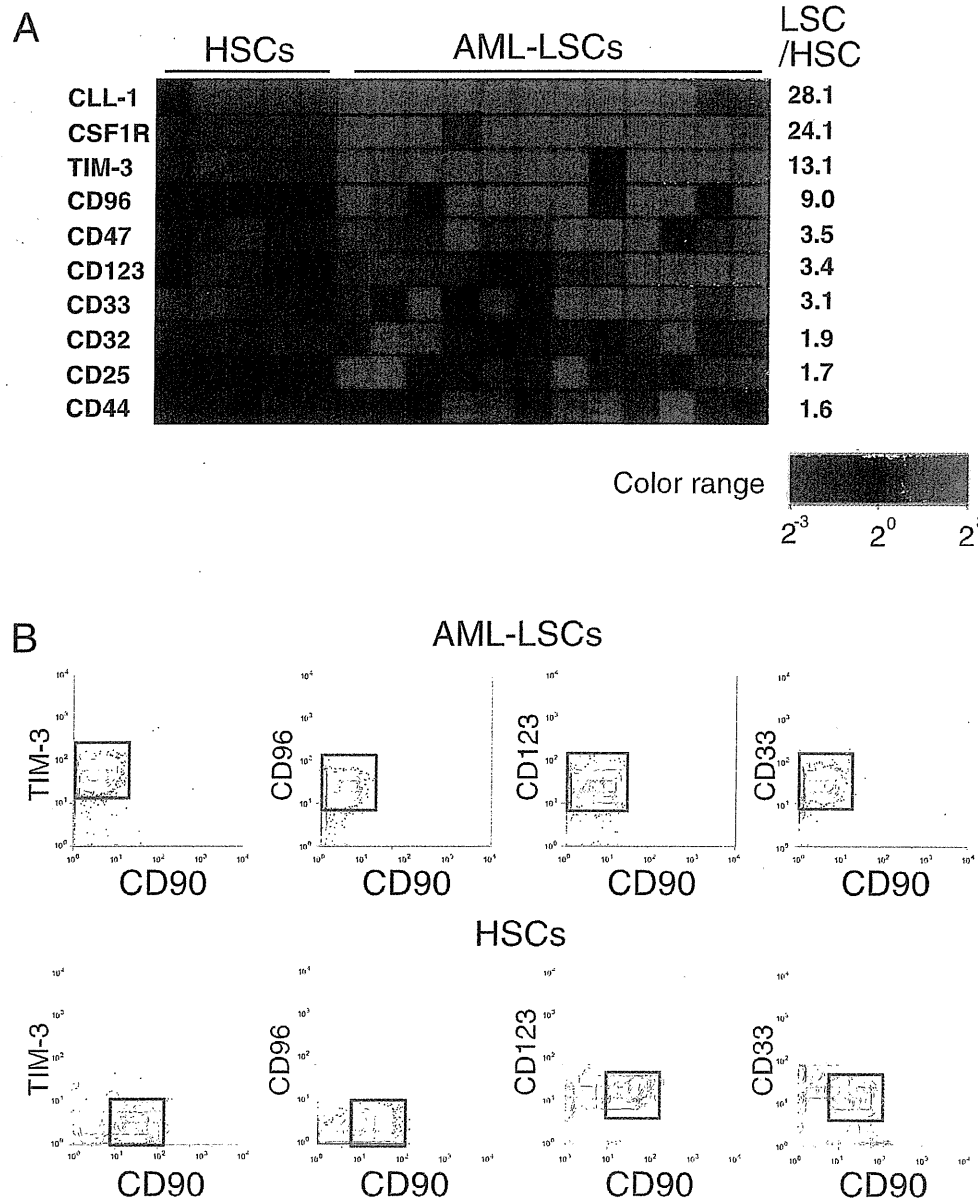

Figure 1. The expression of LSC-specific surface molecules in AML. (A) $CD34^+CD38^-$ HSCs and $CD34^+CD38^-$ AML LSCs were purified and analyzed on cDNA microarray. The surface molecules that are expressed strongly in AML LSCs compared to normal HSCs are listed. (B) The expression of representative surface markers in AML LSCs and normal HSCs.

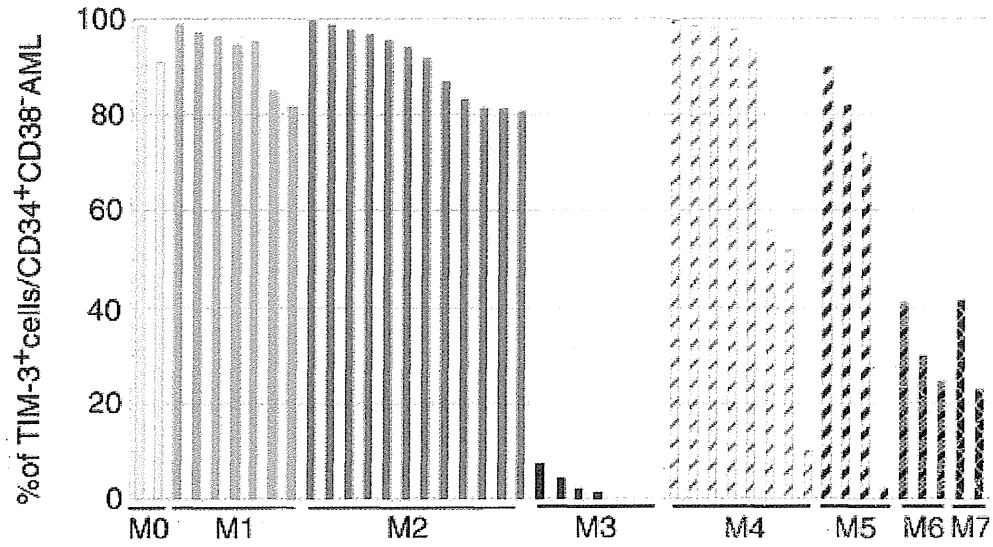

Figure 2. The frequency of TIM-3$^+$ cells in the CD34$^+$CD38$^-$ fraction in each AML subtype. TIM-3 protein is expressed in the vast majority of the CD34$^+$CD38$^-$ LSC population in AML types M0, M1, M2, and M4.

cells, but not in granulocytes, T cells, or B cells.[11] In the bone marrow TIM-3 is not expressed in normal HSCs or the vast majority of the CD34$^+$CD38$^+$ progenitor population. But within the CD34$^+$CD38$^+$ fraction, TIM-3 was expressed in a fraction of granulocyte/monocyte progenitors (GMPs) at a low level, while it is not in common myeloid progenitors (CMPs), megakaryocyte/erythrocyte progenitors (MEPs), or common lymphoid progenitors (CLPs). The vast majority of purified TIM-3$^+$ GMPs gave rise to CFU-M, suggesting that upregulation of TIM-3 mainly occurs in concert with the monocyte lineage commitment at the GMP stage.[11]

TIM-3 expression in human AML

TIM-3 has been identified as an AML LSC–specific marker based on the comparison between expression profiles of CD34$^+$CD38$^-$ AML cells and normal HSCs (Fig. 1A). While TIM-3 protein is not expressed in normal HSCs (Fig. 1B), it is expressed at a high level in the vast majority of CD34$^+$CD38$^-$ LSCs and the CD34$^+$CD38$^+$ progenitor fraction in AML M0, M1, M2, and M4 types in virtually all cases (Fig. 2) and in all cytogenic subgroups.[11,12] In AML M5, M6, and M7, a considerable fraction of CD34$^+$CD38$^-$ cells express TIM-3. However, TIM-3 expression was not seen in the CD34$^+$CD38$^-$ population in M3 cases (Fig. 2). TIM-3 expression tends to decline at the CD34$^-$ leukemic blast stage. Of note, the expression level of TIM-3 was found to be high in AML with core-binding factor translocations or with mutations in CEBPA.[12]

Strikingly, the TIM-3$^+$ population in the bone marrow contains all AML LSCs, and normal HSCs are always included in patients' TIM-3$^-$ populations. TIM-3$^+$ and TIM-3$^-$ AML populations have been transplanted into sublethally-irradiated immunodeficient mice and only mice transplanted with TIM-3$^+$ AML cells developed human AML.[11] In another experiment, the TIM-3$^-$ fraction did not include LSCs but normal HSCs, as evidenced by the fact that normal human hematopoiesis was frequently reconstituted in mice transplanted with the TIM-3$^-$ fraction.[12]

Targeting AML LSCs by TIM-3-specific antibodies in xenograft models

To utilize TIM-3 to target AML LSCs, it is critical to establish human TIM-3 antibodies that can kill TIM-3–expressing cells *in vivo*. In terms of an antibody-based treatment, knowing the antibody-dependent cell-mediated cytotoxicity (ADCC) and the complement-dependent cytotoxicity (CDC) activities is critical in order to eliminate target cells.[21] A TIM-3 monoclonal antibody (IgG2b) was obtained by immunizing Balb/c mice with L929 cells stably-expressing human TIM-3 and soluble TIM-3 protein.[11] For this antibody, the variable portion of the VH regions of the cloned hybridoma that

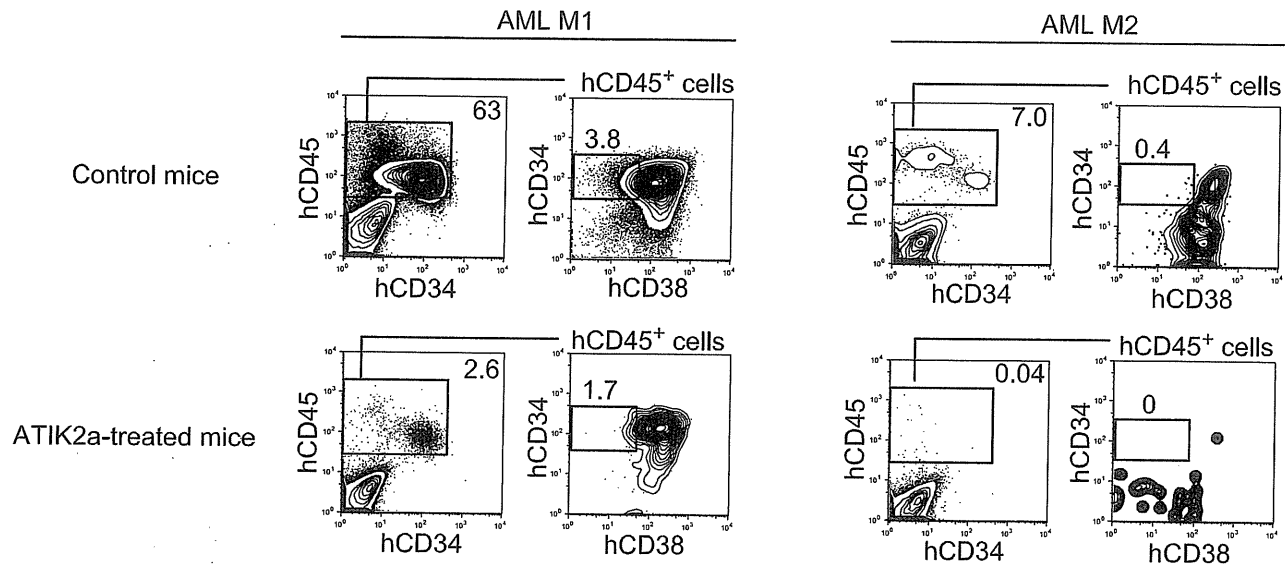

Figure 3. Potent antileukemic effects of the TIM-3 monoclonal antibody in a xenograft model. Mice were transplanted with LSCs purified from patients with AML M1 (left) or M2 (right) and were then treated with ATIK2a or control antibodies. In both experiments, ATIK2a treatment significantly reduced the human $CD45^+$ AML cells and $CD34^+CD38^-$ AML LSCs *in vivo*. Representative results of bone marrow analysis four weeks after treatment are shown.

recognize TIM-3 were grafted onto IgG2a Fc regions because the IgG2a subclass is the most efficient at inducing ADCC activity in mice.[22,23] The established clone, ATIK2a, was effective at killing TIM-3–expressing cell lines via both CDC and ADCC.[11]

The effect of ATIK2a on normal and malignant AML hematopoiesis was tested in xenograft models. NOD-SCID mice transplanted with 10^5 $CD34^+$ cord blood cells with or without ATIK2a injection developed almost equal percentages of human cells. In mice injected with ATIK2a, however, human $TIM\text{-}3^+$ mature monocytes were not found, suggesting that while targeting TIM-3 does not affect hematopoiesis, it does eliminate normal monocytes.

In contrast, ATIK2a exerted profound effects on leukemia development. In mice transplanted with human AML of M0, M1, and M4 types, ATIK2a treatment significantly reduced the human $CD45^+$ AML cell burden, as well as the $CD34^+CD38^-$ LSC cell numbers *in vivo* (Fig. 3). Retransplantation into secondary recipients of the remaining AML cells from primary recipients treated with ATIK2a never gave rise to human AML, indicating that the ATIK2a treatment successfully eradicated the LSCs in the primary recipients. These data suggest that eliminating AML LSCs by using TIM-3–"killing" antibodies may be a practical approach to curing human AML.

Perspective

To use surface markers for targeting AML LSCs, specificity as well as sensitivity are critical. TIM-3 has several advantages over other candidate markers. First, TIM-3 protein is not detectable in normal HSCs or in other myelo-erythroid or lymphoid progenitors, although monocyte-lineage committed progenitors begin to upregulate TIM-3. Second, TIM-3 could mark all LSCs that can reconstitute human AML in immunodeficient mice in the majority of M0, M1, M2, and M4 AML cases, and its expression level is sufficient to eradicate LSCs by antibody-based treatment. The expression level of other candidate molecules, including CD25,[24] CD32,[24] CD44,[25] and CD47,[26] in LSCs was only two- to threefold higher at the mRNA level compared with normal HSCs (Fig. 1A), and in some AML cases, LSCs did not express these molecules. CD33 and CD123 proteins are expressed at relatively high levels in normal HSCs (Fig. 1B) and myeloid progenitors, including CMPs and GMPs,[27] suggesting that targeting these molecules would harm normal hematopoiesis. In fact, prolonged cytopenia has been observed in AML patients treated with gemtuzumab, a recombinant humanized anti-CD33 monoclonal antibody conjugated with the cytotoxic antibiotic calicheamicin.

In contrast, CLL-1,[28] CSF1R,[29] and CD96[30] are molecules specifically expressed by LSCs. CLL-1 is

a transmembrane glycoprotein;[31] the proportion of CLL-1–expressing $CD34^+CD38^-$ AML cells is highly diverse in cases.[28] CD96 is a member of the Ig gene superfamily; it is expressed on activated T cells.[32] Of note, similar to the case of TIM-3, $CD96^+$, but not $CD96^-$, AML cells efficiently reconstitute AML in the immunodeficient mice,[30] suggesting that CD96 can mark all functional AML LSCs. The expression level of CD96 protein is also high enough to clearly separate AML LSCs and normal HSCs (Fig. 1B). However, the sensitivity of TIM-3 is likely to be the highest among these molecules, at least for AML M0, M1, M2, and M4.

To apply our findings to clinic, we have developed a chimeric TIM-3 monoclonal antibody by fusing the variable regions of ATIK2a to the human IgG constant region. Thus far we have injected this antibody to cynomolgus monkeys, which showed no significant adverse effects (unpublished data). A clinical study with further intensive treatment is being planned.

It is important to understand the function of molecules in the maintenance and/or reconstitution capabilities of LSCs. For example, it was shown that anti-CD44 monoclonal antibodies reduced leukemic burden and blocked secondary engraftment in a NOD-SCID model.[25] This effect on LSCs was mediated in part by the disruption of LSC–niche interactions.[25] Anti-CD47 antibodies can block LSC reconstitution in a NOD-SCID model,[26] and this might be due to the activation of phagocytosis by macrophages through inhibition of interaction of CD47 with SIRPA. Since the pathway for eradication of LSCs by anti-CD44 or anti-CD47 treatment is different from that of anti-TIM-3, the combination of these antibodies might be critical for future treatments targeting AML LSCs.

Conflicts of interest

The authors declare no conflicts of interest.

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Revised International Prognostic Scoring System for Myelodysplastic Syndromes

Peter L. Greenberg,[1] Heinz Tuechler,[2] Julie Schanz,[3] Guillermo Sanz,[4] Guillermo Garcia-Manero,[5] Francesc Solé,[6] John M. Bennett,[7] David Bowen,[8] Pierre Fenaux,[9] Francois Dreyfus,[10] Hagop Kantarjian,[5] Andrea Kuendgen,[11] Alessandro Levis,[12] Luca Malcovati,[13] Mario Cazzola,[13] Jaroslav Cermak,[14] Christa Fonatsch,[15] Michelle M. Le Beau,[16] Marilyn L. Slovak,[17] Otto Krieger,[18] Michael Luebbert,[19] Jaroslaw Maciejewski,[20] Silvia M. M. Magalhaes,[21] Yasushi Miyazaki,[22] Michael Pfeilstöcker,[2] Mikkael Sekeres,[20] Wolfgang R. Sperr,[15] Reinhard Stauder,[23] Sudhir Tauro,[24] Peter Valent,[15] Teresa Vallespi,[25] Arjan A. van de Loosdrecht,[26] Ulrich Germing,[11] and Detlef Haase[3]

[1]Stanford University Cancer Center, Stanford, CA; [2]Hanusch Hospital, Boltzmann Institute for Leukemia Research, Vienna, Austria; [3]Georg August Universität, Göttingen, Germany; [4]Hospital Universitario La Fe, Valencia, Spain; [5]The University of Texas, MD Anderson Cancer Center, Houston, TX; [6]Hospital del Mar, Barcelona, Spain; [7]James P. Wilmont Cancer Center, University of Rochester Medical Center, Rochester, NY; [8]St James's University Hospital, Leeds, United Kingdom; [9]Hôpital Avicenne, Assistance Publique–Hôpitaux de Paris (AP-HP)/University Paris XIII, Bobigny, France; [10]Hôpital Cochin, AP-HP University of Paris V, Paris, France; [11]Heinrich-Heine University Hospital, Düsseldorf, Germany; [12]Antonio e Biagio e C Arrigo Hospital, Alessandria, Italy; [13]Fondazione Istituti di Ricovero e Cura a Carattere Scientifico Policlinico San Matteo and University of Pavia, Pavia, Italy; [14]Institute of Hematology and Blood Transfusion, Praha, Czech Republic; [15]Medical University of Vienna, Vienna, Austria; [16]University of Chicago Comprehensive Cancer Research Center, Chicago, IL; [17]Quest Diagnostics Nichols Institute, Chantilly, VA; [18]Elisabethinen Hospital, Linz, Austria; [19]University of Freiburg Medical Center, Freiburg, Germany; [20]Cleveland Clinic, Cleveland, OH; [21]Federal University of Ceara, Fortaleza, Brazil; [22]Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan; [23]University Hospital of Innsbruck, Innsbruck, Austria; [24]University of Dundee, Scotland, United Kingdom; [25]Hospital Universitario Vall d'Hebron, Barcelona, Spain; and [26]VU University Medical Center, Amsterdam, The Netherlands

The International Prognostic Scoring System (IPSS) is an important standard for ssessing prognosis of primary untreated adult patients with myelodysplastic syndromes (MDS). To refine the IPSS, MDS patient databases from international institutions were coalesced to assemble a much larger combined database (Revised-IPSS [IPSS-R], n = 7012, IPSS, n = 816) for analysis. Multiple statistically weighted clinical features were used to generate a prognostic categorization model. Bone marrow cytogenetics, marrow blast percentage, and cytopenias remained the basis of the new system. Novel components of the current analysis included: 5 rather than 3 cytogenetic prognostic subgroups with specific and new classifications of a number of less common cytogenetic subsets, splitting the low marrow blast percentage value, and depth of cytopenias. This model defined 5 rather than the 4 major prognostic categories that are present in the IPSS. Patient age, performance status, serum ferritin, and lactate dehydrogenase were significant additive features for survival but not for acute myeloid leukemia transformation. This system comprehensively integrated the numerous known clinical features into a method analyzing MDS patient prognosis more precisely than the initial IPSS. As such, this IPSS-R should prove beneficial for predicting the clinical outcomes of untreated MDS patients and aiding design and analysis of clinical trials in this disease. (*Blood.* 2012;120(12): 2454-2465)

Introduction

The myelodysplastic syndromes (MDS) consist of a heterogeneous spectrum of myeloid clonal hemopathies. The International Prognostic Scoring System (IPSS) has been an important standard for assessing prognosis of primary untreated adult MDS patients.[1] However, since its publication in 1997, modification of existing parameters and additional prognostic systems have been suggested as providing meaningful differences for patients' clinical outcomes,[2-5] and the World Health Organization (WHO) added morphologic refinement of the French-American-British (FAB) classification.[6,7] In addition, the WHO Prognostic Scoring System (WPSS)[2,3] has provided new insights into prognostic variables, adding red blood cell (RBC) transfusion dependence along with IPSS cytogenetic classification and WHO dysplastic categories. Importantly, recent newer cytogenetic groupings are reported to be prognostically valuable and to refine those features used in the IPSS.[8] Additional variables suggested as providing prognostic information in MDS included serum lactate dehydrogenase (LDH),[9-11] ferritin,[12] and β_2-microglobulin[13,14] as well as marrow fibrosis[15-17] and patient comorbidities and performance status.[5,18-20]

To examine the prognostic impact of these new clinical and cytogenetic variables and attempt to refine the IPSS, coordination of investigators and coalescence of MDS databases from multiple international institutions provided a much larger combined database of patients by the International Working Group for Prognosis in MDS (IWG-PM) project.

The aims of this study were to refine the IPSS by reassessing the prior major predictive features, determining the impact of the newer clinical features for prognostic power, incorporating larger and more differentiated cytogenetic subgroups, and reevaluating

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their prognostic impact. Statistically weighted clinical features were used to generate a prognostic categorization model. This larger combined database permitted better analyses of the specific impact of marrow blast percentage, depth of cytopenias, and of the less frequent features, particularly further evaluating the relatively rare cytogenetic subgroups. In addition, as some features had only been reported from single centers, this combined database extended such findings.

Methods

Under the aegis of the MDS Foundation, MDS databases of primary untreated MDS patients from multiple international institutions from 11 countries, including data from the Spanish, French, Piemonte (Italy) and Brazilian MDS Registries and that from the International MDS Risk Analysis Workshop (IMRAW), were submitted and evaluated by the IWG-PM project. Databases came from both university- and nonuniversity-based hospitals associated with the country's MDS-focused groups. Institutional review board approval was obtained from the respective institutions. After careful vetting for accuracy, a combined IWG-PM database of 7012 patients, classified morphologically by FAB (n = 7000) and, in most cases, by the WHO criteria (n = 5504),[6] was created. Inclusion criteria were: primary MDS patients whose disease had not been treated with disease-altering therapy during their MDS phase (ie, no hypomethylating agents, intensive chemotherapy, or hematopoietic stem cell transplantation). Marrow blasts were required to be $\leq 30\%$, peripheral blood blasts $\leq 19\%$, white blood count (WBC) $\leq 12 \times 10^9$/L, and absolute neutrophil count (ANC) $\leq 8 \times 10^9$/L). The patient's blood counts needed to demonstrate ≥ 2 months of stable disease. Marrow blasts and cytogenetic results, hemoglobin, ANC, and platelet levels at diagnosis were documented, and data regarding patient's survival and development of acute myeloid leukemia (AML) were obtained. The patient ages were ≥ 16 years. Data regarding use of erythropoiesis-stimulating agents or myeloid growth factors were not systematically collected.

The results of cytogenetic analysis of bone marrow were reviewed by the Cytogenetics Committee (D.H., Chair; J.S., C.F., M.M.L.B., F.S., and M.L.S.) using standard ISCN criteria.[21] Specific karyotypic abnormalities and their risk categories were used as per Schanz et al, which required ≥ 10 patients for inclusion as a specific abnormality. Parameters evaluated were cytogenetic risk category,[8] marrow aspirate blast percent, depth of cytopenias, degree of marrow fibrosis (0-1+ vs 2-3+), Eastern Cooperative Oncology Group performance status, serum LDH (normal values defined by each hospital), ferritin and β_2-microglobulin levels, RBC transfusion dependence, and patient age at diagnosis. The database of untreated primary MDS patients from the Medical University of Vienna was used as an external independent validation cohort.

Statistical methods

Modeling of prognostic risk was based on multivariate analysis of survival time and time to AML transformation. Functional relations of bone marrow blasts and cytopenias with prognostic risk were analyzed to define appropriate categories for score calculation.[22] Robust Cox models[23] for survival, time to transformation, and combination of both were built to derive the relative weights within the score. To compensate for possible heterogeneities, analyses were stratified by data source, year of diagnosis, and age. This led to generation of reference scores for these clinical outcomes.

The score was developed following a hierarchical approach. The main score was built based on the initially elaborated categories for bone marrow blasts and cytopenias together with the cytogenetic risk categories.[8] The categorizations of cytopenias were adjusted to clinically relevant cutpoints. To calculate a specific feature's added score, the proportional weight of the score was used. A "combined" score model was effectively used rather than having 2 separate models for survival and AML transformation. Separate specific score variants for survival and AML transformation were considered, but they provided very little gain. Therefore, for ease of communication and implementation, 1 unified model was preferred. This model approximated (statistically) both outcomes adequately, particularly for survival when age was included. Risk-scoring values were rounded to the nearest 0.5 unit with re-estimation of all statistics for the rounded scores. To ease interpretation, boundaries of the 5 risk categories of the final score were chosen to build a scale with approximately equal risk increments between 2 adjacent levels.

The effect of age was modeled as an optional additive feature for overall survival prediction by including age in a model with the already defined main scores. Additional potentially differentiating features were analyzed to estimate their incremental prognostic values, given the already defined main score.

As a measure of prognostic power, the Dxy coefficient for censored data[24] was used. Dxy is a concordance coefficient varying between -1 and 1, with 0 representing no predictive power and 1 perfect concordance of ascribed risk and survival and time to transformation, respectively. For a potentially additive feature to be considered clinically significant, both a $P < .05$ and a gain in prognostic power (Dxy) were required. Dxy's were internally validated by bootstrapping the related Cox models.[24] Two-sided P values $< .05$ were reported as significant. Correlations between ordered categorical variables were measured by Kendall tau. In line with the nature of the project, no adjustment for multiple testing was applied. All analyses were performed using the open source software R Version 2.14.1.[25,26] Kaplan-Meier curves were used to demonstrate clinical outcomes.

Results

Patient characteristics

Data from 7012 patients from multiple institutional databases in the combined IWG-PM database were evaluated. Their median age was 71 years, 77% were > 60 years, the male/female ratio 1.5:1, and median follow-up time 3.9 years. The 7012 patients obtained for evaluation were classified by FAB (n = 7000, 99.8%)[7] and additionally by WHO (n = 5504, 78.5%)[6] and/or WPSS (n = 2325, 33.2%).[9] Table 1 shows the individual clinical variables and outcomes (survival, AML evolution) for our patient cohort, with Dxy concordance coefficients (indicating prognostic power) and univariate P values. Bootstrap-validated Dxy values were almost identical with the sample results given in Table 1 (all differences were at most .01, except the value for β_2-microglobulin).

Identification of significant prognostic variables

As in the IPSS, marrow cytogenetic subset, marrow blast percentage, and cytopenias were considered as the basis of this new prognostic system (the Revised IPSS [IPSS-R]) given their statistical weight compared with the other variables analyzed herein using Cox proportional hazard regression analyses, with overall survival and AML transformation as outcomes. Multivariate analysis of these variables led to their relative statistical weighting, determining their impact on prognostic risk, using Cox proportional hazard regression. In descending order, these 5 major variables for evaluating clinical outcomes were: cytogenetic risk groups, marrow blast percentage, and depth of cytopenias (hemoglobin, platelet, and ANC levels, respectively). The novel components obtained in the current analysis included: 5 rather than 3 cytogenetic prognostic subgroups with specific classification of a number of less common cytogenetic subsets and alteration of others (Table 2)[8]; the < 5% marrow blast category was split between 0%-2% and > 2-< 5%, whereas all patients with > 10% blasts were grouped in the same category; depth of cytopenias at clinically and statistically relevant cutpoints rather