Research Article

The Akt/Mammalian Target of Rapamycin Signal Transduction Pathway Is Activated in High-Risk Myelodysplastic Syndromes and Influences Cell Survival and Proliferation

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Abstract

The Akt/mammalian target of rapamycin (mTOR) signaling pathway is important for both cell growth and survival. In particular, an impaired regulation of the Akt/mTOR axis has been strongly implicated in mechanisms related to neoplastic transformation, through enhancement of cell proliferation and survival. Myelodysplastic syndromes (MDS) are a group of heterogeneous hematopoietic stem cell disorders characterized by ineffective hematopoiesis and by a high risk of evolution into acute myelogenous leukemia (AML). The pathogenesis of the MDS evolution into AML is still unclear, although some recent studies indicate that aberrant activation of survival signaling pathways could be involved. In this investigation, done by means of immunofluorescent staining, we report an activation of the Akt/mTOR pathway in high-risk MDS patients. Interestingly, not only mTOR was activated but also its downstream targets, 4E-binding protein 1 and p70 ribosomal S6 kinase. Treatment with the selective mTOR inhibitor, rapamycin, significantly increased apoptotic cell death of $CD33^+$ (but not $CD33^-$) cells from high-risk MDS patients. Rapamycin was ineffective in cells from healthy donors or low-risk MDS. Moreover, incubation of high-risk MDS patient $CD34^+$ cells with rapamycin decreased the *in vitro* clonogenic capability of these cells. In contrast, the phosphoinositide 3-kinase inhibitor, LY294002, did not significantly affect the clonogenic activity of high-risk MDS cells. Taken together, our results indicate that the Akt/mTOR pathway is critical for cell survival and proliferation in high-risk MDS patients. Therefore, this signaling network could become an interesting therapeutic target for treating more advanced MDS cases. [Cancer Res 2007;67(9):4287–94]

Introduction

The phosphoinositide 3-kinase (PI3K)/Akt signaling pathway is involved in many different cellular processes, including proliferation, differentiation, and apoptosis (1, 2). Akt (also known as protein kinase B) is a 57-kDa serine/threonine protein kinase that is activated through a double phosphorylation mechanism. First, Akt is recruited to the plasma membrane by phosphatidylinositol (3,4,5)-triphosphate, which is synthesized by PI3K, and is then phosphorylated by the phosphoinositide-dependent protein kinase 1 at the Thr^{308} of the activation loop. Subsequently, a still unidentified kinase phosphorylates Akt at the Ser^{473} in the COOH-terminal regulatory region domain.

An impaired regulation of the PI3K/Akt axis has been strongly implicated in carcinogenesis (3–7). In particular, the activation of the PI3K/Akt survival pathway is often associated with hematologic malignancies (8–14), including acute and chronic human leukemias.

One of the downstream targets of Akt is represented by the mammalian target of rapamycin (mTOR), a highly conserved serine/threonine protein kinase that is essential for the regulation of cell growth and proliferation, by controlling these processes at the translational level (15) and by acting on the cell cycle progression. Indeed, mTOR is capable of regulating the synthesis of key proteins, such as retinoblastoma protein, p27Kip1, cyclin D1, c-*myc*, or signal transducer and activator of transcription 3. Furthermore, recent studies have shown that mTOR is also involved in cell death, so that a deregulation of this kinase could lead to the activation of antiapoptotic mechanisms (16, 17).

Akt-mediated regulation of mTOR activity is a complex multistep phenomenon (9). Akt inhibits tuberous sclerosis 2 (TSC2; or hamartin) function through direct phosphorylation. TSC2 is a GTPase-activating protein (GAP) that functions in association with the putative tuberous sclerosis 1 (TSC1; or tuberin) to inactivate the small G-protein Ras homologue enriched in brain (Rheb). TSC2 phosphorylation by Akt represses GAP activity of the TSC1/TSC2 complex, allowing Rheb to accumulate in a GTP-bound state. Rheb-GTP then activates, through a mechanism not yet elucidated, the protein kinase activity of mTOR when complexed with the regulatory-associated protein of mTOR (Raptor) adaptor protein and mLST8 (also known as GβL), a protein homologous to β subunits of heterotrimeric G-proteins. The mTOR/Raptor/mLST8 (also referred to as mTORC1) complex is sensitive to rapamycin and, importantly, in some cases inhibits Akt via a negative feedback loop, which involves, at least in part, p70 ribosomal S6 kinase (p70S6K). The relationship between Akt and mTOR is further complicated by the existence of the mTOR/rapamycin-insensitive companion of mTOR/mLST8 complex (also referred to as mTORC2), which displays rapamycin-insensitive activity (9). Moreover, Akt directly phosphorylates mTOR on Ser^{2448} and activates it.

Downstream of the mTOR are two well-characterized substrates: 4E-binding protein 1 (4E-BP1) and the p70S6K. On the one hand,

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Table 1. Patients' characteristics

Characteristics	
General	
No. patients	20
Male/female ratio	16:4
Median age, y (range)	70.1 (53-79)
Category	
High-risk MDS*	15
Low-risk MDS*	5
Karyotype	
Diploid	13
Monosomy 7	1
Trisomy 8	3
Complex†	3

*Classified according to IPSS.
†More than three chromosomal aberrations.

the phosphorylation of 4E-BP1 by mTOR suppresses its ability to bind the translation-initiation factor eukaryotic initiation factor 4E, a protein that is recruited to the translation initiation complex for regulating protein synthesis and initiating the translation of transcripts encoding genes involved in cell cycle control. On the other hand, mTOR also mediates the phosphorylation and the subsequent activation of p70S6K, which phosphorylates the 40S ribosomal protein S6 to initiate the translation of a 5′-terminal olygopyrimidine tract-containing mRNAs encoding components of the protein synthesis machinery.

The myelodysplastic syndromes (MDS) are a heterogeneous group of bone marrow disorders characterized by a defect in the differentiation of the hematopoietic stem cell that causes anemia, neutropenia, bleeding problems, and infections. The disease can result in a slow decrease in blood cell counts, but it may also have a more aggressive evolution, which is a worsening severe cytopenia or, in ~30% of all the patients, transformation into acute myelogenous leukemia (AML). On evolution of MDS into AML, the progressing clonal cells present an excessive survival and decreased apoptosis (18–21). Thus, the identification of the aberrant signaling pathways responsible for an increased survival of MDS cells is of high importance, as they might represent promising targets for novel forms of therapy aimed at preventing MDS evolution into AML.

Recently, we have shown that patients affected by high-risk MDS frequently show an activation of Akt compared with both low-risk MDS patients and healthy donors (22). However, in that study, we restricted our investigation to Akt. To better assess the relevance of Akt activation for MDS progression, we decided to investigate some of the downstream Akt targets, including mTOR, p70S6K, and 4E-BP1. Here, we show that mTOR, p70S6K, and 4E-BP1 were phosphorylated in high-risk (but not in low-risk) MDS patients, and this correlated with Akt activation. No activating mutations were detected in the PI3K *p110α* catalytic subunit gene of MDS patients. Furthermore, we show that rapamycin (a mTOR pharmacologic inhibitor) decreased the survival of $CD33^+$ cells from high-risk MDS patients and negatively affected the clonogenic ability of high-risk MDS $CD34^+$ precursors. Taken together, our findings indicate a critical role for activated mTOR and its downstream targets as survival factors in patients diagnosed with high-risk MDS. Hence, the Akt/mTOR pathway could become an important target for innovative therapeutic strategies in the treatment of high-risk MDS.

Materials and Methods

Antibodies and reagents. The following antibodies and reagents were purchased from commercial sources. Rabbit polyclonals to Ser^{473} phosphorylated Akt (p-Akt) and Akt, to Ser^{2448} phosphorylated mTOR (p-mTOR) and mTOR, to Ser^{65} phosphorylated 4E-BP1 (p-4E-BP1) and 4E-BP1, and

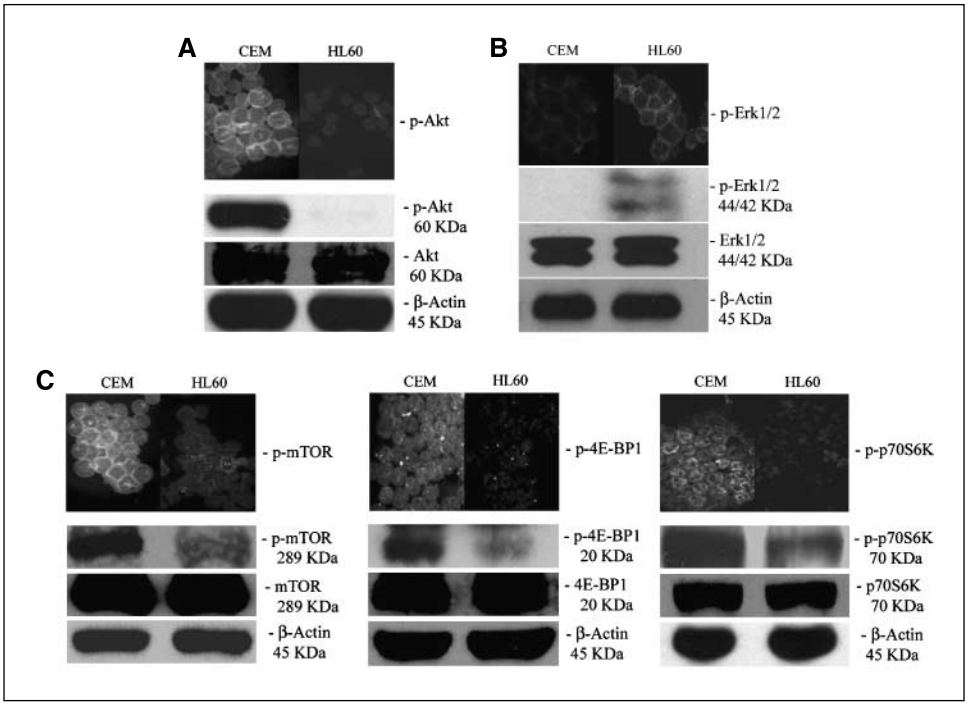

Figure 1. Activation state of the Akt/mTOR pathway in CEM and HL60 cell lines. Representative immunofluorescence and Western blot analysis (75 μg protein lysates) showing total and phosphorylated protein levels in CEM and HL60 cell lines. Original magnification, ×600. *A*, Ser^{473} p-Akt and total Akt. *B*, Thr^{202}/Tyr^{204} p-Erk1/2 and total Erk1/2. *C*, Ser^{2448} p-mTOR and total mTOR; Ser^{65} p-4E-BP1 and total 4E-BP1; and Thr^{389} p-p70S6K and total p70S6K. Under basal conditions, CEM cells displayed undetectable levels of p-Erk1/2, whereas HL60 cells showed higher levels. On the other hand, HL60 cells did not display detectable amounts of p-Akt and showed very low levels of p-mTOR, p-4E-BP1, and p-p70S6K, whereas in CEM cells the Akt/mTOR pathway was activated. β-Actin was used for loading control in Western blot experiment.

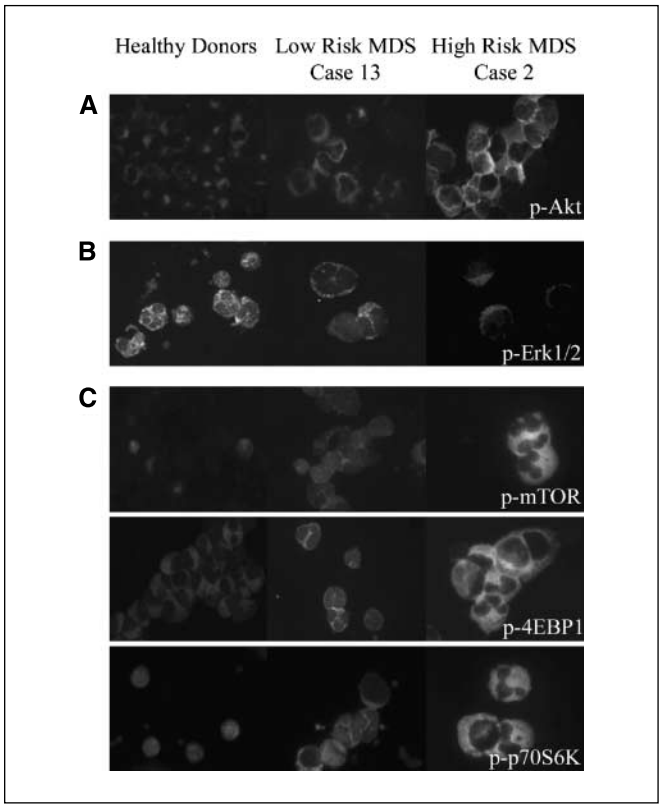

Figure 2. Immunocytochemical analysis of p-Akt, p-mTOR, p-4E-BP1, and p-p70S6K levels in healthy donors and low-risk and high-risk MDS patients. Representative immunofluorescence analysis of BMMCs from healthy donors, low-risk MDS patients (case 13), and high-risk MDS (case 2) patients. Original magnification, ×600. *A,* immunostaining with Ser^{473} p-Akt antibody. *B,* immunostaining with Thr^{202}/Tyr^{204} p-Erk1/2 antibody. *C,* immunostaining with Ser^{2448} p-mTOR antibody, with Ser^{65} p-4E-BP1 antibody, and with Thr^{389} p-p70S6K antibody.

to Thr^{202}/Tyr^{204} phosphorylated extracellular signal-regulated kinase 1/2 (p-Erk1/2) and Erk1/2 and mouse monoclonal to Thr^{389} phosphorylated p70S6K (p-p70S6K) and p70S6K were all from Cell Signaling Technology. Phycoerythrin-conjugated mouse monoclonal to CD33 or CD71 was purchased from Miltenyi Biotec GmbH. Secondary antibodies were FITC-conjugated $F(ab')_2$ fragment of sheep anti-mouse IgG and FITC-conjugated $F(ab')_2$ fragment of goat anti-rabbit IgG (both from Sigma-Aldrich). Anti-β-actin, horseradish peroxidase (HRP)–conjugated antirabbit IgG, HRP-conjugated antimouse IgG, and the Phototope-HRP Western blot Detection System were from Cell Signaling Technology.

Patient characteristics and isolation of mononuclear cells from bone marrow samples. Bone marrow samples were obtained from 20 patients with MDS and from healthy donors who had given informed consent in accordance with institutional guidelines. All the samples were from the Institute of Hematology and Medical Oncology "L. e A. Seràgnoli" of the Policlinico S. Orsola-Malpighi (Bologna, Italy). In all the subjects participating in this study, the diagnosis was defined according to the French American British classification (23), whereas the International Prognostic Scoring System (IPSS; ref. 24) was used to divide the patients into two categories (low- and high-risk MDS). For *in vitro* experiments, bone marrow mononuclear cells (BMMC) were isolated by Ficoll-Paque (Amersham Biosciences) density gradient centrifugation.

Tissue cell cultures. Human T-lymphoblastoid CEM cells and HL60 AML cells were cultured at 37°C with 5% CO_2 in RPMI 1640 (Cambrex BioScience) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and streptomycin/penicillin at an optimal cell density of 0.3×10^6 to 0.8×10^6 cells/mL.

DNA extraction and mutation analysis. Genomic DNA was isolated from total BMMCs by using the QIAamp DNA Blood Mini kit (Qiagen Ltd.) according to the manufacturer's instructions. Then, the DNA samples were sequenced, as described previously (25), to investigate the presence of mutations in the exons 9 and 20 of the PI3K *p110α* subunit gene.

Western blot. Equal number of cells (4×10^6) from CEM and HL60 cells were collected by centrifugation and resuspended in M-PER Extraction reagent (Pierce) according to the manufacturer's protocol. The protein content was quantified using a bicinchoninic acid protein assay (Pierce) and equal protein amounts (75 μg) were separated by SDS-PAGE as described elsewhere (22).

Immunocytochemistry analysis. Freshly isolated BMMCs were collected by centrifugation at a density of 0.3×10^6 cells/mL and immunostaining analysis was done as described previously (22). Slides were incubated with a mixture containing 4′,6-diamidino-2-phenylindole (DAPI) as a counterstaining for nuclei and antifade as a mounting solution (DAPI/antifade, Resnova). Finally, slides were examined under epifluorescent illumination. Images were taken on a Zeiss Axio Imager.Z1 microscope, with 60×/NA 1.40 optics, coupled to a computer-driven Zeiss AxioCAM digital camera (MRm), using the Zeiss AxioVision (version 4.5) software and the Zeiss colocalization module with constant settings of exposure. For quantification of immunoreactivity, at least 50 to 100 cells per slide were counted.

$CD33^+$ and $CD34^+$ cell immunomagnetic positive selection. $CD33^+$ or $CD34^+$ cells were obtained from total BMMCs after immunomagnetic separation using either the CD33 mini-MACS selection kit or the CD34 Micro Beads kit (both from Miltenyi Biotec) according to the manufacturer's instructions.

Flow cytometric analysis of apoptotic cell death. For sub-G_1 (apoptotic cells) peak analysis, $CD33^+$ and $CD33^-$ cells were cultured in EGM-2 BulletKit medium (Cambrex BioScience) for 48 h with rapamycin (Sigma-Aldrich) or for 24 h with LY294002 (Sigma-Aldrich). Then, the cells were harvested by centrifugation and prepared as described previously (22). The subdiploid DNA content was evaluated using an Epics XL flow cytometer with the appropriate software (System II, Beckman Coulter). At least 10,000 events per sample were acquired. Results were statistically analyzed by GraphPad Prism software (version 3.0).

Clonogenic assays. Fresh $CD34^+$ MDS cells were resuspended in Iscove's modified Dulbecco's medium supplemented with 2% FBS at a concentration of 8×10^3 cells/mL and added to the methylcellulose complete medium (MethoCult GF^+ H4535, Stem Cell Technologies). Cells were then plated in 35-mm Petri dishes in the presence of rapamycin or LY294002 and incubated in a humidified CO_2 incubator (5% CO_2, 37°C) for 14 days. Colonies (>50 cells) and clusters (<50 cells) were then evaluated according to Nissen-Druwey's methods (26), scored under an inverted microscope, and statistically analyzed by GraphPad Prism software (version 3.0).

Results

Patient characteristics. BMMC fractions from 20 patients diagnosed with MDS were examined in this study. Patient demographics and disease characteristics are summarized in Table 1. Median age was 70.1 years (range, 53–79 years). MDS patients were classified according to the IPSS (24), with four subgroups showing different clinical outcomes: low risk, intermediate-1 risk, intermediate-2 risk, and high risk. In our study, low risk and intermediate-1 risk were grouped as low-risk MDS (n = 5), whereas intermediate-2 risk and high risk were grouped as high-risk MDS (n = 15). Karyotype alterations were present in ~30% of the patients. Five of the high-risk MDS patients evolved into AML.

Specificity of the antibodies. Because in most MDS cases the number of cells available for analytic purposes represents a limiting factor, we decided to evaluate the activation of the Akt/mTOR pathway in BMMCs by immunocytochemistry. Therefore, a very critical issue concerned the specificity of the antibodies used in our study. For this reason, a series of control experiments

Table 2. The expression of Ser^{473} p-Akt, Ser^{2448} p-mTOR, Ser^{65} p-4E-BP1, and Thr^{389} p-p70S6K in high-risk and low-risk MDS patients

Case	Age	Diagnosis	Karyotype	PI3K mutations	Cells	p-Akt	p-mTOR	p-p4E-BP1	p-p70S6K	p-Erk1/2
1	63	High-risk MDS	46,XY	None	BMMCs	3	3	3	4	1
2	63	High-risk MDS	54,XYY complex	None	BMMCs	4	3	3	4	2
3	72	High-risk MDS	47,XY (+8)	None	BMMCs	3	3	4	5	1
4	70	High-risk MDS	46,XX	None	BMMCs	2	3	4	5	2
5	70	Low-risk MDS	46,XY	None	BMMCs	1	1	1	1	3
6	76	Low-risk MDS	46,XY	None	BMMCs	1	1	2	1	3
7	72	High-risk MDS	46,XY	None	BMMCs	4	5	4	5	2
8	79	High-risk MDS	46,XY complex	None	BMMCs	3	2	3	2	1
9	63	High-risk MDS	46,XY	None	BMMCs	2	3	3	4	2
10*	53	High-risk MDS	45,XX (−7)	None	BMMCs	4	3	4	4	2
11*	69	High-risk MDS	47,XY (+8)	None	BMMCs	5	4	4	5	1
12*	65	High-risk MDS	47,XX (+8)	None	BMMCs	4	4	4	4	1
13	72	Low-risk MDS	46,XY	None	BMMCs	1	1	2	1	3
14	79	High-risk MDS	46,XY complex	None	BMMCs	3	3	3	3	2
15	76	Low-risk MDS	46,XY	None	BMMCs	1	1	2	1	4
16*	78	High-risk MDS	46,XY	None	BMMCs	3	2	2	2	1
17	77	Low-risk MDS	46,XY	None	BMMCs	1	1	1	1	4
18	63	High-risk MDS	46,XY	None	BMMCs	3	4	5	5	2
19*	70	High-risk MDS	46,XY	None	BMMCs	5	4	4	5	1
20	72	High-risk MDS	46,XX	None	BMMCs	3	5	4	5	2

*Patients that evolved from MDS to AML. Quantification of immunofluorescence staining intensity: CEM cells (set as 5) were considered as a positive control, whereas HL60 cells were considered as a negative control (set as 0).

were first done to validate the antibodies used in this investigation, using immunofluorescent staining and Western blot. To this end, we used the human T-lymphoblastoid CEM cell line, having high levels of p-Akt (27), and the HL60 cell line, having none or very low levels of p-Akt (28). About the expression of Akt, CEM cells displayed high levels of Ser^{473} p-Akt, whereas HL60 cells showed very low immunoreactivity toward Ser^{473} p-Akt antibody, as expected (Fig. 1*A*). Control experiments were always done excluding the primary antibody, to verify that the secondary antibody did not result in unspecific immunostaining (data not shown).

Given that a cross-talk between the Akt and Erk1/2 pathway has been shown (29), we also analyzed the activation of Erk1/2 in our cell lines using both immunofluorescence staining and Western blot. Indeed, Akt phosphorylates and down-regulates Raf, which is an upstream activator of the mitogen-activated protein kinase/ERK kinase/Erk1/2 pathway. Accordingly, CEM cells were negative for Thr^{202}/Tyr^{204} p-Erk1/2, whereas HL60 cells showed higher immunoreactivity toward Thr^{202}/Tyr^{204} p-Erk1/2 antibody (Fig. 1*B*).

Control experiments were also done with the other phospho-specific antibodies to Ser^{2448} p-mTOR, Ser^{65} p-4E-BP1, and Thr^{389} p-p70S6K. Immunofluorescence microscopy analysis showed that CEM cells had high levels of these antigens, whereas HL60 had very low levels (Fig. 1*C*). To further validate the results obtained with immunocytochemical staining, we also did Western blot analysis. As shown in Fig. 1*B* and *C*, this technique revealed that CEM cells had an overall activation of the signaling pathway, whereas HL60 displayed absent Akt phosphorylation and extremely low levels of Ser^{2448} p-mTOR, Ser^{65} p-4E-BP1, and Thr^{389} p-p70S6K. All cells analyzed (CEM and HL60) always expressed total Akt, mTOR, 4E-BP1, and p70S6K, as revealed by Western blot (Fig. 1*B* and *C*). Taken together, these results showed the specificity of the antibody used for immunocytochemistry.

Ser^{473} p-Akt levels and Thr^{202}/Tyr^{204} p-Erk1/2 in normal and MDS mononuclear cells. The level of Akt and Erk1/2 activation was investigated in BMMCs from healthy donors and MDS patients. As Fig. 2*A* shows, normal BMMCs and low-risk MDS displayed barely detectable levels of Ser^{473} p-Akt, whereas high-risk MDS showed an activation of Akt. The proportion of p-Akt–positive cells varied for different high-risk MDS cases, with an average of 50% to 70% positive cells per sample. As for Thr^{202}/Tyr^{204} Erk1/2, normal BMMCs and low-risk MDS displayed activation of Erk1/2, whereas high-risk MDS showed much lower levels of p-Erk1/2 (Fig. 2*B*). Results from all patients analyzed by immunocytochemistry ($n = 20$) are summarized in Table 2. Overall, these results confirmed our own previous findings (22).

mTOR and its downstream targets are activated in high-risk MDS BMMCs. The levels of $Ser2^{448}$ p-mTOR, Thr^{389} p-p70S6K, and Ser^{65} p-4E-BP1 were then analyzed by immunocytochemistry in healthy donor BMMCs and compared with those of BMMCs from high- and low-risk MDS patients. High-risk MDS patients always showed enhanced levels of these phosphorylated proteins compared with either low-risk MDS cells or healthy donors (Fig. 2*C*). Results from all patients ($n = 20$) analyzed by immunocytochemistry for these antigens are summarized in Table 2. In agreement with the p-mTOR capability of targeting both p-p70S6K and p-4E-BP1, high-risk MDS patients who showed high levels of p-mTOR (p-mTOR ≥ 3) also displayed enhanced levels of both p-p70S6K and p-4E-BP1. On the contrary, patients with low levels of p-mTOR ($0 \leq$ p-mTOR ≤ 2) showed reduced levels of both p-p70S6K and p-4E-BP1.

Lineage identity of p-Akt– and p-mTOR–positive cells in high-risk MDS patients. Lineage identity of cells presenting a positive staining toward p-Akt and p-mTOR was established by double immunolabeling total BMMCs with a myeloid-specific marker, CD33 (30). Furthermore, to evaluate the lineage specificity of the cells, we tested another marker, CD71, which is specific for the erythroid lineage (31). As shown in Fig. 3, BMMCs from high-risk MDS patients, which were positive for CD33, displayed a high immunoreactivity also toward Ser^{473} p-Akt and Ser^{2448} p-mTOR. On the contrary, cells that were positive for CD71 showed low levels of p-Akt and p-mTOR.

Rapamycin increases apoptotic cell death in $CD33^+$ cells from high-risk MDS patients. $CD33^+$ and $CD33^-$ fractions were purified from total BMMCs and treated for 48 h with rapamycin, to inhibit the activation of the mTOR pathway, or for 24 h with LY294002, which selectively inhibits PI3K. Then, apoptosis was quantified by flow cytometric analysis. Under basal conditions, the percentage of apoptotic cells in control samples was higher in healthy donors and low-risk MDS compared with high-risk MDS patients, as expected. However, on treatment with rapamycin, healthy donors and low-risk MDS patients showed little variations in the percentage of apoptotic cells, in either the $CD33^+$ or $CD33^-$ fraction. On the contrary, high-risk MDS cells were much more sensitive to rapamycin, displaying a significant increase in apoptotic cells in the $CD33^+$ fraction, whereas $CD33^-$ cells were much less responsive to the treatment (Fig. 4*A*). Because a recent study (32) evidenced that rapamycin could result in additional Akt activation through a feedback mechanism, we also analyzed the levels of p-Akt in BMMCs from high-risk MDS patients after treatment with rapamycin. However, under our experimental conditions, the inhibition of mTOR did not result in further Akt activation (Fig. 4*B*). In high-risk MDS cases, LY294002 did not significantly change the number of apoptotic cells, whereas it induced apoptosis in CEM cells when used at the same concentrations (Fig. 4*C*). Considering that LY294002 did not significantly increased the percentage of apoptotic $CD33^+$ cells, we investigated whether the *p110α* catalytic subunit gene of PI3K displayed activating mutations in MDS patients. Indeed, activating mutations of this gene have been discovered in a wide variety of cancers (33, 34). However, we did not find any genomic mutations in all the MDS patients analyzed (Table 2).

Clonogenic assays. The effect of rapamycin or LY294002 on the clonogenic capability of $CD34^+$ cells from high-risk MDS patients was investigated next. MDS cells were plated in methylcellulose medium with increasing concentrations of rapamycin or LY294002 and colonies were scored after 14 days of culture. In accordance with Nissen-Druey's observations (26), high-risk MDS cells originated only few small colonies, characterized by macrophage aggregates and small eosinophilic colonies. In contrast, $CD34^+$ cells from healthy donors showed a normal differentiation, in that every type of lineage (erythroid, lymphoid, and myeloid) could be visible after 14 days of culture. Overall, rapamycin did not influence the clonogenic capability of $CD34^+$ cells from healthy donors nor from low-risk MDS patients (Fig. 5*A* and *B*). In fact, the number of colonies in healthy donors was not affected by the drug, whereas low-risk MDS displayed a slight but nonsignificant decrease in the percentage of colony growth after treatment with rapamycin compared with healthy donors. In contrast, the size and number of colonies from high-risk MDS patients were significantly inhibited by rapamycin, in a dose-dependent manner (Fig. 5*C*). About the effects of LY294002, the drug did not significantly reduced the growth of colonies in high-risk MDS patients (Fig. 5*D*).

Discussion

Several lines of evidence indicate that the PI3K/Akt signaling pathway plays an important role in both cell proliferation and apoptosis, and its activation has frequently been linked with tumor progression. In particular, this signal transduction network could be involved in leukemogenesis (8–10), as several reports showed that the PI3K/Akt activation protects AML blasts from undergoing apoptosis (11–13). Furthermore, recent studies have evidenced that the evaluation of either Akt phosphorylation status (35) or PI3K

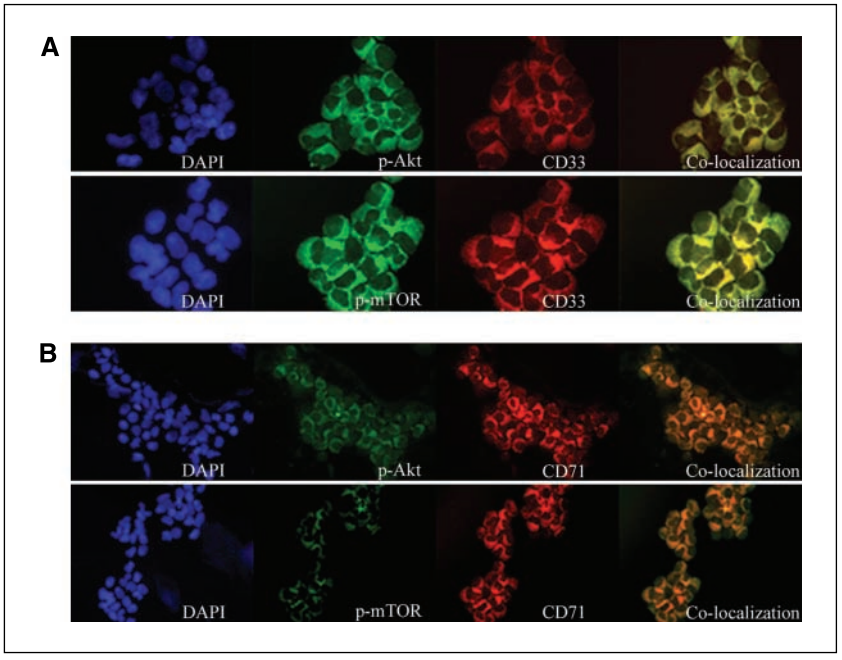

Figure 3. Identification of the lineage identity of BMMCs with p-Akt and p-mTOR activation. Representative immunocytochemical analysis (case 7) of BMMCs from high-risk MDS patients. Original magnification, ×600. Nuclei are visualized by DAPI staining (*blue signal*). *A,* levels of Ser^{473} p-Akt and Ser^{2448} p-mTOR in high-risk MDS (*green signal*). The identification of $CD33^+$ cells was done with a phycoerythrin-conjugated anti-CD33 antibody (*red signal*). The merged image for p-Akt and $CD33^+$ staining indicates colocalization of the two antigens (*yellow signal*). *B,* levels of Ser^{473} p-Akt and Ser^{2448} p-mTOR in high-risk MDS (*green signal*). The identification of $CD71^+$ cells was done with a phycoerythrin-conjugated anti-CD71 antibody (*red signal*). The merged image for p-mTOR and $CD71^+$ staining indicates colocalization of the two antigens (*orange signal*).

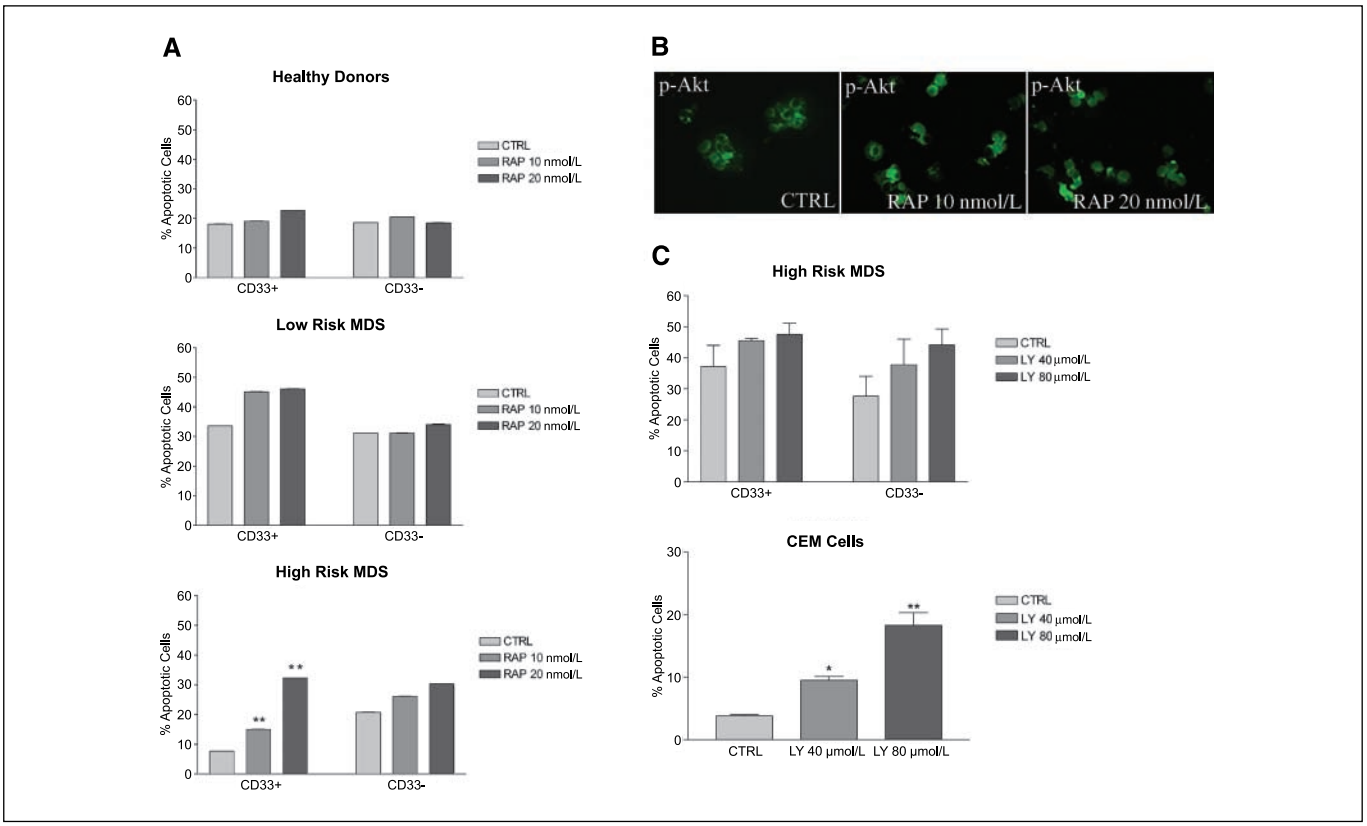

Figure 4. Rapamycin, but not LY294002, selectively increases apoptotic cell death in the CD33$^+$ fraction from high-risk MDS patients. *A,* flow cytometric detection of apoptotic cells in CD33$^+$ and CD33$^-$ fractions of healthy donors and low-risk MDS and high-risk MDS, after treatment for 48 h with increasing concentrations of rapamycin (*RAP*; 0, 10, and 20 nmol/L). No significant difference was seen in the percentage of apoptotic cells in healthy donors or low-risk MDS after treatment with rapamycin. As for high-risk MDS, the percentage of apoptotic CD33$^+$ cells was significantly increased after rapamycin treatment, whereas the CD33$^-$ fraction was not significantly affected. Histograms are representative of three independent experiments. *Columns,* % apoptotic cells; *bars,* SD. *, $P < 0.05$ versus control cells (*CTRL*); **, $P < 0.01$ versus control cells (Dunnett test after ANOVA). *B,* representative immunofluorescence analysis of Ser^{473} p-Akt staining in CD33$^+$ cells after treatment with rapamycin; at the concentrations used, there was no increase in the p-Akt levels, indicating that rapamycin does not further activate Akt through a feedback mechanism. Original magnification, ×600. *C,* flow cytometric detection of apoptotic cells from high-risk MDS patients and CEM cells after a 24-h treatment with increasing concentrations of LY294002. No significant difference was seen in the percentage of apoptotic cells neither in CD33$^+$ nor in CD33$^-$ cells from high-risk MDS, whereas CEM cells were responsive to this treatment. Histograms are representative of three independent experiments. *Columns,* % apoptotic cells; *bars,* SD. *, $P < 0.05$ versus control cells; **, $P < 0.01$ versus control cells (Dunnett test after ANOVA).

p110δ expression (36), and the analysis of PI3K *p110α* catalytic subunit gene somatic mutations (33, 34) in AML blasts may function as prognostic markers for studying the disease progression.

The MDS are a group of hematopoietic stem cell disorders characterized by ineffective hematopoiesis and by a high risk of evolution into AML (37). Our recent studies have suggested that lipid-dependent signal transduction pathways could play an essential role in the progression of MDS (22, 38–40), particularly the activation of the PI3K/Akt pathway, which was shown in high-risk MDS patients (22).

In this study, we investigated the functional status of some downstream targets of Akt (i.e., mTOR, 4E-BP1, and p70S6K). By immunocytochemical analysis, we examined the phosphorylation levels of these proteins in MDS BMMCs, using healthy donors as controls for comparison of staining intensity. We found that not only mTOR was activated but also its downstream targets, 4E-BP1 and p70S6K, which are involved in both cell proliferation and cancer progression. Either high- or low-risk MDS patients showed detectable levels of p-mTOR, p-4E-BP1, and p-p70S6K, but the staining intensity was different. Indeed, high-risk MDS patients displayed high levels of p-mTOR and its downstream targets, p-4E-BP1 and p-p70S6K. On the other hand, low-risk MDS patients were only weakly positive for p-mTOR and its targets, as well as for p-Akt, whereas healthy donors were always negative.

To assess the relevance of the Akt/mTOR pathway activation for the survival and proliferation of MDS cells, we used rapamycin, a macrolide that inhibits the mTOR-dependent downstream signaling pathways and is currently used alone or in combination with cyclosporine as an immunosuppressive drug (41–43). Interestingly, over the last few years, rapamycin has also undergone clinical trials (44, 45) for the treatment of AML and other malignant hematologic disorders. Rapamycin does not directly inhibit mTOR but rather binds to its immunophilin, FK506 binding protein 12 (FKBP12). Then, rapamycin/FKBP12 complex binds to mTOR complexed with Raptor and inhibits downstream signaling events.

We isolated CD33$^+$ and CD33$^-$ cells from healthy donors or MDS cells so that the two fractions could be treated with increasing concentrations of rapamycin. Interestingly, the basal levels of apoptosis in CD33$^+$ cells from healthy donors and low-risk MDS was higher than in the same fraction from high-risk MDS patients and did not change in response to rapamycin treatment. On the contrary, untreated CD33$^+$ cells from high-risk MDS patients showed a lower percentage of apoptotic cells, strongly suggesting that in these patients antiapoptotic mechanisms were activated,

and the treatment with rapamycin inhibited at least some of these prosurvival signals, leading to a significant increase in apoptosis. The findings indicating a selective toxicity of rapamycin on $CD33^+$ cells are fully consistent with the fact that the mTOR activation was detected in $CD33^+$ cells but not in $CD71^+$ cells from high-risk MDS patients. This observation seems to indicate that the activation of the Akt/mTOR pathway is selective for the myeloid lineage and could also explain why MDS evolution into erythroleukemia is an exceptional event (31).

Furthermore, we did methylcellulose-based clonogenic assays with $CD34^+$ MDS cells. Our results show that in $CD34^+$ cells from high-risk MDS, rapamycin significantly inhibited the growth of colonies in a dose-dependent manner, whereas the treatment did not influence the clonogenic ability of $CD34^+$ cells from neither healthy donors nor low-risk MDS.

Another important finding of our study is that rapamycin treatment did not result in further activation of Akt as could be expected because it was initially thought that rapamycin would inhibit mTORC1 but not TORC2, and this could result in additional Akt up-regulation (46). Nevertheless, recent results have highlighted that prolonged treatment with rapamycin could also inhibit mTORC2 and, as a consequence, down-regulate Akt phosphorylation (47).

Interestingly, we showed that in high-risk MDS samples, Erk1/2 was not activated, whereas healthy donors and low-risk MDS showed a higher amount of active Erk1/2. This finding underscores a difference between MDS and AML, where both Erk1/2 and Akt are usually strongly activated in the same patients (29). Given that LY294002 was not effective in inducing apoptosis or reducing clonogenic capability, it is conceivable that in high-risk MDS, the activation of Akt is PI3K independent. This hypothesis was also strengthened by the absence of activating genomic mutations in the PI3K *p110α* subunit gene, which have not been found in our MDS patients. Moreover, our preliminary results showed that a selective inhibitor for PI3Kδ (48), which was highly effective in AML patients to inhibit cell proliferation, had no effect in our high-risk MDS samples. This is another difference between MDS and most AML cases, as far as activation of PI3K/Akt is concerned, although PI3K-independent Akt up-regulation has been reported in a few AML cases (9).

It might be that Akt activation in MDS samples is related to protein kinase C-β, which has been shown to represent a potential alternative pathway for Akt up-regulation in both chronic lymphocytic leukemia (49) and multiple myeloma (50). Alternatively, Akt up-regulation in MDS might be due to decreased activity of protein phosphatases acting on phosphorylated Akt forms (9).

Taken together, our results show that in high-risk MDS patients, the Akt/mTOR pathway is overactivated and that this leads to an imbalance in the apoptotic processes. Therefore, this survival network is likely to play an important role in the MDS pathogenesis and contribute to the malignant growth of MDS cells. Furthermore, our findings indicate that the mTOR pathway is specifically up-regulated in the hematopoietic myeloid progenitors of high-risk MDS patients. In fact, rapamycin, but not LY294002, influenced the clonogenic ability of $CD34^+$ MDS cells by reducing the size and number of the colonies. It is interesting that in a very recent pilot

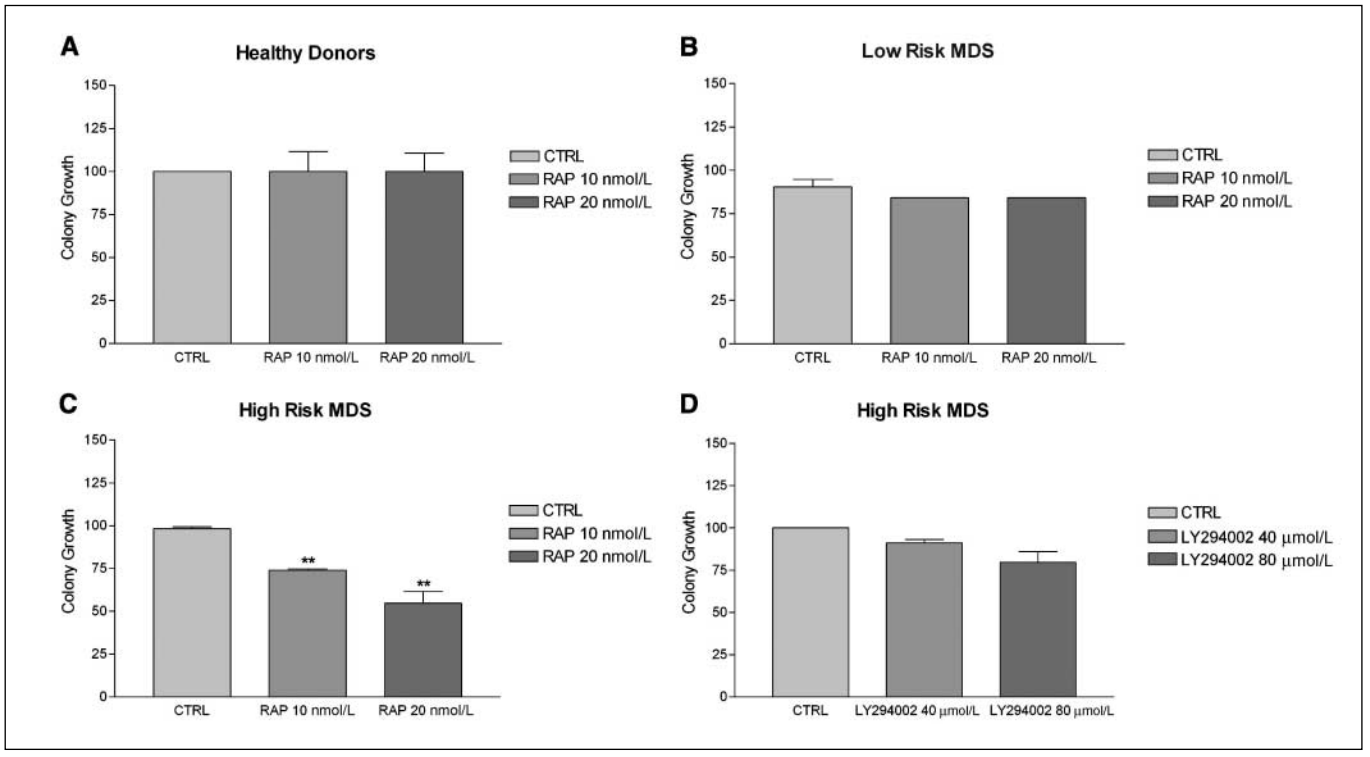

Figure 5. Rapamycin, but not LY294002, negatively affects the clonogenic capability of $CD34^+$ cells from high-risk MDS patients. High- and low-risk MDS and healthy donor $CD34^+$ cells were incubated in the appropriate medium in the presence of increasing concentrations of rapamycin or LY294002. The colonies (>50 cells) were scored at day 14. *A,* healthy donors: rapamycin did not influence the colony growth. *B,* low-risk MDS patients: rapamycin slightly reduced the number and size of the colonies, but the differences were not statistically significant. *C,* high-risk MDS: rapamycin significantly inhibited both the number and the size of the colonies. *D,* high-risk MDS: LY294002 reduced slightly, but not significantly, the number and the size of the colonies. Results are percentage of control. *Columns,* mean of duplicates from three independent experiments; *bars,* SD. **, $P < 0.01$ versus control cells (Dunnett test after ANOVA).

study, sirolimus (rapamycin) was used with some success in advanced MDS patients (44). However, in that investigation, no functional evaluation of the Akt/mTOR axis was done. Therefore, we feel that our data are complementary to those findings and strengthen the concept that the Akt/mTOR axis could become in the future an important target for the development of innovative strategies for the MDS treatment.

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