Coexpression of hyperactivated AKT1 with additional genes activated in leukemia drives hematopoietic progenitor cells to cell cycle block and apoptosis

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The phosphatidylinositol 3-kinase/AKT pathway is an integral component of signaling involved in the development of many cancers, including myeloid leukemias such as chronic myeloid leukemia and acute myeloid leukemia (AML). Increased AKT1 activity is frequently seen in AML patients, providing leukemic cells with growth and survival promoting signals. An important aspect of AKT1 function is its involvement in cellular metabolism and energy production. Under some circumstances, strong activation of AKT1 increases oxidative stress, which can cause apoptosis when cells progressively build up excess free radicals. This has been described in hematopoietic cells overexpressing activated AKT1; however, whether this is true in cells coexpressing other genetic events involved in leukemia is not known. This prompted us to investigate the effect of constitutively active AKT1 (myristoylated AKT1) in hematopoietic progenitor cells expressing constitutively active signal transducer and activator of transcription 5, Fms-related tyrosine kinase 3–internal tandem duplication, or antiapoptotic B-cell lymphoma 2. Surprisingly, myristoylated AKT1 was incompatible with proliferation driven by both signal transducer and activator of transcription 5 and Fms-related tyrosine kinase 3–internal tandem duplication, which triggered cell cycle block and apoptosis. Moreover, transplantable cells of B-cell lymphoma 2–transgenic mice were impaired in their engraftment ability to recipient mice when expressing hyperactivated AKT1. This was linked to AKT1-mediated proapoptotic functions and not to impairment in homing to the bone marrow. Although cells expressing hyperactivated AKT1 displayed higher levels of reactive oxygen species both in vitro and in vivo, the addition of the antioxidant N-acetyl-L-cysteine significantly reduced apoptosis. Taken together, the results indicate that constitutive AKT1 activity is incompatible with growth- and survival-promoting ability of other activated genes in AML. Copyright © 2015 ISEH - International Society for Experimental Hematology. Published by Elsevier Inc.

Aberrant signal transduction enhances survival and proliferation of hematopoietic progenitor cells in acute myeloid leukemia (AML). This occurs through various genetic alterations affecting signaling, as in Fms-like tyrosine kinase 3 (FLT3). Constitutive activation of signaling proteins is frequently demonstrated in AML, involving major signaling cascades such as the mitogen-activated protein kinase pathway, the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, and signal transducer and activator of transcription 5 (STAT5). Because these mutant signaling proteins are frequently present in AML, they have developed as attractive therapeutic targets.

Phosphatidylinositol 3-kinase is a major signaling protein involved in cell growth, survival, and metabolism. Many of the effects are mediated by the serine/threonine kinase AKT (protein kinase B [PKB]) downstream of PI3K. The AKT family comprises AKT1, AKT2, and AKT3 (PKBz, β, and γ), which are mostly redundant in function. AKT1 is more ubiquitously expressed, whereas AKT2 and AKT3 are restricted to certain tissues. In hematopoietic cells, both AKT1 and AKT2 share important functions, although AKT1 seems to be more important in signaling.
AKT activation inhibits apoptosis through a variety of mechanisms, including phosphorylation and inhibition of glycogen synthase kinase 3, the proapoptotic B-cell lymphoma 2 (Bcl-2) family member Bad, caspase 9, and members of the FoxO subfamily of forkhead transcription factors [1].

Dysregulation of the PI3K/AKT pathway has been implicated in many human cancers. The p110α catalytic subunit of PI3K encoded by PIK3CA is one of the most frequently mutated genes in human cancers and promotes carcinogenesis by activation of AKT1 [2]. Mutations in the Akt1 gene have been detected in some tumors as an alternative pathway of transformation [3,4]. Contrarily, studies of AML indicate no recurrent imbalanced regions within the PIK3CA or Akt1 genes [5–7]. Despite the lack of evidence for activating mutations of Akt1, elevated levels of phosphorylated Akt1 have been observed in primary blast cells of most patients [8–10]. The cause of this AKT1 activation remains elusive but appears linked to upstream signaling events or crosstalk to other pathways. Mutations in the FLT3 gene are among the most common genetic alterations found in AML patients. Constitutively activated FLT3 occurs most often as internal tandem duplications (ITDs) within the juxtamembrane domain and/or the first kinase domain and is observed in approximately 20–25% of all AML patients [11,12]. Mutations in FLT3-ITD are associated with activation of the PI3K/AKT pathway similar to wild-type (WT) FLT3 [13,14]. In contrast, STAT5 is phosphorylated by FLT3-ITD and not WT FLT3, which is required for transformation in vivo [15,16].

The importance of phosphorylated STAT5 for hematopoietic malignancies is underlined by observations in lymphoid and myeloid leukemias that have constitutive STAT5 phosphorylation. The introduction of constitutively active STAT5 (caSTAT5) mutants into murine hematopoietic cells suffices to induce multilineage leukemia in mice [17]. In contrast, AKT1 overexpression or constitutive active forms of AKT1 are unable to transform hematopoietic cells in vitro [18], but may, in combination with other pathways, mediate transformation. When activated AKT1 was expressed in a murine bone marrow (BM) transplantation model, recipient mice developed myeloproliferative disease, T-cell lymphoma, or AML [19]. Analysis of hematopoietic stem cells (HSCs) in these mice revealed increased cycling and apoptosis of the transplanted cells, as well as impaired engraftment, implying that HSCs are sensitive to excess AKT signaling.

It was recently reported that permanent AKT1 activation can lead to increased oxidative stress that renders cells susceptible to reactive oxygen species (ROS)-triggered apoptosis [20,21]. In these studies, AKT1 was overexpressed without the presence of other genetic alterations such as would be expected in primary leukemic cells. This raises questions about the function and involvement of activated AKT1 in leukemogenesis when coexpressed with other genes involved in leukemia. Herein, we present evidence that constitutively activated AKT1 (myristoylated AKT1 [myrAKT1]) forces hematopoietic progenitors into a quiescent state of cell cycle and induces apoptosis when coexpressed with caSTAT5, FLT3-ITD mutation, or antipoptotic Bcl-2. Transplanted cells from Bcl-2-transgenic mice expressing constitutively active AKT1 were impaired in engraftment of recipient mice. Although cells expressing active AKT1 displayed higher levels of ROS, the addition of the antioxidant N-acetyl-L-cysteine (NAC) significantly reduced apoptosis. These findings suggest that increased oxidative stress by hyperactivated AKT1 is detrimental to hematopoietic cells. Thus, it is possible that enhancing AKT1 activity could lead to strategies in eradicating leukemic cells, even in the presence of other oncogenes or activated pathways.

Materials and methods

Mice

C57BL/6/J (WT; CD45.2), B6.SJL (CD45.1), or C57BL/6/J-vav-Bcl-2 (Bcl-2) mice [22] were housed in the Animal Facility at Linköping University (Linköping, Sweden). Bone marrow of FLT3-ITD+/– mice (B6.129-Flt3tm1Dgg/J) [23] was provided by Ewa Sitnicka (Lund, Sweden). The study was approved by the Animal Ethics Committee at Linköping University and carried out in accordance with Swedish legislation for care and use of laboratory animals.

Cell lines and Western blots

Ba/F3 cells were cultured in Roswell Park Memorial Institute 1640 medium (PAA Laboratories, Les Mureaux, France) with 10% fetal calf serum (FCS; Hyclone, Logan, UT), 25 nmol/L Hepes, and 50 μmol/L 2-mercaptopethanol (Sigma-Aldrich, St Louis, MO), whereas FDCP-1 cells were cultured in Iscove’s modified Dulbecco’s medium (IMDM; PAA Laboratories) with 10% FCS and 2 mmol/L L-glutamine. Both cell lines were supplemented with 1% penicillin-streptomycin and 5% interleukin (IL)-3-containing supernatant when routinely maintained. Western blots were done with pAKT1 (Ser 473) and total AKT (Cell Signaling Technology, Danvers, MA).

Isolation and culture of c-kit+ cells

Mononuclear BM cells were harvested by crushing femurs and tibiae from 8–14-week-old mice. Magnetic-activated cell sorting immunomagnetic cell separation using magnetically labeled anti-CD117 beads (Miltenyi, Cologne, Germany) was used to isolate c-kit+ cells. After selection, cells were cultured in IMDM supplemented with 20% FCS, 10 U/mL penicillin-streptomycin, 50 ng/mL murine stem cell factor (SCF), human IL-6, and human thrombopoietin (TPO; all from Peprotech, Rocky Hill, NJ) before retroviral infections.

Retroviral infections

Transduction with caSTAT5 and/or myrAKT were done by spinoculation with retrovirus supernatants of pMX-puro-caSTATS [24] and pLZRS-myrAKT1-EGFP [21]. As controls, empty vectors...
were used. Retroviral supernatants were produced by transient transfection of 293T cells using calcium phosphate coprecipitation with 4 μg cDNA. Cells were plated in 3.5-cm dishes, and 2.5 mL IMDM was refreshed 18 hours after transfection. After an additional 24 and 48 hours, viral supernatants were collected, pooled, and filtered through a 0.45-μm filter. Transduction was performed by spinoculation for 90 min at 1,800 g cDNA. Cells were plated in 3.5-cm dishes, and 2.5 mL IMDM supplemented with 20% FCS; 50 mg/mL each SCF, IL6, and TPO; and 5 μg/mL polybrene (American Bioanalytical, Natick, MA). When c-kit+ cells of FLT3-ITD mice were used, IL-6 was substituted by human FLT3 ligand (FL). Three days posttransduction, green fluorescent protein (GFP)-positive cells were sorted on FACS Aria (BD Biosciences, San Jose CA) and cultured in IMDM with 20% FCS in the presence or absence of cytokines as indicated.

**Phosflow analysis**

Cells were stimulated with IL-3 (50 ng/mL) for 30 min, then fixed with 1% paraformaldehyde (EMS, Hatfield, PA) for 10 min and permeabilized using 0.3% saponin (Sigma-Aldrich) in phosphate-buffered saline (PBS) for 45 min on ice, followed by incubation with ice-cold methanol for 30 min on ice. After washing, cells were stained with anti-phospho-AKT antibody (pS473, BD Biosciences) and conjugated with Alexa-647 for flow cytometry on FACS Canto (BD Biosciences). Acquired data were analyzed on Cytobank (Fluidigm, San Francisco, CA).

**Cell-cycle analysis**

Cultured cells were fixed with Cytofix/Cytoperm (BD Biosciences) for 30 min at 4°C, followed by washing twice with Perm/Wash (BD Biosciences) buffer. After washing, cells were stained with phycoerythrin (PE)-conjugated anti-Ki67 antibody (Sigma-Aldrich) and 4,6-diamino-2-phenylindole (0.5 μg/mL; Sigma-Aldrich). Cell-cycle status was determined using 4,6-diamino-2-phenylindole versus Ki-67 on FACSAria SORP (BD Biosciences), and the amount of cells in G0/G1 and S-phase was calculated using FlowJo software (TreeStar, Ashland, OR).

**Apoptosis assays**

For apoptosis measurements, cells were collected at the indicated time points, washed with cold PBS +5% FCS twice, resuspended in Annexin-V binding buffer, and incubated with Annexin V-allophycocyanine and 7-aminoactinomycin D (7-AAD; 5 μg/mL; both from BD Biosciences) at room temperature for 15 min. Cells were analyzed on FACS Canto (BD Biosciences) within 1 hour. Acquired data were analyzed by FlowJo software.

**Transplantations**

Before transplantation, 8–12-week-old CD45.1 mice were lethally irradiated with 9 Gy. For intravenous transplantation, recipient mice received 10,000 sorted GFP+ cells of CD45.2 mice via tail vein injections in 200 μL. For intraperitoneal transplantations, after subcutaneous injection with Tengemiscin (0.1 mg/kg), a hole was drilled of the right femur by a 30-gauge needle (BD Biosciences), and 5,000 GFP+ cells were injected directly in the BM cavity with a Hamilton syringe with a 31-gauge needle (Hamilton Bonaduz AG, Bonaduz, Switzerland) in 10 μL. After transplantation, mice were maintained under sterile conditions in microisolater cages and provided with autoclaved food and water containing 111 mg/L ciprofloxacin (Ciprofaxin; Fresenius Kabi, Uppsala, Sweden). Peripheral blood was collected by lateral tail vein bleeding and analyzed 1, 2, 4, 8, or 12 weeks posttransplant, depending on the experiment, by tracking CD45.1, CD45.2, and GFP expression on FACS Canto using anti-CD45.1-PE and anti-CD45.2-PE–cyamine 7 (BioLegend, San Diego, CA). Erythrocytes were lysed with ammonium chloride (Stem Cell Technologies, Vancouver Canada) and the leukocytes resuspended in PBS with 5% FCS. Data were analyzed using FlowJo software.

**Measurements of reactive oxygen species**

Reactive oxygen species were measured by flow cytometry, as indicated by the manufacturers, using either 10 μmol/L 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA; Molecular Probes, Eugene, OR) or CellROX Deep Red Flow Cytometry assay kit (Life Technologies, Waltham, MA). We inhibited ROS activity by the addition of NAC (Sigma-Aldrich).

**Statistical analysis**

Statistical testing was performed using InStat software (Graphpad Software, San Diego, CA). Experiments performed with sorted GFP+ cells were done at least in triplicate. Data were expressed as mean values and SDs. Statistical significance of differences observed between groups were determined by Student’s t test.

**Results**

**Constitutively active AKT1 is incompatible with STAT5-driven proliferation**

To elucidate whether hyperactivated AKT1 causes apoptosis in hematopoietic cells coexpressing additional oncogenes present in primary AML cells, we overexpressed myrAKT together with caSTAT5, mutated FLT3-ITD, or antiapoptotic Bcl-2 in hematopoietic progenitors. In leukemia, STAT5 is an indispensable downstream target, and cells transformed by bcr-abl [25,26] or FLT3-ITD [16,27,28] are addicted to STAT5 for maintaining the leukemic state. In this study, we have used myristoylated AKT1 (myrAKT) to mimic constitutively activated AKT1. When fused to the Src myristylation signal, AKT1 is targeted to the cell membrane, where it becomes activated and phosphorylated [29], as demonstrated in the hematopoietic progenitor cell line FDCP-1 (Fig. 1A).

We transduced cells by retroviral gene transfer with myrAKT1 and/or caSTAT5 into two IL-3-dependent hematopoietic progenitor cell lines: Ba/F3 pro B-cells and myeloid FDCP-1 cells. In agreement with previous reports [30,31], myrAKT1 was insufficient for cell growth and survival of Ba/F3 cells deprived of cytokines, whereas caSTAT5 sustained proliferation and cells increased in numbers during the 5 days analyzed (Fig. 1A). By contrast, hyperactivated AKT1 disabled STAT5-mediated proliferation (Fig. 1B). This was also true in FDCP-1 (Fig. 1C). To generate high numbers of transduced cells for analysis, FDCP-1 cells were first selected in puromycin in the presence of IL-3 for 4 days, then tested for effects of caSTAT5 and myrAKT1 after IL-3 starvation. We observed the same effect of myrAKT1
as with BaF3; thus, hyperactivated AKT1 suppressed STAT5-mediated proliferation and cell viability. These results are surprising, since previous studies have implied that both AKT1 and STAT5 can be simultaneously activated in leukemic cells, for instance via FLT3-ITD [32,33].

Constitutively active AKT1 induces cell-cycle block and apoptosis of FLT3-ITD-expressing bone marrow progenitor cells

Bearing in mind that AKT1 and STAT5 are downstream targets of FLT3-ITD, but that myrAKT1 was incompatible with caSTAT5-driven proliferation, we decided to study the consequence of overexpressing myrAKT1 in hematopoietic progenitors from BM of FLT3-ITD-transgenic mice. We first determined whether myrAKT1 was phosphorylated in transduced cells. Western blot (Fig. 2A) and Phosflow analyses (Fig. 2B) of myrAKT1-transduced cells showed that AKT1 was phosphorylated in the absence of external stimulus as strongly as endogenous AKT1 protein in IL-3-stimulated control cells (WT/Puro). Expression of FLT3-ITD in progenitor cells induced constitutive AKT1 phosphorylation, and coexpression of myrAKT led to even stronger phosphorylation (Figs. 2A and 2B).

Next, we transduced c-kit+ BM progenitor cells from FLT3-ITD-transgenic mice with myrAKT1/GFP in the presence of cytokines (SCF+FL or SCF+TPO+FL). Two days after infection, GFP+ cells were sorted with fluorescence-activated cell sorting (FACS), recultured with or without cytokines, and analyzed for cell-cycle status and Annexin V/7-AAD staining for apoptosis. In the absence of cytokines, the proportion of cells in G0 from WT mice after 48 hours increased to 46.6% when expressing myrAKT1 compared with control GFP cells, of which only 18.8% cells were in G0 (Fig. 2C). This proportion increased further to 66.4% when myrAKT1 was expressed in progenitor cells of FLT3-ITD mice. Surprisingly, the same effect was also seen when cells were cultured with cytokines. In this case, the numbers of c-kit+ progenitors in G0 after stimulation with SCF+FL increased to 53.1% in FLT3-ITD cells expressing myrAKT1, compared with 22.8% in control GFP-infected cells. Similarly, myrAKT1-infected cells of FLT3-ITD mice cultured in SCF+TPO+FL accumulated in G0 to 59.7%, compared with 31.9% for control cells. With some delay (72 hours), the numbers of apoptotic cells, detected by Annexin V/7-AAD staining of cells, increased to some extent (20–25%) in cultures of cells expressing myrAKT1 compared with GFP-infected cells (Fig. 2D), indicating that AKT1-driven cell-cycle exit and apoptosis are not abrogated by the strong prosurvival and proliferative activity of FLT3-ITD.

Hyperactivated AKT1 induces apoptosis in progenitor cells expressing B-cell lymphoma 2

In FLT3-ITD+ cell lines and primary cells from AML patients, we observed elevated levels of the antiapoptotic Bcl-2 family member myeloid cell leukemia-1 (MCL-1). Furthermore, MCL-1 is dependent on FLT3-ITD signaling, because the expression is reversible upon pharmacologic inhibition of FLT3 activity by tyrosine kinase inhibitors.
Recently, it was observed that FLT3-ITD-dependent upregulation of MCL-1 was linked to STAT5\(^{[28]}\). Considering our results that myrAKT1 interferes with STAT5- and FLT3-ITD-driven proliferation, we decided to coexpress myrAKT1 with antiapoptotic Bcl-2. Progenitor cells from BM of Bcl-2 mice were transduced with myrAKT1/GFP, and, after sorting GFP\(^+\) cells, cell cycle and apoptosis were analyzed over 72 hours. After 24 hours, Bcl-2 cells coexpressing myrAKT1 accumulated to a higher extent in G\(_0\) of cell cycle when grown without cytokines compared with cells infected with control virus (31.4\% vs. 17.6\%; \(n = 3\); Fig. 3A). This was followed by increased numbers of apoptotic cells, as measured by Annexin-V binding and 7-AAD staining, after 72 hours, with 22.0\% for WT/GFP, 53.5\% for WT/myrAKT1, 30.9\% for Bcl-2/GFP, and 54.9\% for Bcl-2/myrAKT1 (\(n = 3\); Fig. 3B). One representative experiment for detection of apoptotic cells, as measured by Annexin-V binding and 7-AAD staining, is shown in Figure 3C. The results demonstrate that Bcl-2 fails to rescue cell death by hyperactivated AKT1.

Figure 2. myrAKT1 induces cell-cycle exit and apoptosis in hematopoietic bone marrow progenitor cells of FLT3-ITD transgenic mice. (A) Western blot analysis (\(n = 2\)) for pAKT1 (pS473) in WT or FLT3-ITD\(^+\) progenitor cells transduced with myrAKT1 or control retrovirus, either unstimulated or stimulated for 30 min with IL-3 (50 ng/mL), to detect endogenous AKT1 phosphorylation. (B) Detection of AKT1 (pS473)-phosphorylation by Phosflow analysis in the same cells as in (A). One representative experiment out of three is shown. (C, D) BM c-kit\(^+\) cells from C57BL6/J WT control or FLT3-ITD\(^+\)/C0 transgenic mice were cultured overnight before retroviral infections. Forty-eight hours later, GFP\(^+\) cells were cultured in duplicates without cytokines or with SCF+FL or SCF+TPO+FL. (C) After an additional 48 hours, the cell cycle status was analyzed by flow cytometry. Data represent the average number of cells/mL \(\pm\) SD (\(n = 3\)). (D) Flow cytometric analysis of apoptotic cells (Annexin V binding and 7-AAD staining) after 72 hours. Data presented as mean \(\pm\) SD (\(n = 3\)). Statistical analysis was performed using a Student’s \(t\) test. *\(p \leq 0.05\); **\(p \leq 0.01\); ***\(p \leq 0.001\).
Hyperactivated AKT1 impairs engraftment of B-cell lymphoma 2–expressing progenitor cells

Next, we decided to test whether myrAKT1 overexpression has proapoptotic effects in vivo. Recipient mice were transplanted with BM progenitor cells enriched for c-kit expression from WT or Bcl-2-transgenic mice transduced with myrAKT1/GFP or GFP control virus. Forty-eight hours postinfection, $1 \times 10^4$ GFP$^+$ cells were injected to lethally irradiated mice in a noncompetitive setting. Peripheral blood was then analyzed for GFP expression after 4, 8, and 12 weeks. Already after 4 weeks, reconstitution was negatively affected in mice receiving

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**Figure 3.** Activated AKT1 induces cell-cycle exit and increases apoptosis of BM progenitors from Bcl-2 transgenic mice. c-kit$^+$ BM progenitors from WT or Bcl-2 transgenic mice were infected with retrovirus containing control GFP or myrAKT1/GFP. Forty-eight hours later, GFP$^+$ cells were cultured without cytokines (black bars) or with SCF$^+$TPO$^+$IL-6 (white bars). (A) After 24 hours, cell-cycle status was analyzed by flow cytometry. (B) Flow cytometric analysis of apoptotic cells (Annexin V binding and 7-AAD staining) after 72 hours. Data are presented as mean ± SD ($n = 3$). (C) Representative flow cytometry data of cells stained for Annexin V and 7-AAD. Statistical analysis was performed using a Student's t test. **$p \leq 0.01$; ***$p \leq 0.001$. ns = not significant.
Figure 4. Short-term and long-term hematopoietic stem cells coexpressing activated AKT are impaired of engraftment potential even when expressing Bcl-2. (A) Level of engraftment in B6.SJL (CD45.1) recipient mice (n = 5) transplanted with $1 \times 10^4$ c-kit+ BM progenitor cells of C57BL6/J (CD45.2) from WT or Bcl-2-transgenic mice. Cells were infected for 2 days with control GFP or myrAKT1/GFP retrovirus, then FACS sorted for GFP expression before lateral tail injections. The level of reconstitution of donor cells in recipient mice was analyzed for CD45.1, CD45.2, and GFP expression after 4, 8, and 12 weeks by flow cytometry on peripheral blood. Each dot represents one mouse, and the data are presented as mean (horizontal line) ± SD. (B) Survival curves during 85 days posttransplant of mice (n = 10) transplanted with $1 \times 10^4$ c-kit+ BM cells of C57BL6 WT or Bcl-2-transgenic mice infected with control GFP or myrAKT1/GFP retrovirus. (C) Engraftment levels as measured 2 weeks posttransplant for GFP expression in peripheral blood of individual mice (n = 5) transplanted with c-kit+ BM cells of C57BL6 or Bcl-2-transgenic mice infected with control GFP or myrAKT1/GFP retrovirus injected either by intravenous tail injections or intrafemoral injections. Each dot represents one mouse, and the data are presented as means (horizontal line). Statistical analysis was performed using a Student’s t test. **p ≤ 0.01; ***p ≤ 0.001. ns = not significant.
myrAKT1-expressing BM cells. The level of donor-cell reconstitution in peripheral blood was 54.9% for WT/GFP, 17.1% for WT/myrAKT, 67.2% for Bcl-2/GFP, and 25.2% for Bcl-2/myrAKT (Fig. 4A). At longer time points, reconstitution remained high in mice transplanted with GFP control virus but was severely impaired in mice transplanted with myrAKT1-expressing cells (Fig. 4A). Several of these mice succumbed to hematopoietic failure during the first 8 weeks (Fig. 4B).

Impaired engraftment of bone marrow cells expressing hyperactivated AKT1 does not correlate to disturbed homing

Previous studies have demonstrated that AKT1 can regulate trafficking of hematopoietic progenitors by influencing expression of adhesion molecules and homing receptors [21]. Since we could not distinguish whether the effects of myrAKT1 were due to apoptotic induction or the ability of AKT1 to disable homing and/or adhesion of progenitor cells to the BM, we decided to compare intravenous injections to direct delivery to the BM by intrafemoral injections. As seen in Figure 4C, engraftment was impaired when myrAKT1-expressing cells were injected directly to the marrow, demonstrating that hyperactivated AKT1 negatively affected hematopoietic reconstitution, but not by homing defects.

Hyperactivated AKT1 increases the levels of intracellular reactive oxygen species, but antioxidant N-acetyl-L-cysteine protects cells from activated AKT1-induced apoptosis

Previous studies have shown that activation of AKT1 can lead to accumulated levels of intracellular ROS, which could be detrimental to hematopoietic cells [30]. To investigate whether this was the cause of the proapoptotic effects mediated by activated AKT1 in cells expressing FLT3-ITD, STAT5, or Bcl-2, we analyzed ROS levels in cells overexpressing myrAKT1. To measure the ROS levels, cells infected with a puromycin-containing myrAKT1 virus were stained with DCFDA and analyzed by flow cytometry. In cells infected with myrAKT1, the levels of ROS increased significantly 48 hours postselection with puromycin (Fig. 5A). Treatment of infected GFP+ FACS-sorted cells with NAC, a well-known antioxidant, partially prevented apoptosis (Figs. 5B and 5C), suggesting that the detrimental effects by hyperactivated AKT1 are linked to accumulation of increased levels of ROS. This was further confirmed in myrAKT1/GFP-transplanted progenitor cells from WT mice after injection to recipient mice. Seven days posttransplant, GFP+ donor cells in peripheral blood were analyzed for ROS level by staining with CellRox Deep Red and flow cytometry. Cells with myrAKT1 in recipient mice expressed twofold higher ROS level than cells transduced with control virus (Fig. 5D).

Discussion

Despite evidence of elevated levels of phosphorylated AKT1 in primary leukemic blast cells, reoccurring mutations in the Akt1 gene are absent in patients with AML, raising the question of whether permanent AKT1 activation is incompatible with leukemogenesis. As a model to investigate the consequence of hyperactivated AKT1 in hematopoietic cells expressing additional oncogenic events, we used FLT3-ITD signaling, present in approximately one fourth of all AML patients, as a model. We used a myristoylated form of AKT1 that is localized at the cell membrane and becomes constitutively activated where the protein is expected to execute normal signaling functions. Our data demonstrate that myrAKT1 disables STAT5-mediated proliferation of Ba/F3 and FDCP-1 cells. In addition, myrAKT1 had detrimental effects on cell proliferation and induced apoptosis when overexpressed in BM-derived progenitors from FLT3-ITD transgenic mice. Although a cell-cycle block was more evident in cells cultured in the absence of cytokines, FLT3-ITD cells coexpressing myrAKT1 cultured in cytokines displayed significantly more cells in G0 after 24 hours in culture and were approximately 20% more apoptotic after 72 hours. This indicates that AKT1-driven cell-cycle block and apoptosis are not inhibited by FLT3-ITD activity. Although AKT1 is a downstream target of FLT3-ITD, we interpret the results as an overstimulation of cells expressing both FLT3-ITD and hyperactivated AKT1. Previous reports have indicated that AKT1 may facilitate, rather than inhibit, cell death under certain conditions [20,35,36]. However, to our knowledge, our data are novel with regard to the proapoptotic function of AKT1 in the presence of other growth-promoting and antiapoptotic oncogenic events.

There are other examples of oncogenes promoting proliferation that trigger apoptosis when delivering a strong mitogenic signal, such as c-MYC [37]. However, in contrast to the concept that constitutive oncogenic-driven apoptosis is due to cell-cycle entry sensitizing cells to apoptosis, myrAKT1, together with the three oncogenes we have tested (caSTAT5, FLT3-ITD, and Bcl-2), led to cell-cycle block before cell death. The reason for this is presently not known.

Previous reports have indicated that proapoptotic function of hyperactivated AKT1 could be linked to suppression of antioxidant enzymes and an increase in ROS levels [20,38]. In our study, hematopoietic progenitor cells expressing myrAKT1 contained higher levels of ROS both in vitro and in vivo, which was counteracted in vitro by addition of NAC, known as a scavenger of excess levels of ROS. A recent study in mice double deficient in AKT1 and AKT2 indicated that HSCs lacking AKT function persisted in G0 and that the intracellular content of ROS was decreased [39]. This implies that AKT1 regulates ROS for proper HSC maintenance and differentiation. It is also possible that AKT1 hyperactivity has different effects in
Figure 5. Activated AKT1 increases the levels of intracellular ROS, but NAC protects cells from activated AKT1-induced apoptosis. (A) c-kit<sup>+</sup> BM progenitors cells of C57BL6 WT or Bcl-2 transgenic mice were infected with a puromycin-retrovirus-containing myrAKT1 or empty virus. After 2 days of puromycin selection (2.0 μg/mL), cells were analyzed for ROS by flow cytometry. Data are presented as mean ± SD (n = 3; in duplicates) of DCFDA mean fluorescence intensity. (B) c-kit<sup>+</sup> BM progenitors cells of C57BL6 WT or Bcl-2 transgenic mice were infected for 2 days with myrAKT1/GFP retrovirus. GFP<sup>+</sup> cells were recultured with or without 1.0 and 2.0 nmol/L NAC for 72 hours and analyzed for apoptosis (Annexin V binding and 7-AAD staining) by flow cytometry. Data are presented as mean ± SD (n = 3). (C) Representative flow cytometry data of Annexin V/7-AAD binding of myrAKT1/GFP-transduced cells treated with NAC, as in (B). (D) c-kit<sup>+</sup> BM progenitors cells of C57BL6 WT mice were infected with control GFP or myrAKT1/GFP retrovirus and then FACS-sorted for GFP expression before lateral tail injections. ROS level as mean fluorescence intensity was determined in GFP<sup>+</sup> cells from peripheral blood after 1 week by CellRox Deep Red staining and flow cytometry (WT/GFP: 741 ± 112; WT/myrAKT: 1,541 ± 138). Each dot represents one mouse (n = 6), and the data are presented as mean (horizontal line) ± SD. Statistical analysis was performed using a Student’s t test. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001. ns = not significant.
progenitors, driving them toward cell-cycle block due to harmful effects by increased ROS levels. For instance, Ba/F3 cells expressing hyperactivated AKT1 are short-lived upon growth factor deprivation and are induced to apoptosis [30].

The inability of Bcl-2 to prevent myrAKT1-mediated proapoptotic effects is surprising considering previous findings that Bcl-2 is involved as an antioxidant agent to overcome ROS effects [40]. However, in previous studies where AKT1 has been shown to have harmful effects [20,30], AKT1 was expressed as a fusion protein to the estrogen receptor. AKT1 activity was then controlled by tamoxifen treatment where the estrogen receptor fusion protein translocated to the nucleus [30]. In contrast, myrAKT1 mimics the novel Akt1 pleckstrin homology domain mutation (AKT1-E17K) found in solid tumors and is localized at the cell membrane [41]. This could mean that AKT1 executes different functions when nucleus localized versus targeted to the cell membrane.

In summary, several studies suggest that AKT1 is harmful to hematopoietic cells when constitutively expressed. Here we show that hyperactivity of AKT1 is harmful owing to an improper balance of ROS. This could be one reason that activating mutations of Akt1 leading to permanent activation are absent in AML, in contrast to several other cancers. Phosphorylation and activation of AKT1 are under tight regulation where the AKT1 protein is transiently oscillating between active and inactive states [42]. Since activating mutations of FLT3-ITD and other signaling proteins translocated to the nucleus [30]. In contrast, myrAKT1 mimics the novel Akt1 pleckstrin homology domain mutation (AKT1-E17K) found in solid tumors and is localized at the cell membrane [41]. This could mean that AKT1 executes different functions when nucleus localized versus targeted to the cell membrane.

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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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