Cytokine-regulated expression of *survivin* in myeloid leukemia

Bing Z. Carter, Michele Milella, Dario C. Altieri, and Michael Andreeff

***Survivin*, a member of the inhibitors-of-apoptosis gene family, is expressed in a cell-cycle–dependent manner in all the most common cancers but not in normal differentiated adult tissues. *Survivin* expression and regulation were examined in acute myeloid leukemia (AML). *Survivin* was detected by Western blot analysis in all myeloid leukemia cell lines and in 16 of 18 primary AML samples tested. In contrast, normal CD34$^+$ cells and normal peripheral blood mononuclear cells expressed no or very low levels of *survivin*. Cytokine stimulation increased *survivin* expression in leukemic cell lines and in primary AML samples. In cultured primary samples, single-cytokine stimulation substantially increased *survivin* expression in comparison with control cells, and the combination of G-CSF, GM-CSF, and SCF increased *survivin* levels even further. Conversely, all-trans retinoic acid significantly decreased *survivin* protein levels in HL-60, OCI-AML3, and NB-4 cells within 96 hours, parallel to the induction of myelomonocytic differentiation. Using selective pharmacologic inhibitors, the differential involvement of mitogen-activated protein kinase kinase (MEK) and phosphatidylinositol-3 kinase (PI3K) pathways were demonstrated in the regulation of *survivin* expression. The MEK inhibitor PD98059 down-regulated *survivin* expression in both resting and GM-CSF–stimulated OCI-AML3 cells, whereas the PI3K inhibitor LY294002 inhibited *survivin* expression only on GM-CSF stimulation. In conclusion, these results demonstrate that *survivin* is highly expressed and cytokine-regulated in myeloid leukemias and suggest that hematopoietic cytokines exert their antiapoptotic and mitogenic effects, at least in part, by increasing *survivin* levels. (Blood. 2001; 97:2784-2790)**

Introduction

Survivin, a member of the inhibitors-of-apoptosis (IAP) family of proteins, is present during fetal development but is undetectable in terminally differentiated adult tissues. However, *survivin* is prominently expressed in transformed cell lines, in all the most common human cancers, and in approximately 50% of high-grade non-Hodgkin lymphomas.[1-4] *Survivin* suppresses apoptosis induced by Fas, Bax, caspases, and anticancer drugs.[5] Conversely, the down-regulation of *survivin* by antisense oligonucleotides induces apoptosis in vitro.[6,7] Although *survivin* protein lacks the ability to directly inhibit caspase-3,[8] it binds quantitatively to a new IAP-inhibiting protein, Smac/Diablo,[9,10] raising the possibility that it might suppress caspases indirectly by freeing other IAP family members from the constraints of this protein. Taken together, these studies support the notion that *survivin* exerts an antiapoptotic effect.

Survivin expression is cell-cycle–dependent. In proliferating cells, *survivin* is expressed at high levels in the G_2/M phase and is rapidly down-regulated after cell-cycle arrest.[11] Recent studies suggest that *survivin* also plays a role in cell cytokinesis, and the same function has been observed for the *survivin*-homologous ancient baculovirus IAP repeat (BIR)–family proteins in *Caenorhabditis elegans* and yeast.[12-14] The role of *survivin* in cell division control is thought to involve caspase-dependent loss of p21 and deregulation of mitotic transition.[12] Moreover, BIR-family proteins are required for the targeting of members of the Aurora family of kinases to metaphase chromosomes, thereby controlling chromosome segregation and cytokinesis.[15,16] On the basis of these collective findings, therefore, *survivin* is considered to play a pivotal role in linking cell death and cell proliferation.[17,18]

Survival and growth of hematopoietic cells exquisitely depend on the presence of appropriate cytokines that can be provided through either autocrine production or paracrine secretion by stromal cells in the bone marrow micro-environment.[19] Cytokines contribute to the regulation of the apoptotic threshold of normal and leukemic cells by modulating the expression and function of different families of pro- and antiapoptotic proteins.[20,21] In particular, granulocyte macrophage–colony-stimulating factor (GM-CSF) exerts its biologic activity by binding its receptor, which in turn activates multiple intracellular signal transduction pathways through the common β subunit.[22] Among these pathways, both the mitogen-activated protein kinase kinase/extracellular-signal regulated kinase (MEK/ERK) and the phosphatidylinositol-3 kinase (PI3K) pathways have been linked to the induction of resistance to apoptosis and the ability of hematopoietic cells to grow autonomously.[20,23] Although both pathways regulate the expression and function of several Bcl-2 family members, such as Mcl-1,[24,25] the downstream events linking GM-CSF–initiated biochemical events to either proliferation or survival of hematopoietic cells are incompletely elucidated. Growth factor–mediated regulation of IAP expression has recently been demonstrated in endothelial cells.[26,27] However, the ability of hematopoietic cytokines to affect

From the University of Texas M. D. Anderson Cancer Center, Houston, TX; the Division of Medical Oncology I, Regina Elena Cancer Institute, Rome, Italy; and Yale University School of Medicine, New Haven, CT.

Submitted June 20, 2000; accepted January 5, 2001.

Supported in part by grants from the National Institutes of Health (PO1 CA55164, PO1 CA49639, CA78810, and HL54131); by the Keck Foundation; and by an American Cancer Society International Fellowship for Beginning Investigators (M.M.). M.A. holds the Stringer Professorship for Cancer Treatment and Research.

Reprints: Michael Andreeff, Section of Molecular Hematology and Therapy, Dept of Blood and Marrow Transplantation, University of Texas M. D. Anderson Cancer Center, Box 448, 1515 Holcombe Blvd, Houston, TX 77030; e-mail: mandreef@notes.mdacc.tmc.edu.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

survivin expression in myeloid cells has not been studied. Moreover, unlike other IAPs, *survivin* gene expression is not influenced by NF-κB signaling,[17,28] and little is known about other potentially involved signal transduction pathways.

In the study reported here, we examined the regulation of *survivin* expression in acute myeloid leukemia (AML). Our results demonstrate that *survivin* is expressed in AML cell lines and in primary AML samples and that expression is up-regulated by hematopoietic cytokines and inhibited by all-trans retinoic acid (ATRA). We further demonstrate that *survivin* expression is regulated through MEK/ERK and PI3K pathways and can be modulated by selective signal transduction inhibitors.

Materials and methods

Cell lines and primary samples

Human leukemia cell lines were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 1 mM L-glutamine, and 50 μg/mL penicillin–streptomycin. For Mo7e cells, medium was supplemented with 100 U/mL GM-CSF (Immunex, Seattle, WA). Bone marrow and peripheral blood were obtained from patients with AML and normal donors after informed consent was obtained according to institutional guidelines. Mononuclear cells were purified by Ficoll-Hypaque (Sigma Chemical, St Louis, MO) density-gradient centrifugation and cultured in AIM-V medium (Gibco-BRL, Gaithersburg, MD) supplemented with cytokines (Amgen, Thousand Oaks, CA). Blast percentages and other characteristics of primary AML samples are listed in Table 1.

Cell culture studies

OCI-AML3 or HL-60 cells (0.5×10^6 cells/mL) and mononuclear cells purified from the bone marrow of patients with AML (1×10^6 cells/mL) were treated with cytokines for 48 hours in serum-free RPMI 1640 or AIM-V medium, respectively. To block the MEK/ERK and the PI3K pathways, OCI-AML3 cells were washed twice with serum-free RPMI 1640 medium, resuspended at 0.2×10^6 cells/mL in the presence of PD98059 (2′-amino-3′-methoxyflavone[29,30]; CalBiochem, La Jolla, CA), LY294002 (2-[4-morpholinyl]-8-phenyl-[4H]-1-benzopyran-4-one;[31] Sigma), or the appropriate concentration of vehicle (dimethyl sulfoxide [DMSO]) for 2 hours at 37°C before the addition of GM-CSF (100 U/mL). In other experiments, HL-60, OCI-AML3 cells (0.3×10^6 cells/mL), and NB-4 cells (0.1×10^6 cells/mL) were cultured in the presence of ATRA (1 μM) for up to 96 hours. Cells were harvested at different times; live cells were counted by trypan blue exclusion, and the morphologic characteristics were evaluated under a light microscope after staining with HEMA quick stain solution (Biochemical Sciences, Swedesboro, NJ).

Western blot analysis

Cells were washed twice with phosphate-buffered saline (PBS) buffer and lysed at 4×10^4 cells/μL in cell lysis buffer (20 mM HEPES, pH 7.4, 0.25% NP-40 containing protease inhibitor cocktail; Boehringer Mannheim, Indianapolis, IN) for 10 minutes on ice. Equal amounts of lysate (equivalent to 5×10^5 cells) were subjected to SDS-PAGE to 12% polyacrylamide gels. Proteins were transferred to Hybond-P (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) membranes and reacted with polyclonal antibody against *survivin* for 2 hours at room temperature. After they were washed, membranes were probed with a horseradish peroxidase–conjugated secondary antibody and reacted with ECL reagent (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Anti–β-actin blot was made in parallel as a loading control. Signals were detected by a PhosphorImager (Storm 860, version 4.0; Molecular Dynamics, Sunnyvale, CA) and quantified by Scion Image software (Scion, Frederick, MD). Results were expressed as *survivin*/β-actin ratios.

Table 1. Characteristics and relative *survivin* protein levels of AML and normal CD34⁺ samples analyzed

AML patient no.	FAB	BM blast (%)	Cytogenetics	Relative *survivin* level
1	M2	77	47,XY,+19[18]	0.28
2	M2	97	46,XY[20]	0.76
3	M5B	73	46,XX[25]	0.41
4	M1	80	46,XX,inv(9)(p11q12)[19]	1.17
5	M1	78	46,XX,inv(16) (p13q22), idic(22)(q11)[20]	0.29
6	M5B	89	46,XY[29]	0.36
7	M1	86.6	46,XY,iso17(q10)[8] 46,XY[12]	0.73
8	M5	71	46,XY[30]	0.16
9	M1	90.4	46,XX[20]	0.67
10	AML-prior	43	46,XX[19]	0.23
11	M1	51	45,XY,−7[20]	0
12	M1	51	47,XY,+4[18] 48,XY,+4,+8[1]	0.12
13	M2	71	46,XY,add(2)(q37),−5,−22, +2mar[20]	0.25
14	M1	89	47,XY,+8[16]	0.55
15	M2	76	46,XY[20]	0.78
16	M1	73	40-4,XY,add(1)(p36), del(1)(q22),−2,−4,−5, add(5) (q35),−7,−11, −12,−12,−13,−15, add(16)(p13),−17, add(19)(q13),−A	0.21
17*	M3-microgranular type	78	46,XY,inv(9)(p11q12), t(15;17)(q22;q21)[20]	0
18	M5A	88	47,XY,+11[9] 48,XY,+11, +13[10] 46,XY[1]	0.75
Normal CD34⁺				
1	BM			0.25
2	BM			0.09
3	BM			0.23
4	BM			0
5	PB			0
6	PB			0
7	PB			0

FAB, French-American-British classification; BM blast (%), percentage of leukemic blasts before Ficoll-Hypaque separation; relative *survivin* level, ratio of *survivin*/actin relative to that of OCI-AML3.

*77% promyeloblast, 1% blast.

Reverse transcription–polymerase chain reaction

OCI-AML3 cells were treated with either PD98059 or LY294002, as described above, and RNA was isolated with STAT-60 solution (Tel-Test, Friendswood, TX). One microgram total RNA was reverse-transcribed with *survivin* reverse primer (5′TCTCCTTTCCTAAGACATT3′) by AMV reverse transcriptase (Boehringer Mannheim) at 42°C for 1 hour. Polymerase chain reaction (PCR) amplification reaction mixtures (25 μL) contained cDNA, *survivin* forward primer (5′CACCACTTCCAGGGTTTA3′), the reverse primer, *survivin* probe (5′TGGTGCCACCAGCCTTCCTGTG3′), and TaqMan Universal PCR master mix (PE Applied Biosystems, Foster City, CA). Thermal cycle conditions included holding the reactions at 50°C for 2 minutes and at 95°C for 10 minutes and cycling for 40 cycles between 95°C for 15 seconds and 60°C for 1 minute. Results were collected and analyzed by an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems).

Cell-cycle analysis

OCI-AML3 and HL-60 cells (0.5×10^6), cultured under various conditions, were harvested at different times, washed twice with cold PBS, and

fixed with 2 mL ice-cold ethanol (70% vol/vol in water) for 1 hour at 4°C. After centrifugation, fixed cells were exposed to 500 μL propidium iodide (PI) staining solution (25 μg/mL PI, 180 U/mL RNase, 0.1% Triton X-100, and 30 mg/mL polyethylene glycol in 4 mM citrate buffer, pH 7.8; all from Sigma) for 1 hour at 4°C and analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Cell-cycle distribution was then analyzed using the ModFit LT software (Verity Software House, Topsham, ME).

Results

Expression of *survivin* protein in leukemic cell lines and primary AMLs

Survivin expression in myeloid leukemias has not yet been studied, though preliminary evidence that *survivin* mRNA is present in leukemic cell lines and in primary AML samples was reported from our group.[32] Here we examined *survivin* protein expression by Western blot analysis. As shown in Figure 1, all leukemic cell lines tested (RC-2a, KG-1, HL-60, HL-60 DOX, OCI-AML3, Mo7e, NB-4, and K562) expressed *survivin* protein at comparable levels (1.26 ± 0.32, *survivin*/β-actin ratio; survivin/β-actin ratio for OCI-AML3 = 1). Sixteen of 18 AML bone marrow samples showed variable levels of *survivin* (Figure 1). The mean *survivin*/β-actin ratio related to OCI-AML3 in these samples (± SD) was 0.44 ± 0.31 (range, 0 to 1.17; Figure 1, Table 1). Hence, as in other malignancies, *survivin* protein is widely expressed in leukemic cell lines and primary AML blasts. We also examined *survivin* protein expression in normal CD34$^+$ cells obtained by magnetic-bead sorting of 4 bone marrows from normal donors and 3 peripheral blood mononuclear cell samples obtained after G-CSF mobilization. Three bone marrow samples were weakly positive, and all others were negative for *survivin* expression (0.08 ± 0.11; Figure 1, Table 1). Likewise, *survivin* protein expression was not detectable in 3 unseparated peripheral blood mononuclear cell samples (data not shown).

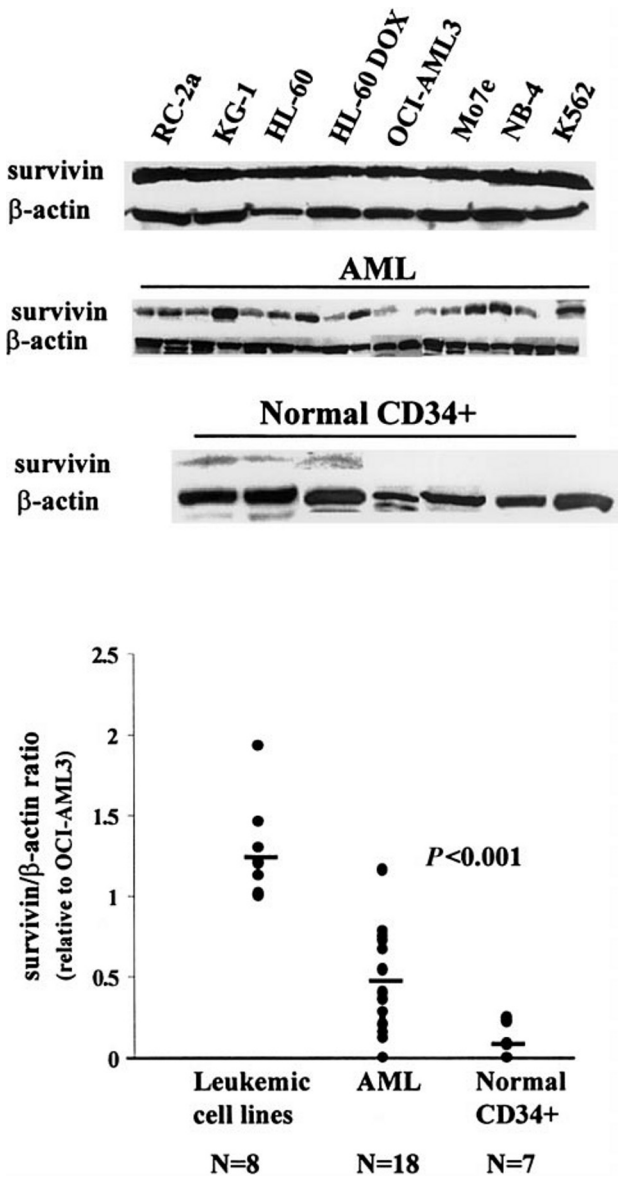


Figure 1. Western blot analysis of *survivin* expression in leukemic cell lines, primary AML samples, and normal CD34$^+$ cells. Cell lysates equivalent to 0.5×10^6 cells were loaded on each lane. The experimental conditions are described in "Materials and methods."

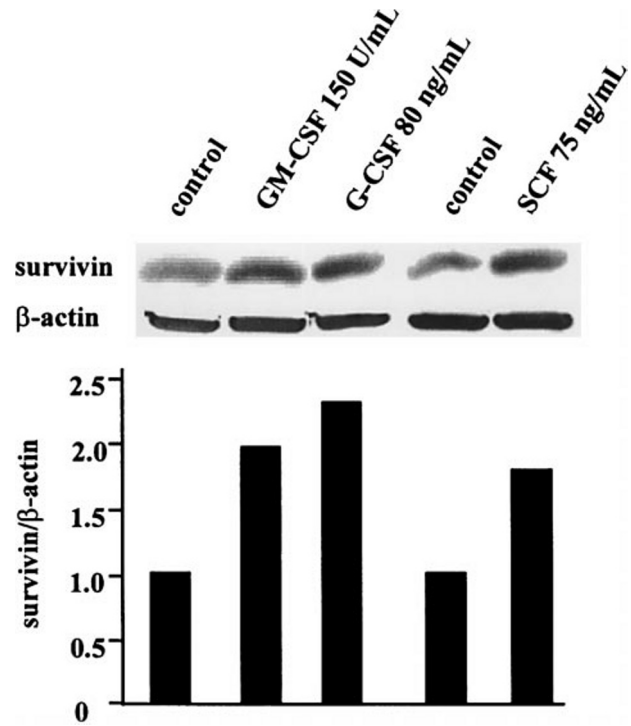


Figure 2. Cytokines stimulate *survivin* protein expression in OCI-AML3 cells. Cells were cultured in serum-free RPMI 1640 medium with GM-CSF (150 U/mL), G-CSF (80 ng/mL), or SCF (75 ng/mL). After 48 hours, cells were lysed, and *survivin* levels were compared with those in untreated cells by Western blot analysis.

Induction of *survivin* protein expression by cytokines

Because survival and growth of leukemic cells largely depend on the presence of appropriate cytokines, we also tested the effect of different hematopoietic cytokines on *survivin* expression in human leukemic cell lines and primary AML blasts. As shown in Figure 2, *survivin* protein levels were 2-fold in GM-CSF (150 U/mL), 2.3-fold in G-CSF (80 ng/mL), and 1.8-fold in stem cell factor (SCF; 75 ng/mL)–treated OCI-AML3 cells compared to cells cultured in cytokine- and serum-free medium for 48 hours. Similar results were obtained in HL-60 cells (data not shown). In 3 AML bone marrow samples studied (patients 15, 17, 18; Table 1) *survivin* protein level decreased dramatically after 48-hour culture in cytokine- and serum-free medium (Figure 3). Treatment with GM-CSF (100 U/mL) and, to a lesser extent, G-CSF (50 ng/mL) and SCF (100 ng/mL) substantially increased *survivin* protein levels compared to control cells, and combinations of these cytokines resulted in the induction of higher *survivin* levels (Figure 3).

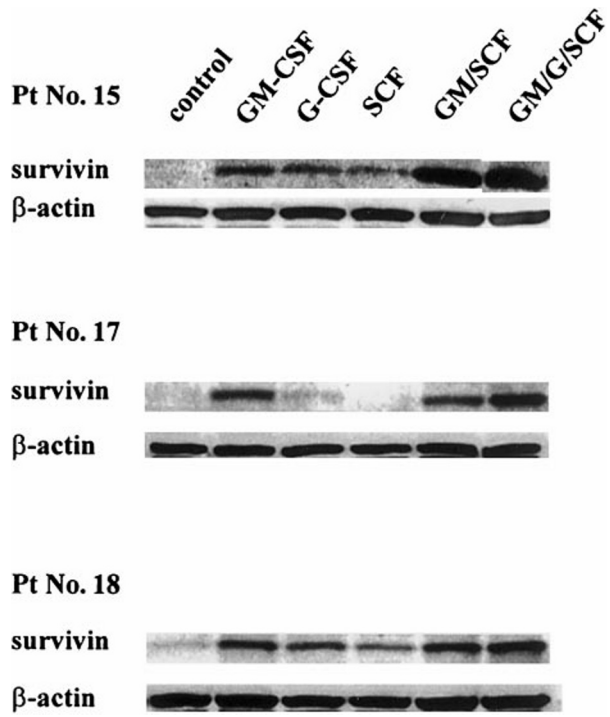


Figure 3. Effect of cytokines on *survivin* expression in primary AML blast cells. Cells were cultured in serum-free AIM-V medium with GM-CSF (100 U/mL), G-CSF (50 ng/mL), SCF (100 ng/mL), or various combinations of these cytokines for 48 hours. Then they were lysed, and *survivin* protein levels were assessed by Western blot analysis. Results were quantitated by PhosphorImager. GM, GM-CSF; G, G-CSF; and Pt No., patient number as shown in Table 1.

ATRA-induced differentiation and inhibition of *survivin* protein expression

Like other antiapoptotic proteins, such as Bcl-2 and Mcl-1,[33,34] *survivin* expression is likely to be differentially regulated during differentiation. We therefore examined the effect of ATRA-induced leukemia cell differentiation on *survivin* expression. HL-60 and OCI-AML3 cells were treated with ATRA (1 μM) for up to 96 hours, and *survivin* protein levels, cell-cycle status, and differentiation were determined. *Survivin* protein levels were significantly decreased in the ATRA-treated leukemic cells studied (Figure 4). In HL-60 cells, no significant effect was observed at 48 hours; however, at 72 hours, *survivin* levels were decreased by 67% and at 96 hours they were decreased by 96% compared to the levels in untreated control cells (Figure 4A). In OCI-AML3 cells, *survivin* levels decreased by 80% at 96 hours (Figure 4B). Concomitant with *survivin* down-regulation, ATRA-treated OCI-AML3 and HL-60 cells showed inhibition of cell-cycle progression and morphologic features of myelomonocytic differentiation (data not shown). Similarly, ATRA decreased *survivin* protein expression and induced differentiation in NB-4 cells. After 96 hours of culture in 1 μM ATRA, *survivin* protein levels were decreased by 65% compared to untreated cells (data not shown).

Regulation of s*urvivin* expression by the MEK/ERK and the PI3K signal transduction pathways

We next examined the role of the MEK/ERK and the PI3K signal transduction pathways in the regulation of basal and cytokine-stimulated *survivin* expression in OCI-AML3 cells using their respective pharmacologic inhibitors PD98059 and LY294002.[29-31] Treatment with PD98059, but not with LY294002, significantly decreased *survivin* levels in the absence of cytokine stimulation (Figure 5A). However, on stimulation with GM-CSF (100 U/mL), both PD98059 and LY294002 partially inhibited *survivin* expression (Figure 5A), suggesting that both pathways are involved in GM-CSF–mediated regulation of *survivin*. To examine whether *survivin* expression is regulated at the transcriptional level, RNAs from untreated and GM-CSF–stimulated OCI-AML3 cells, cultured in the presence or absence of PD98059 or LY294002, were analyzed by quantitative real-time RT-PCR with *survivin*-specific primers. Consistent with the protein expression data, *survivin* mRNA levels were reduced only by the MEK inhibitor PD98059 under basal conditions and by both the MEK and the PI3K inhibitors on GM-CSF stimulation (Figure 5B). *Survivin* expression in OCI-AML3 cells was also increased by treatment with the phosphatase inhibitor sodium orthovanadate (25 to 50 μM for 48 hours), and this increase was abrogated by pretreatment with either

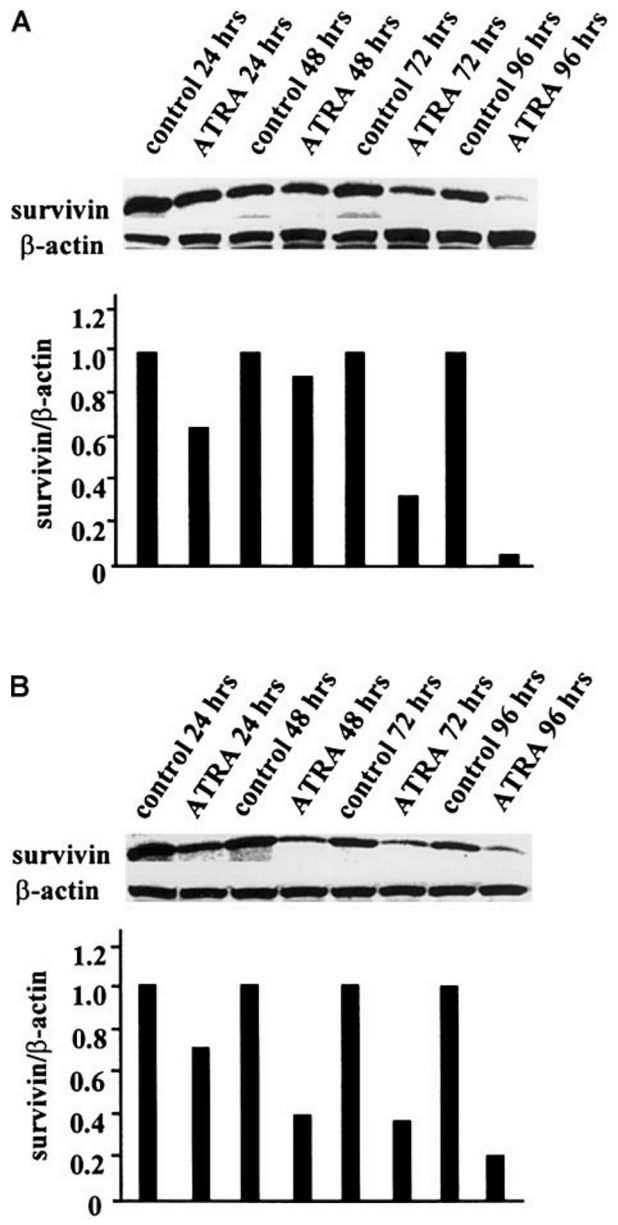
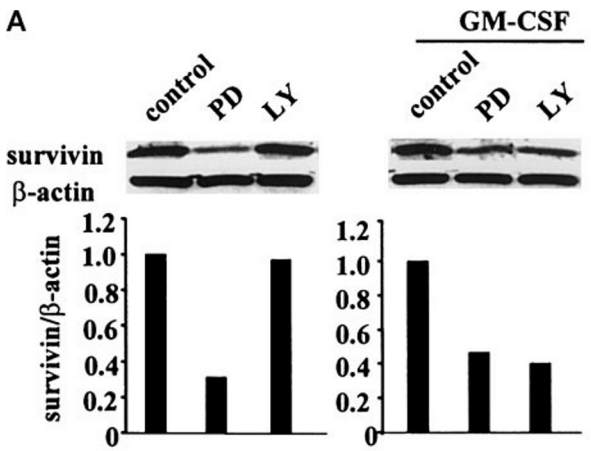


Figure 4. ATRA-induced down-regulation of *survivin* protein expression in HL-60 and OCI-AML3 cells. HL-60 (A) and OCI-AML3 (B) cells were treated with 1 μM ATRA for up to 96 hours, as described in "Materials and methods." Cells were lysed, and *survivin* protein levels were determined at 24, 48, 72, and 96 hours by Western blot analysis. The experiment was performed 3 times, and the results shown here are representative.

PD98059 or LY294002 (data not shown), further supporting a role for the MEK/ERK and the PI3K pathways in the regulation of *survivin* expression. We also assessed the effect of signal transduction inhibitors on the cell-cycle distribution of unstimulated and GM-CSF–stimulated OCI-AML3 cells. PD98059 profoundly inhibited the G_1/S transition in both unstimulated and GM-CSF–stimulated cells (78% and 79% reduction in S phase, respectively), whereas treatment with LY294002 only slightly affected cell-cycle distribution in either culture condition (33% and 36% reduction in S phase, respectively) (data not shown). PD98059-induced cell-cycle arrest was time- and dose-dependent, and its kinetics and dose-response curve paralleled those of *survivin* expression (Figure 6). Taken together, our data indicate that *survivin* expression is differentially regulated by the MEK/ERK and the PI3K signal transduction pathways under basal and GM-CSF–stimulated conditions and suggest that the effect of MEK/ERK blockade may be mediated, at least in part, by the inhibition of cell-cycle progression.



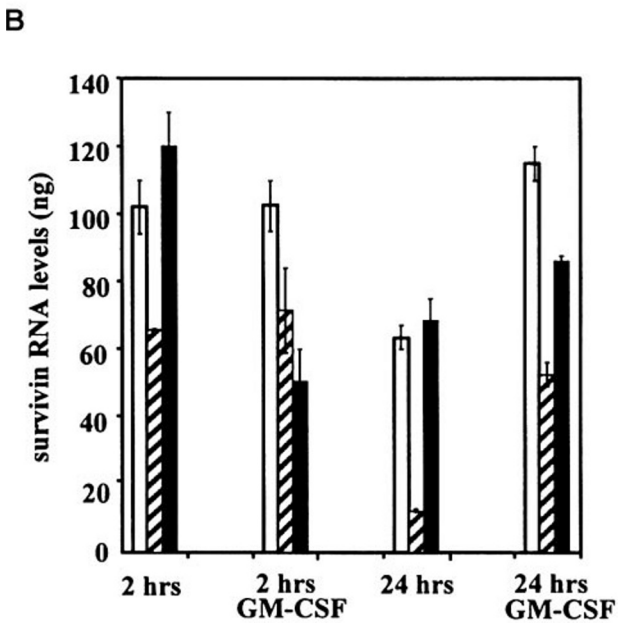


Figure 5. Regulation of *survivin* expression in OCI-AML3 cells by MEK and PI3K inhibitors. (A) Western blot shows *survivin* protein expression in cells treated with 20 μM PD98059 (PD) or 10 μM LY294002 (LY) for 48 hours without or with GM-CSF (100 U/mL). Results of 1 of 3 independent experiments are shown. (B) Quantitative RT-PCR demonstrates the regulation of *survivin* mRNA expression in response to the inhibitors at 2 and 24 hours (Taqman PCR; see "Materials and methods"). □ indicates control; ▨, PD; and ■, LY.

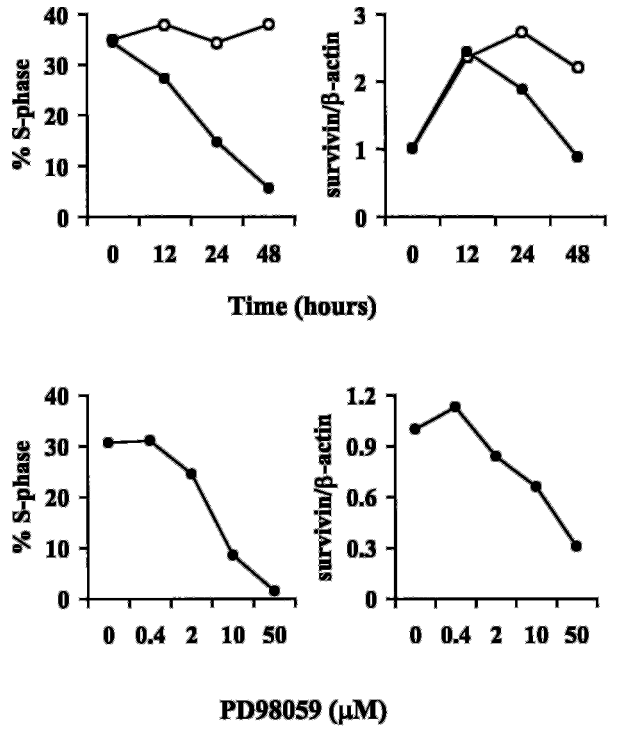


Figure 6. Kinetics and dose-response of PD98059-induced cell-cycle arrest and down-regulation of *survivin* protein expression. OCI-AML3 cells were treated with either vehicle (control) or PD98059, harvested and stained with PI to determine DNA content (left panels) or analyzed by Western blot with a *survivin*-specific antiserum (right panels). Top panels show the kinetics of cell-cycle arrest and down-regulation of *survivin* expression in response to 20 μM PD98059. Bottom panels show the dose-response curves for PD98059 at 48 hours. Results are expressed as the percentage of cells in S phase (calculated using the ModFit LT software, left panels) and *survivin*/β-actin ratios (as quantitated by a PhosphorImager, right panels). Results of 1 of 3 independent experiments are shown. ○ indicates control; ●, PD98059 (20 μM).

Discussion

In this study, we provide evidence that the recently described IAP family member *survivin* is widely expressed in myeloid leukemia cell lines and in almost all primary AML samples tested. Furthermore, we demonstrate that *survivin* expression in leukemic cells is regulated by cytokines and differentiation-inducing agents and that the modulation of major signal transduction pathways, such as the MEK/ERK and the PI3K pathways, can contribute to the regulation of *survivin* expression at the mRNA and protein levels.

The induction of programmed cell death is the common outcome of successful cytotoxic therapy for many different types of cancer, including AML.[35-40] Multiple genetic alterations that result in the disruption of the physiological regulation of apoptosis are thought to account for the ability of leukemic cells to grow autonomously and for their clinical resistance to therapy.[41-44] Recently, a new family of downstream inhibitors of caspases, the IAP family, has emerged as a potential key player in the regulation of apoptosis in cancer,[45,46] and we have already demonstrated that XIAP is expressed and has prognostic relevance in AML.[47] Our present results demonstrate that another IAP family member, *survivin,* is constitutively expressed in both myeloid leukemia cell lines and in primary AML blasts and at significantly lower levels ($P < .001$) in normal $CD34^+$ cells from bone marrow or G-CSF–stimulated peripheral blood. The latter finding extends to the hematopoietic progenitors the previous report of lack of *survivin* expression in normal bone marrow.[17] Because *survivin* has been

demonstrated to efficiently inhibit apoptosis induced by a variety of stimuli in vitro[5] and its presence has been correlated in vivo with reduced apoptotic indices and poor prognosis in solid tumors,[3,4,48-50] we are currently investigating its functional and prognostic relevance in AML.

Acute myeloid leukemia is a heterogeneous disease characterized by the accumulation of leukemic blasts arrested at various stages of granulocytic and monocytic differentiation. Transcriptional modulation aimed at restoring the ability of AML cells to regulate the expression of genes resulting in differentiation is, therefore, an attractive therapeutic strategy that has proved effective in the treatment of patients with acute promyelocytic leukemia (APL).[51] Our study shows that ATRA significantly down-regulates *survivin* expression in AML (HL-60, OCI-AML3) and APL (NB-4) cell lines, concomitant with the induction of cell differentiation. Whether other differentiation inducers such as DMSO, hexamethylene bisacetamide, vitamin D, and butyrate are also able to down-regulate *survivin* expression is unknown. Further studies are required to elucidate whether ATRA directly inhibits *survivin* transcription or whether it affects *survivin* expression primarily because of the cell-cycle arrest that accompanies differentiation. Regardless, together with previous evidence indicating that ATRA transcriptionally down-regulates Bcl-2 and Bcl-X_L expression in leukemias,[52] our findings suggest that ATRA may lower the apoptotic threshold by modulating multiple pathways, eventually rendering AML cells more susceptible to cytotoxic agents.

The present study also provides unequivocal evidence that hematopoietic cytokines such as GM-CSF, G-CSF, and SCF, alone or in combination, strongly increase *survivin* expression in myeloid leukemia cell lines and, most important, in primary AML samples. Further evidence that IAP family members may function as growth factor–inducible antiapoptotic genes comes from the recent observation that *survivin* and XIAP expression are increased in endothelial cells in response to mitogenic growth factors, resulting in a decreased sensitivity to apoptotic stimuli.[26,27] Previous studies from our group have also shown that quiescent, but not proliferating, leukemic progenitors overexpress Bcl-2 and Bcl-X_L.[53] This observation, together with the present finding of increased *survivin* expression in response to cytokines, raises the intriguing possibility that Bcl-2 and *survivin* may represent complementary survival pathways that are differentially regulated by the cell-cycle status of leukemic progenitors. Quiescent progenitors are protected from apoptosis and are restrained from entering the cell cycle by the expression of Bcl-2 (and possibly Bcl-X_L). However, once recruited into the cell cycle, proliferating cells could switch to a *survivin*-mediated survival pathway that enables them to successfully complete mitosis and avoid a "default" induction of apoptosis at cell division.[17] Consistent with this hypothesis, preliminary data indicate that AML cells that survive Bcl-2 antisense treatment in vitro express high levels of *survivin* (B.Z.C., unpublished results, December 1999). Interestingly, in the IL-3–dependent cell line BaF3, cytokine withdrawal-induced apoptosis was inhibited by the forced expression of either Bcl-2 or *survivin*,[1] suggesting that, though they act at different levels, these 2 survival pathways may indeed function in concert to prevent cell death. Cytokine-mediated up-regulation of a *survivin*-dependent survival pathway might also explain the conflicting clinical results reported for cytokine "priming" strategies for the therapy of AML.[54]

The binding of GM-CSF to its receptor activates multiple signaling pathways, which in turn lead to the proliferation, differentiation, and survival of various hematopoietic cells.[19,20,22] Here we provide the first evidence that, in addition to modulating the expression and function of Bcl-2 family members, GM-CSF–mediated activation of both the MEK/ERK and the PI3K signal transduction pathways regulates the expression of *survivin* at both the mRNA and the protein level. This finding is consistent with the notion that GM-CSF antiapoptotic activity relies on multiple and, in part, redundant pathways.[55] Interestingly, we found that disruption of the MEK/ERK, but not of the PI3K, pathway also inhibited the constitutive expression of *survivin* (Figure 5), suggesting that the MEK/ERK pathway might be constitutively active in the cytokine-independent OCI-AML3 cell line. Consistent with this hypothesis, recent data from our group demonstrate that active ERK species are indeed detectable in OCI-AML3 cells in the absence of cytokine or serum stimulation (M.M., manuscript in preparation, May 2000).

Regulation of mouse *survivin* expression requires integration of typical CDE/CHR G_1 repressor elements and basal transcriptional activity by Sp1 sites, which results in a cell-cycle–regulated expression in the G_2/M phase.[56] Our data indicate that pharmacologic disruption of the MEK/ERK kinase module in unstimulated and GM-CSF–stimulated cells profoundly inhibits the G_1/S transition, suggesting that the observed down-regulation of *survivin* expression may be, at least in part, secondary to the inhibition of cell-cycle progression. However, the early inhibition of *survivin* mRNA transcription (at 2 hours) in the absence of any detectable cell-cycle changes suggests that a direct transcriptional effect might also take place. Likewise, a cell-cycle–independent transcriptional effect is the most likely explanation for the inhibition of GM-CSF–stimulated *survivin* expression observed in response to PI3K inhibition. Support for this hypothesis comes further from the recent observation that, in endothelial cell lines, treatment with angiopoietin-1 up-regulates *survivin* expression in a PI3K/AKT-dependent fashion in the absence of any effect on cell proliferation.[57] Further studies are under way to elucidate the potential involvement of PI3K- and MEK/ERK-dependent transcription factors in *survivin* gene expression. The results reported here—the effects of cytokines, ATRA, and signal transduction inhibitors on *survivin* expression—may be of help in the development of novel strategies for the treatment of leukemia and other cancers by targeting antiapoptotic *survivin*.

Acknowledgments

We thank Rosemarie Lauzon for assisting in the manuscript preparation and Teresa McQueen for magnetic-bead sorting normal $CD34^+$ cells.

References

1. Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med. 1997;3:917-921.
2. Adida C, Crotty PL, McGrath J, Berrebi D, Diebold J, Altieri DC. Developmentally regulated expression of the novel cancer antiapoptosis gene survivin in human and mouse differentiation. Am J Pathol. 1998;152:43-49.
3. Kawasaki H, Altieri DC, Lu CD, Toyoda M, Tenjo T, Tanigawa N. Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer. Cancer Res. 1998;58:5071-5074.
4. Lu CD, Altieri DC, Tanigawa N. Expression of a novel antiapoptosis gene, survivin, correlated with tumor cell apoptosis and p53 accumulation in gastric carcinomas. Cancer Res. 1998;58: 1808-1812.
5. Tamm I, Wang Y, Sausville E, et al. IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. Cancer Res. 1998;58: 5315-5320.
6. Ambrosini G, Adida C, Sirugo G, Altieri DC. Induction of apoptosis and inhibition of cell proliferation by survivin gene targeting. J Biol Chem. 1998;273:11177-11182.

7. Grossman D, McNiff JM, Li F, Altieri DC. Expression and targeting of the apoptosis inhibitor, survivin, in human melanoma. J Invest Dermatol. 1999;113:1076-1081.
8. Verdecia MA, Huang H, Dutil E, Kaiser DA, Hunter T, Noel JP. Structure of the human anti-apoptotic protein survivin reveals a dimeric arrangement [see comments]. Nat Struct Biol. 2000;7:602-608.
9. Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell. 2000;102:33-42.
10. Verhagen AM, Ekert PG, Pakusch M, et al. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell. 2000;102:43-53.
11. Li F, Ambrosini G, Chu EY, et al. Control of apoptosis and mitotic spindle checkpoint by survivin. Nature. 1998;396:580-584.
12. Li F, Ackermann EJ, Bennett CF, et al. Pleiotropic cell-division defects and apoptosis induced by interference with survivin function. Nat Cell Biol. 1999;1:461-466.
13. Fraser AG, James C, Evan GI, Hengartner MO. *Caenorhabditis elegans* inhibitor of apoptosis protein (IAP) homologue BIR-1 plays a conserved role in cytokinesis. Curr Biol. 1999;9:292-301.
14. Li F, Flanary PL, Altieri DC, Dohlman HG. Cell division regulation by BIR1, a member of the inhibitor of apoptosis family in yeast. J Biol Chem. 2000;275:6707-6711.
15. Speliotes EK, Uren A, Vaux D, Horvitz HR. The survivin-like *C elegans* BIR-1 protein acts with the Aurora-like kinase AIR-2 to affect chromosomes and the spindle midzone. Mol Cell. 2000;6:211-223.
16. Reed JC, Bischoff JR. BIRinging chromosome through cell division-and survivin' the experience. Cell. 2000;102:545-548.
17. Altieri DC, Marchisio PC, Marchisio C. Survivin apoptosis: an interloper between cell death and cell proliferation in cancer [published erratum appears in Lab Invest 1999;79:1543]. Lab Invest. 1999;79:1327-1333.
18. Reed JC, Reed SI. Survivin' cell-separation anxiety. Nat Cell Biol. 1999;1:E199–E200.
19. Arai KI, Lee F, Miyajima A, Miyatake S, Arai N, Yokota T. Cytokines: coordinators of immune and inflammatory responses. Annu Rev Biochem. 1990;59:783-836.
20. Blalock WL, Weinstein-Oppenheimer C, Chang F, et al. Signal transduction, cell cycle regulatory, and antiapoptotic pathways regulated by IL-3 in hematopoietic cells: possible sites for intervention with anti-neoplastic drugs. Leukemia. 1999;13:1109-1166.
21. Lisovsky M, Estrov Z, Zhang X, et al. Flt3 ligand stimulates proliferation and inhibits apoptosis of acute myeloid leukemia cells: regulation of Bcl-2 and Bax. Blood. 1996;88:3987-3997.
22. Sato N, Sakamaki K, Terada N, Arai K, Miyajima A. Signal transduction by the high-affinity GM-CSF receptor: two distinct cytoplasmic regions of the common beta subunit responsible for different signaling. EMBO J. 1993;12:4181-4189.
23. McCubrey JA, May WS, Duronio V, Mufson A. Serine/threonine phosphorylation in cytokine signal transduction. Leukemia. 2000;14:9-21.
24. Townsend KJ, Trusty JL, Traupman MA, Eastman A, Craig RW. Expression of the antiapoptotic MCL1 gene product is regulated by a mitogen activated protein kinase-mediated pathway triggered through microtubule disruption and protein kinase C. Oncogene. 1998;17:1223-1234.
25. Huang HM, Huang CJ, Yen JJ. Mcl-1 is a common target of stem cell factor and interleukin-5 for apoptosis prevention activity via MEK/MAPK and PI-3K/Akt pathways [In Process Citation]. Blood. 2000;96:1764-1771.
26. Tran J, Rak J, Sheehan C, et al. Marked induction of the IAP family antiapoptotic proteins survivin and XIAP by VEGF in vascular endothelial cells. Biochem Biophys Res Commun. 1999;264:781-788.
27. O'Connor DS, Schechner JS, Adida C, et al. Control of apoptosis during angiogenesis by survivin expression in endothelial cells. Am J Pathol. 2000;156:393-398.
28. Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS Jr. NF-κB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science. 1998;281:1680-1683.
29. Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. J Biol Chem. 1995;270:27489-27494.
30. Dudley DT, Pang L, Decker SJ, Bridges AJ, Saltiel AR. A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc Natl Acad Sci U S A. 1995;92:7686-7689.
31. Vlahos CJ, Matter WF, Hui KY, Brown RF. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J Biol Chem. 1994;269:5241-5248.
32. Segall H, Zhao S, Xie Z, et al. Expression of the inhibitor of apoptosis protein (IAP) family in acute and chronic leukemia blasts [abstract]. Blood. 1998;92:201.
33. Delia D, Aiello A, Soligo D, et al. Bcl-2 proto-oncogene expression in normal and neoplastic human myeloid cells. Blood. 1992;79:1291-1298.
34. Kozopas KM, Yang T, Buchan HL, Zhou P, Craig RW. MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. Proc Natl Acad Sci U S A. 1993;90:3516-3520.
35. Barry MA, Behnke CA, Eastman A. Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia. Biochem Pharmacol. 1990;40:2353-2362.
36. Sen S, D'Incalci M. Apoptosis: biochemical events and relevance to cancer chemotherapy. FEBS Lett. 1992;307:122-127.
37. Ling YH, Priebe W, Perez-Soler R. Apoptosis induced by anthracycline antibiotics in P388 parent and multidrug-resistant cells. Cancer Res. 1993;53:1845-1852.
38. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. Science. 1995;267:1456-1462.
39. Los M, Herr I, Friesen C, Fulda S, Schulze-Osthoff K, Debatin KM. Cross-resistance of CD95- and drug-induced apoptosis as a consequence of deficient activation of caspases (ICE/Ced-3 proteases). Blood. 1997;90:3118-3129.
40. Hannun YA. Apoptosis and the dilemma of cancer chemotherapy. Blood. 1997;89:1845-1853.
41. Russell NH, Hunter AE, Bradbury D, Zhu YM, Keith F. Biological features of leukemic cells associated with autonomous growth and reduced survival in acute myeloblastic leukemia. Leuk Lymphoma. 1995;16:223-229.
42. Smith BD, Bambach BJ, Vala MS, et al. Inhibited apoptosis and drug resistance in acute myeloid leukaemia. Br J Haematol. 1998;102:1042-1049.
43. Bailly JD, Skladanowski A, Bettaieb A, Mansat V, Larsen AK, Laurent G. Natural resistance of acute myeloid leukemia cell lines to mitoxantrone is associated with lack of apoptosis. Leukemia. 1997;11:1523-1532.
44. Norgaard JM, Jensen PD, Bendix K, Clausen N, Palshof T. Relevance of in vitro leukemia cell survival to short- and long-term clinical outcome in AML. Leuk Lymphoma. 1999;32:327-337.
45. LaCasse EC, Baird S, Korneluk RG, MacKenzie AE. The inhibitors of apoptosis (IAPs) and their emerging role in cancer. Oncogene. 1998;17:3247-3259.
46. Uren AG, Coulson EJ, Vaux DL. Conservation of baculovirus inhibitor of apoptosis repeat proteins (BIRPs) in viruses, nematodes, vertebrates and yeasts. Trends Biochem Sci. 1998;23:159-162.
47. Tamm I, Segall H, Kitada S, et al. Expression and prognostic significance of IAP-family genes in human cancers and myeloid leukemias. Clin Cancer Res. 2000;6:1796-1803.
48. Adida C, Berrebi D, Peuchmaur M, Reyes-Mugica M, Altieri DC. Anti-apoptosis gene, survivin, and prognosis of neuroblastoma [letter]. Lancet. 1998;351:882-883.
49. Tanaka K, Iwamoto S, Gon G, Nohara T, Iwamoto M, Tanigawa N. Expression of survivin and its relationship to loss of apoptosis in breast carcinomas. Clin Cancer Res. 2000;6:127-134.
50. Islam A, Kageyama H, Takada N, et al. High expression of survivin, mapped to 17q25, is significantly associated with poor prognostic factors and promotes cell survival in human neuroblastoma. Oncogene. 2000;19:617-623.
51. Warrell RP Jr. Pathogenesis and management of acute promyelocytic leukemia. Annu Rev Med. 1996;47:555-565.
52. Andreeff M, Jiang S, Zhang X, et al. Expression of Bcl-2-related genes in normal and AML progenitors: changes induced by chemotherapy and retinoic acid. Leukemia. 1999;13:1881-1892.
53. Konopleva M, Zhao S, Jiang S, Snell V, Zhang X, Reed JC. Overexpression of antiapoptotic Bcl-XL and Bcl-2 contributes to chemoresistance of quiescent leukemic progenitors and can be selectively reversed by ATRA [abstract]. Blood. 1998;92:600.
54. Buchner T, Hiddemann W, Wormann B, et al. Hematopoietic growth factors in acute myeloid leukemia: supportive and priming effects. Semin Oncol. 1997;24:124-131.
55. Liu R, Itoh T, Arai KI, Watanabe S. Two distinct signaling pathways downstream of Janus kinase 2 play redundant roles for antiapoptotic activity of granulocyte-macrophage colony-stimulating factor. Mol Cell Biol. 1999;10:3959-3970.
56. Li F, Altieri DC. The cancer antiapoptosis mouse survivin gene: characterization of locus and transcriptional requirements of basal and cell cycle-dependent expression. Cancer Res. 1999;59:3143-3151.
57. Papapetropoulos A, Fulton D, Mahboubi K, et al. Angiopoietin-1 inhibits endothelial cell apoptosis via the Akt/survivin pathway. J Biol Chem. 2000;275:9102-9105.