The antiapoptosis protein survivin is associated with cell cycle entry of normal cord blood CD34+ cells and modulates cell cycle and proliferation of mouse hematopoietic progenitor cells

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The inhibitor of the apoptosis protein (IAP) survivin is expressed in proliferating cells such as fetal tissues and cancers. We previously reported that survivin is expressed and growth factor regulated in normal adult CD34+ cells. Herein, we examined survivin expression in CD34+ cells before and after cell cycle entry and demonstrate a role for survivin in cell cycle regulation and proliferation. Analysis of known human IAPs revealed that only survivin is cytokine regulated in CD34+ cells. Survivin expression is coincident with cell cycle progression. Up-regulation of survivin by thrombopoietin (Tpo), Flt3 ligand (FL), and stem cell factor (SCF) occurred in underphosphorylated-retinoblastoma protein (Rb)positive, KI-67negative, and cyclin Dnegative CD34+ cells. Quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR) and multivariate flow cytometry demonstrated that Tpo, SCF, and FL increase survivin mRNA and protein in quiescent G0 CD34+ cells without increasing KI-67 expression, indicating that cytokine-stimulated up-regulation of survivin in CD34+ cells occurs during G0, before cells enter G1. Selective inhibition of the PI3-kinase/AKT and mitogen-activated protein kinase (MAPKp42/44) pathways blocked survivin up-regulation by growth factors before arresting cell cycle. Retrovirus transduction of survivin-internal ribosome entry site–enhanced green fluorescent protein (survivin-IRES-EGFP) in primary mouse marrow cells increased granulocyte macrophage–colony-forming units (CFU-GM) by 1.7- to 6.2-fold and the proportion of CFU-GM in S phase, compared to vector control. An antisense survivin construct decreased total and S-phase CFU-GM. These studies provide further evidence that survivin up-regulation by growth factors is not a consequence of cell cycle progression and strongly suggest that survivin is an important early event for cell cycle entry by CD34+ cells.

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Introduction

Apoptosis and cell cycle regulation are tightly orchestrated processes involving multiple effector molecules.1-3 Pathways that regulate cell cycle and cell survival overlap, but distinct mechanisms are involved.4,5 The inhibitor of apoptosis protein (IAP) family proteins inhibit apoptosis by inactivating several caspsases. There are 7 known IAP family proteins: NAIP,6 XIAP,7 c-IAP1,7,9 c-IAP2,7,8 survivin,10,11 livin,12 and murine Bruce13 and its human homolog, Apollon.14 Survivin and livin are frequently overexpressed in cancer cells and fetal tissues but are barely detectable in adult quiescent tissues.10-12,15 While most IAPs block apoptosis,5,8,12,16-18 their roles in cell cycle regulation are unclear.

We recently reported that survivin is expressed and cytokine regulated in normal adult marrow CD34+ cells, umbilical cord blood (UCB) CD34+ cells, and adult peripheral blood T cells.18 Survivin blocks caspase-3 activity and inhibits apoptosis in cancer cells.11,15,16,19-21 and an inverse correlation between survivin and active caspase-3 expression was observed in CD34+ cells.18 Survivin expression in cytokine-stimulated CD34+ cells was associated with cell cycle progression, being highest in G2/M. However, in contrast to expression occurring only during G2/M in cancer cells,15 survivin is expressed in all phases of the cell cycle in cytokine-stimulated CD34+ cells.18 These studies indicate that survivin is not a cancer-specific protein and suggest that survivin plays a role in the proliferation and survival of normal hematopoietic cells.

Since most proliferating cells express survivin, including cancer cells,10,11,15 normal T cells,18 and normal CD34+ cells,18 it is unclear whether survivin is expressed simply because cells are dividing or whether survivin expression directly affects cell cycle progression and proliferation. Since survivin interacts with the cdk4/cyclin D complex and enhances Rb phosphorylation22,23 and overexpression of survivin enhances cell cycle progression in hepatoma cells, survivin may be involved in cell cycle regulation, at least in cancer cells.24 Our previous findings that survivin is found in G0 CD34+ cells after growth factor stimulation for 48 hours18 raised the question of whether up-regulation of survivin by growth factors occurs in quiescent G0 CD34+ cells before they enter G1.

In this study, we examined whether IAPs in general, and survivin in particular, are expressed and growth factor regulated in normal CD34+ cells. Using real-time reverse transcription–polymerase chain reaction (RT-PCR) and multivariate flow cytometry, we now demonstrate that up-regulation of survivin expression by growth factors occurs in quiescent (G0) CD34+ cells before entering G1 and that survivin expression is not a consequence of cell cycle progression. Overexpression of survivin in normal
primary mouse bone marrow cells enhanced granulocyte macrophage–colony-forming unit (CFU-GM) production and cell cycle, suggesting that survivin plays a regulatory role in cell cycle entry and proliferation of normal hematopoietic cells.

**Materials and methods**

### Animals

Specific pathogen-free female C57Bl/6 mice, 8 to 12 weeks of age, were purchased from Harlan Sprague-Dawley, Indianapolis, IN. Mice were provided continuous access to rodent chow and acidified water. The Institutional Animal Care and Use Committee of Indiana University School of Medicine approved all experimental procedures.

### Growth factors, antibodies, and reagents

Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF), Flt3 ligand (FL), and thrombopoietin (Tpo) were provided by Immunex, Seattle, WA. Recombinant human and mouse stem cell factor (SCF) was a gift from Dr Karl Nocka, UCB Research (Cambridge, MA). Recombinant murine GM-CSF was purchased from BioVisto (Palo Alto, CA). Affinity purified antihuman survivin polyclonal antibody (AF886) and mouse IgG1 were purchased from R&D Systems (Minneapolis, MN). We previously described the specificity of the AF886 survivin antibody for intracellular staining in CD34+ cells.18 Monoclonal antihuman survivin antibody (6E4) was purchased from Cell Signaling (Beverly, MA). Anti-human retinoblastoma protein (Rb) monoclonal antibody (mAb) (G3-245), fluorescein isothiocyanate (FITC)–conjugated anti–underphosphorylated-Rb mAb (clone G99-549), FITC–anti-human cyclin D1/D2/D3 mAb (clone G124-259), FITC–anti-Ki-67 mAb (clone B56), FITC–mouse IgG1 and 2-sequential positive selection with immunomagnetic beads (Miltenyi) Biotech, Auburn, CA. Anti-human cyclin D1/D2/D3 mAb and 2 sequential positive selections with immunomagnetic beads (Miltenyi) Biotech, Auburn, CA. Anti-human cyclin D1/D2/D3 mAb and 2 sequential positive selections with immunomagnetic beads (Miltenyi) Biotech, Auburn, CA.

### Isothermal cDNA cells and cell culture

Normal UCB was obtained with institutional review board approval. Low-density mononuclear cells were separated on Ficoll-Paque (1.077 g/mL) (Amersham Pharmacia Biotech, Piscataway, NJ) and CD34+ cells isolated with antihuman CD34 mAb (QBEND/10) and 2-sequential positive selection with immunomagnetic beads (Miltenyi) Biotech, Auburn, CA. Anti-human cyclin D1/D2/D3 mAb and 2 sequential positive selections with immunomagnetic beads (Miltenyi) Biotech, Auburn, CA. Anti-human cyclin D1/D2/D3 mAb and 2 sequential positive selections with immunomagnetic beads (Miltenyi) Biotech, Auburn, CA.

### Isolation of cord blood CD34+ cells and cell culture

Normal UCB was obtained with institutional review board approval. Low-density mononuclear cells were separated on Ficoll-Paque (1.077 g/mL) (Amersham Pharmacia Biotech, Piscataway, NJ) and CD34+ cells isolated with antihuman CD34 mAb (QBEND/10) and 2-sequential positive selection with immunomagnetic beads (Miltenyi) Biotech, Auburn, CA. Anti-human cyclin D1/D2/D3 mAb and 2 sequential positive selections with immunomagnetic beads (Miltenyi) Biotech, Auburn, CA. Anti-human cyclin D1/D2/D3 mAb and 2 sequential positive selections with immunomagnetic beads (Miltenyi) Biotech, Auburn, CA.

### Quantitative real-time RT-PCR

Primers and probes for human survivin, Ki-67, and glycoldehyde-3-phosphate dehydrogenase (GAPDH) were designed using Primer Express Software (Applied Biosystems, Foster City, CA) and purchased from Applied Biosystems. For survivin, the forward and reverse primers were 5′-TGAACCTCGTTTCGAGGAGGA-3′ and 5′-GTCTAACACAACAGGCA-3′. For GAPDH, the forward and reverse primers were 5′-ACTGCTTCTCCGCTCGCTGCTGC-3′ and 5′-AACTGACCACACTGAGGAA-3′. Amplification of all IAPs was carried out for 30 to 40 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, and elongation for 1 minute at 72°C, followed by final extension for 7 minutes at 72°C. For GAPDH, annealing was carried out at 60°C. PCR products were visualized in 2% agarose gels stained with ethidium bromide.

### Intracellular staining and flow cytometry

Multivariate intracellular staining of CD34+ cells with anti–total-Rb, phosphorylated-Rb, underphosphorylated-Rb, cyclin-D, Ki-67, and survivin antibodies in combination with DNA staining were performed as previously described18 with minor modifications. CD34+ cells were fixed with 4% paraformaldehyde, washed with 0.1% bovine serum albumin/phosphate-buffered saline (BSA/PBS), resuspended in ice-cold 80% ethanol, and incubated for 24 hours at −20°C. Cells were washed with PBS containing 0.25% Triton X-100 and 1% BSA and stained with either FITC–anti-underphosphorylated-Rb, FITC–anti-Ki-67, or FITC–anti-cyclin D mAbs and antihuman survivin antibody. DNA staining was performed using 7-AAD. Stained cells were analyzed using a FACScan and ModFIT (for cell cycle) and CellQuest software (Becton Dickinson, San Jose, CA).

### Retrovirus production and infection of mouse bone marrow progenitor cells

Human survivin cDNA was obtained from Dr Hari Nakshatri (Indiana University School of Medicine, Indianapolis, IN). Mouse survivin cDNA was amplified by RT-PCR from total RNA harvested from NIH3T3 cells.
using random hexamers and the primers 5′-GGTGGTCGCTCTGAGCCG-GAAGTTTGGTTGAGCCATC-3′ and 5′-CTCAAGTTCAAGGTTACTT-CAGCAAGGGCTACGA-3′. The bicistronic retrovirus plasmid MIEG3 containing internal ribosome entry site–enhanced green fluorescent protein (IRES-EGFP)30 was obtained from Dr David Williams (Indiana University). Full-length human and mouse survivin cDNAs were cloned into the MIEG3 plasmid. The orientation and sequence of every construct were confirmed before transfection. A clone showing a reverse direction was used as an antisense construct. Ecotropic retrovirus containing human, mouse, and antisense-mouse survivin and MIEG3 backbone were produced using Phoenix eco cells (ATCC, Manassas, VA) as described.30 Briefly, 5 × 10^6 Phoenix eco cells were seeded onto 100-mm dishes and transfected with 8 μg plasmid using Lipofectamine and Plus Reagent ( Gibco BRL/Invitrogen) 16 hours later. Transfected cells were incubated in serum-free media for 3 hours, followed by readdition of serum. After 24 hours, cells were exposed to 50 mM sodium butyrate for 8 hours, followed by sequential washing with PBS. Cells were fed with IMDM medium, 10% FBS and 2 mM glutamine, incubated at 32° C for 24 hours, and the supernatant was collected, filtered, and stored at −70° C. Virus titer was usually 1-2 × 10^6 plaque-forming units (pfu)/mL. Mouse bone marrow cells were harvested, and mononuclear cells were isolated on Lympholyte-M (Cedarlane Laboratories, ON, Canada) and stimulated with 100 ng/mL each human Tpo, murine SCF, and human G-CSF for 48 hours.30 Media were replaced with freshly thawed retrovirus supernatant containing the identical cytokine cocktail, and the cells were cultured on wells precoated with recombiant fibronectin fragment CH296 (Takara Shuzo, Otsu, Japan) for 48 hours.

**CFU-GM and thymidine suicide assay**

Mouse bone marrow cells cultured with retrovirus supernatant were collected and FACS sorted based on green fluorescence protein (GFP) expression. Ten thousand GFP+ cells were plated in 0.3% agar (Difco Laboratories, Detroit, MI) containing supplemented McCoy 5a medium with 15% HI-FBS, 10 ng/mL rmGM-CSF, and 50 ng/mL rmSCF.31 CFU-GMs were scored after 7 days’ incubation at 37° C in a humidified 5% CO₂, 5% O₂ air atmosphere. S-phase CFU-GMs were quantitated by thymidine suicide as previously described.32 Briefly, GFP+ cells were incubated with either 5 mg/mL thymidine (Sigma Chemical, St Louis, MO) or 50 μCi (1.85 MBq) [methyl-3H]thymidine (20 Ci/mmol [740 GBq/ mmol], New England Nuclear, Boston, MA) at 37° C for 30 minutes. Reactions were terminated by adding 100-fold excessive thymidine (300 μg/mL). Cells were washed twice with IMDM and CFU-GM/I × 10^6 cells quantitated as described above. In some experiments, growth factor addition was delayed for up to 48 hours and colonies scored after 10 days.

**Western blot analyses**

Western blot analyses were performed on lysates from 5 × 10^6 GFP+ cells from each transduced group using the rabbit polyclonal antihuman survivin (AF886) antibody. Preliminary flow cytometry and Western blot analyses experiments demonstrated the cross-reactivity of this antibody for murine survivin.

**Results**

**Expression of mRNA for inhibitor of apoptosis proteins in cord blood CD34+ cells**

Since we previously demonstrated that survivin is expressed and cytokine regulated in normal CD34+ cells, we examined expression and growth factor regulation of all other human IAPs. Comparison of mRNA before and after culture with Tpo, SCF, and FL by RT-PCR for 48 hours demonstrated that in addition to survivin, XIAP, c-IAP1, c-IAP2, and Apollon are expressed in fresh CD34+ cells (Figure 1). Livin and NAIP were not detectable. Only survivin mRNA expression was up-regulated after cytokine stimulation, whereas expression of c-IAP1 and c-IAP2 decreased. Identical results were observed in CD34+ cells from 5 UCB samples, although in 2 of 5 samples, c-IAP1 remained unchanged after culture with Tpo, SCF, and FL, while decreasing in 3 of 5 samples.

**Survivin, Rb protein, cyclin D, and Ki-67 expression in CD34+ cells**

A number of proteins have been linked to cell cycle entry. D cyclins are induced upon mitogenic stimulation in quiescent cells,33,35 and Ki-67 is a nuclear antigen found exclusively in proliferating cells,33,34,36,37 Upon mitogenic stimulation, Rb becomes phosphorylated, allowing cells to transit from G₁ to S phase.5,35,38-40 In unstimulated lymphocytes, underphosphorylated Rb predominates, and the proportion of cells with underphosphorylated Rb decreases within 3 to 8 hours of mitogenic stimulation.39 To address whether survivin up-regulation by growth factors in CD34+ cells occurs before or after cell cycle entry, survivin, total Rb, underphosphorylated Rb, phosphorylated Rb (Ser780, Ser795, and Ser807/811), D cyclins, Ki-67, and cell cycle status were measured by multivariate intracellular flow cytometry following cytokine stimulation. Survivin mRNA was also quantitated by real-time RT-PCR. Within 2 hours, cytokine-stimulated up-regulation of survivin mRNA and protein was observed coincident with up-regulation of D cyclins and Ki-67 (Table 1). Total Rb protein gradually increased, whereas phosphorylated Rb increased dramatically. Underphosphorylated-Rb protein remained constant or marginally decreased (not shown). The ratio of phosphorylated Rb to total Rb protein was dramatically elevated following growth factor addition, whereas the ratio of underphosphorylated Rb to total Rb gradually declined (Table 1). The increase in the percentage of S + G2/M phase cells correlated with up-regulation of survivin, Ki-67, D cyclins, and phosphorylated Rb. Because cells that express underphosphorylated Rb and are negative for Ki-67 and D cyclins are believed to be quiescent, expression of survivin in underphosphorylated-Rbnegative, Ki-67negative, and cyclin Dnegativenegative CD34+ cells was examined before and after growth factor stimulation (Table 2). Survivin protein was up-regulated by growth factors in underphosphorylated-Rbnegative, Ki-67negative, and cyclin Dnegative CD34+ cells as well as in underphosphorylated-Rbnegative, Ki-67negative, and cyclin Dpositive CD34+ cells, suggesting that survivin expression is up-regulated in quiescent cells before cell cycle entry.

**Survivin expression in G0 CD34+ cells before and after growth factor stimulation**

We previously demonstrated that survivin expression is elevated in G0 cells after incubation of unseparated CD34+ cells with cytokines.18 However, these studies did not address the issue of whether
these cells had yet to enter cell cycle or had already completed mitosis and returned to Go. We therefore investigated whether survivin up-regulation by growth factors observed in Go cells is specifically regulated before cells enter cell cycle. Fresh Go CD34+ cells were isolated based upon Hoechst 33342/pyronin Y (Hst/PY) staining (Figure 2A, gate R1) and incubated for 12 hours with 100 ng/mL each of Tpo, SCF, and FL. After culture, cells were stained with Hst/PY, and Hstlow, PPylow cells (gate R2), representing cells in Go, were collected by FACS sorting. Replicate freshly isolated Go cells were stained with CFSE before culture to monitor cell division. No cell division occurred during the 12-hour culture period as defined by CFSE analysis before sorting (Figure 2A). The purity of the Go population (Figure 2A, gate R2) assessed by Hst/PY sorting was 98% (Figure 2B, gate R2) and was 97% ± 2% in 5 experiments. No cell division in the Go CD34+ population (gate R2) was observed based on CFSE analysis. Because Ki-67 is undetectable in Go cells at 4 hours after growth factor incubation was verified at the mRNA level using quantitative real-time RT-PCR (Figure 2F; Table 3). Cells in the R1 and R2 gates were harvested and total RNA analyzed for survivin and Ki-67 expression. Ki-67 expression was essentially negative both before and after growth factor stimulation, confirming the quiescent nature of these cells. The threshold CT values for survivin were 34.7 and 37.8 in R2 and R1 cells, respectively, representing a 2.4 ± 0.7-fold increase in survivin mRNA in R2 cells compared to R1 cells (2 experiments). No increase in Ki-67 mRNA was observed in R2 cells compared with cells in R1 (0.8 ± 0.3-fold, 2 experiments), indicating that survivin is up-regulated in quiescent Go cells after cytokine stimulation. Real-time RT-PCR analysis of freshly isolated Go cells and CFSElow Go cells cultured for 48 hours with growth factors produced similar results (not shown).

**PI3-kinase/AKT and MAP kinase pathway inhibitors block survivin up-regulation by growth factors before cell cycle arrest**

Activation of the PI3-kinase and MAPKp42/44 pathways correlate with survival of CD34+ cells stimulated by hematopoietic growth factors.

**Table 1. Survivin, Ki-67, cyclin D, phosphorylated-Rb, underphosphorylated-Rb, and % S + G2/M cells in Tpo-, SCF-, and FL-stimulated CD34+ cells**

<table>
<thead>
<tr>
<th>Cell population</th>
<th>No. of experiments</th>
<th>2 hours after growth factor addition</th>
<th>4 hours after growth factor addition</th>
<th>6 hours after growth factor addition</th>
<th>18 hours after growth factor addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survivin protein</td>
<td>4</td>
<td>1.4 ± 0.2</td>
<td>1.8 ± 0.2†</td>
<td>2.4 ± 0.5‡</td>
<td>6.2 ± 2.4‡</td>
</tr>
<tr>
<td>Survivin mRNA</td>
<td>3</td>
<td>1.1 ± 0.1</td>
<td>1.2 ± 0.1†</td>
<td>2.1 ± 0.5‡</td>
<td>10.3 ± 3.8‡</td>
</tr>
<tr>
<td>%S + G2/M phase</td>
<td>4</td>
<td>7.5 ± 1.2</td>
<td>6.9 ± 1.0</td>
<td>8.5 ± 1.2‡</td>
<td>19.9 ± 3.1†</td>
</tr>
<tr>
<td>Ki-67</td>
<td>2</td>
<td>1.3 ± 0.01§</td>
<td>1.3 ± 0.02§</td>
<td>1.2 ± 0.01§</td>
<td>1.5 ± 0.2§</td>
</tr>
<tr>
<td>Cyclin D</td>
<td>5</td>
<td>1.1 ± 0.1†</td>
<td>1.2 ± 0.1‡</td>
<td>1.2 ± 0.1‡</td>
<td>1.5 ± 0.2‡</td>
</tr>
<tr>
<td>Phosphorylated Rb/Total Rb</td>
<td>2</td>
<td>1.6 ± 0.7†</td>
<td>1.5 ± 0.5†</td>
<td>1.8 ± 0.7†</td>
<td>2.3 ± 0.6†</td>
</tr>
<tr>
<td>Underphosphorylated Rb/Total Rb</td>
<td>3</td>
<td>0.8 ± 0.1†</td>
<td>0.7 ± 0.1†</td>
<td>0.7 ± 0.1†</td>
<td>0.5 ± 0.1§</td>
</tr>
</tbody>
</table>

Survivin, cyclin D, Ki-67, and underphosphorylated-Rb protein levels were determined by intracellular flow cytometry in the CD34+ cell population from UCB. Data are expressed as means ± SEM from 3 experiments.

*100 ng/mL each rhTpo, rhFL, rhSCF.
†P < .05.
‡P < .01.
§P < .005 compared with time 0.

**Table 2. Survivin expression in CD34+ cell populations following growth factor addition**

<table>
<thead>
<tr>
<th>Cell population</th>
<th>2 hours after growth factor addition</th>
<th>4 hours after growth factor addition</th>
<th>6 hours after growth factor addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34+, cyclin Dnegative</td>
<td>1.8 ± 0.3‡</td>
<td>2.2 ± 0.4‡</td>
<td>2.4 ± 0.4‡</td>
</tr>
<tr>
<td>CD34+, cyclin Dpositive</td>
<td>1.7 ± 0.2‡</td>
<td>2.3 ± 0.5‡</td>
<td>2.7 ± 0.5‡</td>
</tr>
<tr>
<td>CD34+, Ki-67negative</td>
<td>2.4 ± 0.2§</td>
<td>3.2 ± 0.2§</td>
<td>4.2 ± 0.2§</td>
</tr>
<tr>
<td>CD34+, Ki-67positive</td>
<td>3.9 ± 1.0‡</td>
<td>5.4 ± 1.8‡</td>
<td>6.5 ± 2.0‡</td>
</tr>
<tr>
<td>CD34+, underphosphorylated Rbmpositive</td>
<td>2.2 ± 0.2§</td>
<td>3.2 ± 0.3§</td>
<td>3.7 ± 0.6§</td>
</tr>
<tr>
<td>CD34+, underphosphorylated Rbmnegative</td>
<td>1.9 ± 0.3§</td>
<td>3.2 ± 0.5§</td>
<td>3.5 ± 0.6§</td>
</tr>
</tbody>
</table>

Survivin, cyclin D, Ki-67, and underphosphorylated-Rb protein levels were determined by intracellular flow cytometry in the CD34+ cell population from UCB. Data are expressed as means ± SEM from 3 experiments.

*100 ng/mL each rhTpo, rhFL, rhSCF.
†P < .05.
‡P < .01.
§P < .005 compared with time 0.
Survivin and cell cycle status were analyzed over time by flow cytometry. In control cultures, multistain- ing of survivin and DNA in CD34+ cells demonstrated that survivin expression was up-regulated in a time-dependent manner coincident with cell cycle progression (not shown). The PI3-kinase/AKT pathway inhibitor LY294002 (5 µM) reduced survivin expression by 15.2% ± 5.9% and 35.7% ± 11.7% (means ± SEM, 3 experiments) at 4 and 8 hours after cytokine stimulation, respectively, with no effect on cell cycle progression. At 18 hours, survivin expression was reduced by 44.7% ± 10.3% with a concomitant 57.3% ± 15.5% increase in the number of CD34+ cells in G0/G1 phase (Figure 3A). Similar results were observed with 1 µM LY294002. CD34+ cell viability was greater than 90% in all groups up to 8 hours after cytokine addition, but was ~85% in cells treated with 1 or 5 µM LY294002 at 18 hours. Inhibition of survivin was observed in both G0/G1 and S + G2/M cells (data not shown). The MAPK inhibitor PD98059 at 20 µM reduced survivin expression in CD34+ cells by 19.1% ± 8.0% at 8 hours, without any effect on the ability of CD34+ cells to progress through cell cycle. However, at 18 hours, survivin expression was reduced by 37.0% ± 9.5%, concomitant with an 18.6% ± 11% increase in G0/G1 cells (Figure 3B). Similar results were obtained using 40 µM PD98059. Forward and side scatter analysis indicated that greater than 90% of cells in all groups remained viable. Incubation of CD34+ cells with 20 and 40 µM PD98059 for longer than 24 hours or at concentrations greater than or equal to 60 µM for up to 20 hours inhibited survivin up-regulation and reduced cell cycle (not shown). Similar to LY294002, inhibition
Survivin transduced cells were positive for survivin (Figure 4A). Approximately 54.9% of human and mouse survivin, respectively, were survivin positive compared with 33.9% vector control cells, whereas 14.5% of antisense-mouse survivin reduced the proportion of S-phase CFU-GM to 42.1% ± 4.7% (P < .05, 3 experiments). Delayed addition of growth factors for 24 and 48 hours to vector or human (h)-survivin–transduced marrow cells resulted in progressive apoptosis of CFU-GM. However, after normalization for CFU-GM enhancement at time 0, 16% to 77% and 61% to 244% more CFU-GM (2 experiments) were observed in h-survivin–transduced cells after 24 and 48 hours of delayed growth factor addition, respectively, than in vector control cultures, suggesting that survivin overexpression blocked CFU-GM apoptosis caused by cytokine starvation.

Discussion

The IAP proteins are the only known endogenous caspase inhibitors that suppress apoptosis. The IAPs XIAP, c-IAP1, and c-IAP2 directly bind to and inhibit caspases 3, 7, and 9 via their BIR domains. In this report, we demonstrate that in addition to survivin, XIAP, c-IAP1, and c-IAP2 are expressed in CD34+ cells; however, survivin is the only cytokine-regulated IAP in these cells and therefore is the only likely IAP mediating suppression of apoptosis by hematopoietic cytokines. Similarly, survivin is the only IAP up-regulated by CD40 ligation in B-CLL cells. The relationship between survivin and other antiapoptotic molecules that play a role in hematopoietic cells, such as Bcl2, is not known. Both proteins are up-regulated in breast cancer cells, and their expression can be regulated by the tumor suppressor gene p53. Whether there is a direct link between the Bcl2 family members and survivin, or given the importance of apoptosis, that their effects are independent, remains to be determined.

Since the original reports that cancer cells and embryonic tissues, but not normal adult tissues, express survivin, others and we have described survivin expression in normal adult cells. Survivin expression is not observed in resting endothelial cells but is up-regulated in a cell cycle–dependent manner by vascular endothelial growth factor or angiopoietin-1. The murine homolog of survivin, Tap, is induced in T lymphocytes activated by mitogens. We previously reported that survivin is expressed and growth factor regulated in normal adult CD34+ cells and T lymphocytes in all phases of cell cycle. Because it is apparent that survivin is expressed in proliferating cells, the questions of whether survivin expression in CD34+ cells is cytokine regulated or simply reflects cell division and cell cycle progression and whether survivin expression affects proliferation and cell cycle of normal hematopoietic cells are raised. To address these questions, we examined survivin expression in CD34+ cells relative to the cell cycle markers Ki-67, cyclin D, and underphosphorylated-Rb and in Hstlow, PYlow, CFSEbright G0 CD34+ cells relative to Ki-67 expression. We also investigated the effects of PI3-kinase/AKT and MAPKp42/p44 pathway inhibitors on survivin expression and cell cycle in CD34+ cells. Finally, we introduced survivin cDNA into primary mouse bone marrow cells and examined the effects of modulating survivin expression on cell cycle and proliferation of
myeloid progenitor cells. Up-regulation of survivin expression in CD34+ cells by hematopoietic growth factors was coincident with up-regulation of phosphorylated Rb, D cyclins, and Ki-67, indicating that survivin expression parallels cell cycle progression. Multivariant intracellular staining of survivin, Rb, Ki-67, cyclin D, and DNA in CD34+ cells upon growth factor stimulation demonstrated that survivin is up-regulated not only in underphosphorylated-Rb-negative, cyclin D-positive, and Ki-67-positive cells, but also in the underphosphorylated-Rb-positive, cyclin D-negative, and Ki-67-negative cells. Isolation of G0, CD34+ cells sorted based on Hoechst 33342/Pyronin Y and staining with CFSE before incubation with growth factors indicated that survivin mRNA and protein are up-regulated in cytokine-stimulated G0, CD34+ cells that had not yet up-regulated Ki-67 and had not yet divided. These data demonstrate that survivin is up-regulated in CD34+ cells by growth factors during G0 before cells enter G1 and that survivin expression is specifically regulated by growth factors in CD34+ cells and not merely a consequence of cell cycle progression.

We have previously shown that like cancer cells, survivin expression in CD34+ cells is highest during G2/M.18 Our present study clearly demonstrates that unlike cancer cells, survivin expression is up-regulated in quiescent CD34+ cells following growth factor stimulation before cell cycle entry. This raises the question of whether survivin expression in quiescent CD34+ cells is unique to normal hematopoietic cells. Murine survivin (TIAP) mRNA has been demonstrated in quiescent T cells, though its expression was low.11 In addition, survivin up-regulation in synchronized NIH3T3 cells following 12 hours' serum stimulation was observed, where greater than 90% of the cells still remained in G0/G1 without any increase in S + G2/M cells.11 These studies indicate that survivin expression is observed before cell cycle entry in nonhematopoietic cells. Li et al, who reported the specific expression of survivin during G2/M in HeLa cells, used cell cycle synchronization and Northern and Western analyses15 to demonstrate that treatment of HeLa cells with mimosine reduced survivin expression coincident with G1 arrest. Failure to detect survivin expression in G0-arrested cells might be due to the methodology employed. We used real-time RT-PCR and intracellular flow cytometry to demonstrate G0 expression of survivin mRNA and protein, which are more sensitive. Furthermore, survivin inhibition by mimosine could be due to a direct effect on survivin expression rather than an effect on cell cycle arrest.

The selective PI3-kinase/AKT pathway inhibitor LY294002 and MAPK(p42/p44) pathway inhibitor PD98059 significantly blocked up-regulation of survivin expression by growth factors in CD34+ cells. Survivin protein expression decreased in the absence of any effect on cell cycle progression or cell viability, at least during the initial 8 hours, although there was reduction of cell cycle progression after longer exposure. Most importantly, these compounds inhibited up-regulation of survivin expression before an arresting effect on cell cycle was observed. If survivin expression were a result of cell cycle progression, reduction of survivin would be accompanied by cell cycle arrest. Although we did not analyze changes in the proportion of G0 and G1 cells, these data strongly suggest that survivin expression in CD34+ cells is regulated by growth factors and is not merely a consequence of cell cycle progression. These findings also indicate that both the PI3-kinase/AKT and MAPK(p42/p44) pathways are involved in cytokine regulation of survivin expression in normal CD34+ cells, which is consistent with the involvement of these pathways in regulating survivin expression in AML cells.46

Overexpression of human or mouse survivin cDNA in primary mouse bone marrow cells dramatically enhanced CFU-GM proliferation and the proportion of CFU-GM in S phase of the cell cycle. An antisense-mouse survivin construct had the opposite effect. These findings indicate that modulating survivin expression modulates proliferation and cell cycle of primary hematopoietic progenitor cells. Although we did not quantitate survivin protein levels in survivin or antisense survivin-transduced CD34+ cells due to technical limitations, an increase in survivin-positive marrow cells could be quantitated by flow cytometry following transduction with human or mouse survivin. A decrease in survivin-positive cells was observed following transduction with antisense mouse survivin. A similar trend in survivin protein expression in transduced primary marrow cells was observed by Western analysis. In addition, following transduction of these same MIEG3 constructs into BaF3 cells, elevated protein levels of h-survivin (237%–466% increase, 3 experiments), mouse (m)-survivin (118%–287% increase, 3 experiments), and antisense-m-survivin (9%–25% decrease in survivin protein levels, 3 experiments) were observed.
Survivin has been shown to interact with cdk4, and overexpression of survivin enhances Rb phosphorylation in hepatoma cells by releasing p16\(^{INK4a}\) and p21\(^{WAF1/CIP1}\) from the cdk4/p16\(^{INK4a}\) and cdk4/p21\(^{WAF1/CIP1}\) complexes, respectively.\(^1^2-2^3\) This suggests that survivin promotes cell cycle progression by inactivating the p16\(^{INK4a}/\)Rb pathway, a finding consistent with our observation that overexpression of survivin enhances the cell cycle rate of primary mouse CFU-GM. Because survivin is an antiapoptotic protein, it is possible that the enhanced CFU-GM proliferation observed following survivin transduction results from increased CFU-GM survival. This is consistent with reduced apoptosis of survivin-transduced CFU-GM observed in cultures in which growth factor addition was delayed. However, this does not adequately explain the increase in the proportion of S-phase CFU-GMs observed following survivin transduction. There is no reason to assume that enhanced CFU-GM survival means that the additional surviving cells are in S phase.

In conclusion, we have provided evidence demonstrating that survivin expression in CD34\(^+\) cells is growth factor regulated and not a consequence of cell cycle progression. First, survivin is up-regulated in CD34\(^+\) cells that are underphosphorylated Rb\(^{hyp}\), Ki-67\(^{negative}\), and cyclin-D\(^{negative}\), that is, quiescent cells. Second, survivin up-regulation by growth factors occurs in G0 CD34\(^+\) cells before these cells enter G1. Third, inhibition of survivin by selective PI3-kinase/AKT and MAPK\(^{42,44}\) pathway inhibitors occurs before cell cycle arrest. Finally, survivin overexpression enhances and antisense survivin reduces cell cycle rate and proliferation of CFU-GM. Taken together, these data demonstrate that survivin expression is specifically regulated by growth factors in quiescent CD34\(^+\) cells and strongly suggest that up-regulation of survivin is an early and important event for cell cycle entry and proliferation of normal hematopoietic stem and progenitor cells.

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References


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The antiapoptosis protein survivin is associated with cell cycle entry of normal cord blood CD34+ cells and modulates cell cycle and proliferation of mouse hematopoietic progenitor cells

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