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Serum Suppresses Myeloid Progenitor Apoptosis by Regulating Iron Homeostasis

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Abstract The growth and survival of committed hematopoietic progenitors is dependent upon cytokine signaling. However, serum is also required for optimal growth of these progenitors in culture ex vivo. Here we report that serum withdrawal leads to myeloid progenitor cell apoptosis. Although serum deprivation-induced cell death has many hallmarks typical of apoptosis, these cell deaths were not inhibited by hemopoietins, survival factors such as IGF-I, or treatment with a broad-spectrum caspase inhibitor. Rather, apoptosis due to serum withdrawal was associated with damage to mitochondria. Surprisingly the serum factor required for myeloid cell survival was identified as iron, and loss of iron led to marked reductions in ATP production. Furthermore, supplementing serum-deprived myeloid cells with bound or free iron promoted cell survival and prevented mitochondrial damage. Therefore, serum suppresses hematopoietic cell apoptosis by providing an obligate source of iron and iron homeostasis is critical for proper myeloid cell metabolism and survival. J. Cell. Biochem. 82: 171–186, 2001.

Key words: serum; apoptosis; myeloid; mitochondria; iron

Apoptosis is an evolutionary conserved and innate process by which cells systematically inactivate, dissemble, and degrade their own structural and functional components to induce their suicide [Wyllie et al., 1980]. Apoptosis is achieved through activation of genetically defined developmental programs, or can be induced by external factors such as cytokines, hormones, radiation, oxidative stress or growth factor deprivation. The ability of a cell to undergo apoptosis is related to its proliferative status, cell cycle position, and to the regulated expression of genes that promote, inhibit or affect the death program.

Upon receipt of an apoptotic signal, cells activate numerous signaling pathways that serve to regulate the cell death process [Bortner and Cidlowski, 1999]. Important targets of these pathways are the caspase family of aspartate-directed, cysteine-dependent, proteases that cleave key targets required for cellular integrity. These substrates include poly-ADP-ribose polymerase (PARP), which is cleaved by caspases-3 and -7, and the nuclear lamins [Lazebnik et al., 1994; Fernandes-Alnemri et al., 1995], which are cleaved by caspase-6 [Orth et al., 1996; Takahashi et al., 1996]. Many caspases are present in cells as proenzyme forms that are inactive zymogens. However, pro-caspases themselves harbor caspase-recognition sites and are substrates for initiator caspases such as caspase-8 and caspase-9 [Cohen, 1997]. Therefore, there is hierarchical activation of caspases following the receipt of signals that induce apoptosis.

The initiation and efficient completion of apoptosis is generally dependent upon upstream regulators of the cell death program. Key controllers of apoptosis include the Bcl-2 family of proteins that serve to either suppress (e.g., Bcl-2, Bcl-X_L, and Mcl-1) or activate (e.g., Bax, Bak, and Bad) the cell death program [Gross et al., 1999]. Bcl-2 family proteins appear to regulate apoptosis by controlling the integrity of key organelles such as mitochondria and the

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endoplasmic reticulum. For example, most anti-apoptotic Bcl-2 family members are localized to the outer membrane of mitochondria and to the endoplasmic reticulum membrane, and most pro-apoptotic family members disrupt the integrity of these membranes [Gross et al., 1999].

Changes in mitochondrial biogenesis and function are a hallmark of almost all forms of apoptosis. An initial event is a drop in mitochondrial membrane potential (ψA_m), which is required to maintain an asymmetric distribution of charges between the inner and outer mitochondrial membranes [Vayssiere et al., 1994; Zamzami et al., 1995]. The ψA_m facilitates exchange between ATP and ADP, between the cytosol and the mitochondrial matrix and is necessary for ATP synthesis. Reductions in ψA_m result in the loss of outer mitochondrial membrane permeability [Vander Heiden et al., 2000] and to concomitant reductions in mitochondrial gene transcription and translation. There is also a rapid uncoupling of the respiratory chain from oxidative phosphorylation [Wolvetang et al., 1994]. The loss of anion permeability appears to cause changes in the conductance of the voltage-dependent anion channel (VDAC), which has been proposed as the key regulatory target of anti-apoptotic Bcl-2 family proteins [Shimizu et al., 1999]. If disruption of the ψA_m persists, the integrity of the outer membrane is compromised and results in the release of pro-apoptotic factors. The latter include cytochrome *c*, which activates the caspase-9 regulator Apaf-1 [Jiang and Wang, 2000], and apoptosis-inducing factor (AIF), which can induce death independent of caspase activation [Susin et al., 1999].

Because of their key regulatory role, the Bcl-2 family is targeted in many signaling pathways that regulate apoptosis. For example, survival factors such as hemopoietins, which are continuously required to suppress the apoptosis of hematopoietic progenitors [Williams et al., 1990], appear to target the Bcl-2 family at least at three levels. First, cytokine signaling activates the serine/threonine kinase AKT/PKB, which phosphorylates Bad [del Peso et al., 1997] a pro-apoptotic family member [Yang et al., 1995], disrupting its association with Bcl-X_L and thus promoting survival [Zha et al., 1996]. Second, the subcellular localization of at least some Bcl-2 family members is a regulated process [Hsu et al., 1997; Gross et al., 1998]. For example, following the withdrawal of cytokines, Bax re-localizes from the cytosol to the mitochondria [Putcha et al., 1999], where it inserts and induces expansive disruption of the outer membrane [Basanez et al., 1999]. Finally, the expression of many family members is cytokine dependent. For example, in hematopoietic progenitors cytokines are required for the selective induction of Bcl-X_L [Packham et al., 1998] and for the repression of the pro-apoptotic member Hrk [Sanz et al., 2000].

In addition to the requirement for cytokines and mitogens, the ex vivo proliferation and survival of most cells including hematopoietic progenitors, requires serum components. Serum contains several polypeptides, principally insulin-like growth factor-I (IGF-I) and platelet derived growth factor (PDGF), which suppress the apoptotic program [Barres et al., 1992; Harrington et al., 1994]. Furthermore, these survival factors are important antagonists of cell death pathways induced by the inappropriate expression of oncoproteins such as c-Myc [Harrington et al., 1994; Jung et al., 1996; Tateishi and Yamaizumi, 1997]. Here we report that serum deprivation of cytokine-dependent hematopoietic progenitors results in an unusual form of apoptosis. Although many hallmarks of typical apoptosis are observed, serum withdrawal overrides the protective effects of hemopoietins or of a broad-spectrum caspase inhibitor. Rather, serum deprivation is associated with deficits in iron that cause rampant damage to mitochondria that appear due to the depletion of ATP levels. These results underscore the requirements for serum-derived sources of iron for cell growth and survival, and demonstrate that regimens that disrupt iron homeostasis compromise hematopoietic, and thus perhaps leukemic, cell survival.

MATERIALS AND METHODS

Cell Culture and Retroviral Infections

The 32D.3 and FDC-P1.2 cell lines are diploid, IL3-dependent, and non-tumorigenic myeloid cell lines derived from normal mouse bone marrow [Greenberger et al., 1983; Valtieri et al., 1987; Askew et al., 1991; Packham and Cleveland, 1997]. 32D.3 cells engineered to overexpress human Bcl-2 were as previously described [Nip et al., 1997]. Cells were grown in liquid culture in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) (Bio-Whittaker, Walkersville, MD), 2 mM L-

glutamine (BioWhittaker), and 20 U of IL-3 /ml at 37°C, unless otherwise specified. To ensure cells were in exponential growth phase before analysis they were passaged for two sequential days at 5×10^5 cells/ml, and on the third day were treated as indicated in the text, again at a final density of 5×10^5 cells/ml [Askew et al., 1991]. In experiments addressing cell death, viability was assessed using the trypan blue dye exclusion. To assess effects of IL-3 and/or serum withdrawal on cell death, cultures were washed three times in RPMI-1640 medium and then cultured in medium with or without IL-3 and 10% or 0.1% FCS.

Fetal liver-derived myeloid progenitors were isolated from day E15.5 embryos. Cells were cultured for 3–4 weeks in RPMI-1640 medium supplemented with IL-3 (20 U/ml), IL-6 (10 ng/ml, R&D Systems), SCF (10 ng/ml, R&D systems) and 10% FCS. The phenotype of the cells as determined by FACS was uniformly $CD34^+$, c-Kit^+, $ScaI^+$ and negative for all more mature myeloid (Mac1, Mac2, and GR1), erythroid (Ter119), and lymphoid markers (B220, CD4, CD8). To assess the effects of cytokine and/or serum withdrawal on cell death, cultures were washed three times in RPMI-1640 medium and then plated in medium with or without the three cytokines and 10% or 0.1% FCS.

Iron, Transferrin and Iron Chelators

Desferrioxamine (DFO, Desferal) was obtained as a lyophilized mesylate from Novartis (Basle, Switzerland). It was dissolved as a 10-fold concentrated stock in PBS and added to cells at the indicated concentration. Hexadentate chelators such as DFO coordinate iron in a 1:1 ratio [Hider et al., 1996]. Chelator-iron complexes were fully saturated by achieving a 1:1 ratio of DFO:iron using ferric ammonium citrate (FAC). Cells were resuspended in media as described and incubated for the indicated intervals at 37°C with the following: (1) DFO (100 μM) alone or saturated with FAC (100 μM) (Sigma), (2) FAC alone (100 μM), (3) iron-bound transferrin (50 μM) (Sigma), or (4) iron-free transferrin, (50 μM) (T7786, Sigma).

Apoptosis Assays

The cells cultured as described in the text were analyzed for their morphology following cytospin and Wright-Geimsa staining. For electrophoretic analysis of DNA fragmentation, 1×10^6 cells were spun at 2,000 rpm for 5 min, and the cell pellets resuspended in 20 μl of lysis buffer (containing 500 mM EDTA, 1M Tris [pH 7.4], 10% sodium lauryl sarkosyl and 10 mg/ml proteinase K) and incubated at 4°C overnight. After incubation at 50°C for 1 h, RNase (0.5 mg/ml) was added for 1 h and the samples then warmed to 70°C. Ten μl of loading buffer was then added and the DNA loaded into dry wells of a 2% agarose gel containing 0.1 μg/ml ethidium bromide. The samples were run in 1× TBE at 100 V until the marker dye had migrated approximately 4 cm. DNA laddering was visualized using a UV trans-illuminator [Smith et al., 1989].

Annexin-V-FITC staining was carried out as described by the manufacturer (BioWhittaker Inc. Walkersville. MD). Briefly $2–5 \times 10^5$ 32D.3 cells/ml were washed in PBS and resuspended in 190 μl binding buffer (10 mM HEPES [pH 7.4], 140 mM NaCl, 2.5 mM CaCl). Ten μl Annexin V-FITC was then added, the samples incubated for 10 min, and the cells were washed once and resuspended in 190 μl binding buffer. To this was added 10 μl of 20 μg/ml propidium iodide stock solution. Cells were then analyzed for the presence of apoptotic cells using FACS analysis.

Apoptosis was also quantified by a loss of mitochondrial membrane potential (ψA_m). This was assessed with the fluorescent probe JC-1 (Molecular Probes) which was added to the cells (50 μM) for 30 min, the samples washed twice in PBS and analyzed by flow cytometry as previously described [Vanags et al., 1996; Bortner and Cidlowski, 1999; Dinsdale et al., 1999].

To assess whether caspase inhibition would prevent serum deprivation-induced cell death 32D.3 cells were pre-treated for 1 h with 400 μM zVAD-fmk (Calbiochem) prior to the exposure of the apoptotic stimulus. This concentration of zVAD-fmk effectively delays death of 32D.3 cells when they are deprived of IL-3 [Dai et al., 1999].

Immunoblot Analysis

Whole cell protein extracts from 32D.3 cells were isolated as previously described [Eischen et al., 1999]. Protein (50 μg/lane) was electrophoretically separated in 10% polyacrylamide gels containing SDS. Proteins were transferred to nitrocellulose membranes (Protran, Schleicher & Schuell, Dassel, Germany) and blotted with antibodies specific for murine Bcl-2 (15021, PharMingen, 1:250), Bcl-X_L (B2260, Transduc-

tion Labs, 1:250), Mcl-1 (B54020, Transduction Labs, 1:500), caspase-3 (Transduction Labs, 1:2500) and caspase-9 (kindly provided by Dr. D. Green, 1:1000) and β-Actin (Sigma, 1:2500). Incubation with primary antibodies was then followed either by anti-mouse or anti-rabbit secondary antibodies (DAKO). Bound immunocomplexes were detected by enhanced chemiluminescence (Amersham) or Supersignal (Pierce).

Measurement of ATP Levels

ATP levels were measured by the luciferin/luciferase method using the ATP Bioluminescence Assay Kit HS II (Roche Molecular Biochemicals). Luciferase reagent (100 μL) was injected into 100 μL of lysate, and the luminescence was analyzed after a 10 s delay with a 2 s integration on a Tropix TR717 Luminometer. A standard curve was generated from known concentrations of ATP (in the range of 10^{-6} and 10^{-12}M) and used to calculate the subsequent concentration of ATP in each sample. Luminescence increased linearly with the negative log of the ATP concentration in the samples over the range of concentrations measured. For the test samples, 32D.3 cells were diluted to a concentration of 10^5 cells/ml and ATP measured as outlined by the manufacturer.

RESULTS

Serum is Required for Myeloid Progenitor Cell Survival

IL-3 is required for the growth and survival of primary myeloid progenitors and established myeloid cell lines [Williams et al., 1990; Spooncer and Dexter, 1997]. However, ex vivo culture medium also typically includes serum and it is unclear whether serum also plays a role in regulating hematopoietic progenitor cell survival. We initially assessed the requirement for serum in FDC-P1.2 and 32D.3 murine myeloid progenitor cells, which provide excellent in vitro models, as both cell lines are cultured in IL-3 and serum and when deprived of IL-3 undergo cell cycle arrest and die by apoptosis [Askew et al., 1991; Packham and Cleveland, 1997]. To assess the effects of serum withdrawal, 32D.3 and FDC-P1.2 cells were grown in medium supplemented with IL3 (20 U/ml) and either 10% fetal calf serum (FCS) or 0.1% FCS. Culture of 32D.3 or FDC-P1.2 cells in IL-3 medium having low serum induced a time dependent decrease in live cell number and a concomitant increase in cell death, as judged by the failure of cells to exclude the dye trypan blue (Fig. 1A and B). There was a progressive increase in dead cells, such that by 72 h in serum deprived media 75% of 32D.3 cells and 63% of FDCP.1 cells were dead (Fig. 1A and B). In contrast, cells cultured in complete medium displayed no loss in viable cell number. The rate of death of myeloid cells was somewhat protracted versus rate of death when these cells were deprived of IL-3. However, rate of death were markedly accelerated when cells were deprived of both IL-3 and serum (Fig. 1A and B), confirming that both were independently required for survival.

FDC-P1.2 and 32D.3 cells are immortal cell lines derived from bone marrow culture [Dexter et al., 1979; Greenberger et al., 1983]. Immortalizing events associated with culture usually involve inactivation of the ARF-p53 tumor suppressor pathway [Harvey and Levine, 1991; Kamijo et al., 1997; Sherr and DePinho, 2000], and in accord with this notion both of these myeloid cell lines fail to express ARF RNA or protein (JLC, unpublished data). Since these events could theoretically impact the regulation of cell survival, we isolated primary myeloid progenitors by culture of embryonic day E15.5 murine fetal liver hematopoietic cells in medium containing IL-3, IL-6, SCF, and 10% FCS. After three weeks in culture these cells were $>98\%$ cD34+, c-Kit+, Sca-1+ progenitors, and these cells can be maintained in culture for 2–3 months before undergoing replicative crisis [Packham et al., 1998]. As expected, withdrawal of the cytokines led to myeloid cell death, but a comparable rate of cell death was also observed when the cells were cultured in medium with the full complement of cytokines but having only 0.1% FCS (Fig. 1C). Therefore serum contains a factor(s) that is required for both primary and immortal myeloid cell survival.

FACS analysis of all three myeloid cells deprived of only serum demonstrated a hypodiploid peak of sub-G1 (<2N) DNA (data not shown), indicating that DNA degradation was occurring in dying cells. This was confirmed by isolating total cellular DNA from cultures of 32D.3 cells cultured in the absence of serum for 48 h, which revealed a typical "DNA ladder" of genomic DNA (Fig. 2A) that is a hallmark of apoptosis [Wyllie et al., 1980]. Furthermore, analysis of cytospins of cells deprived of serum revealed typical morphological changes

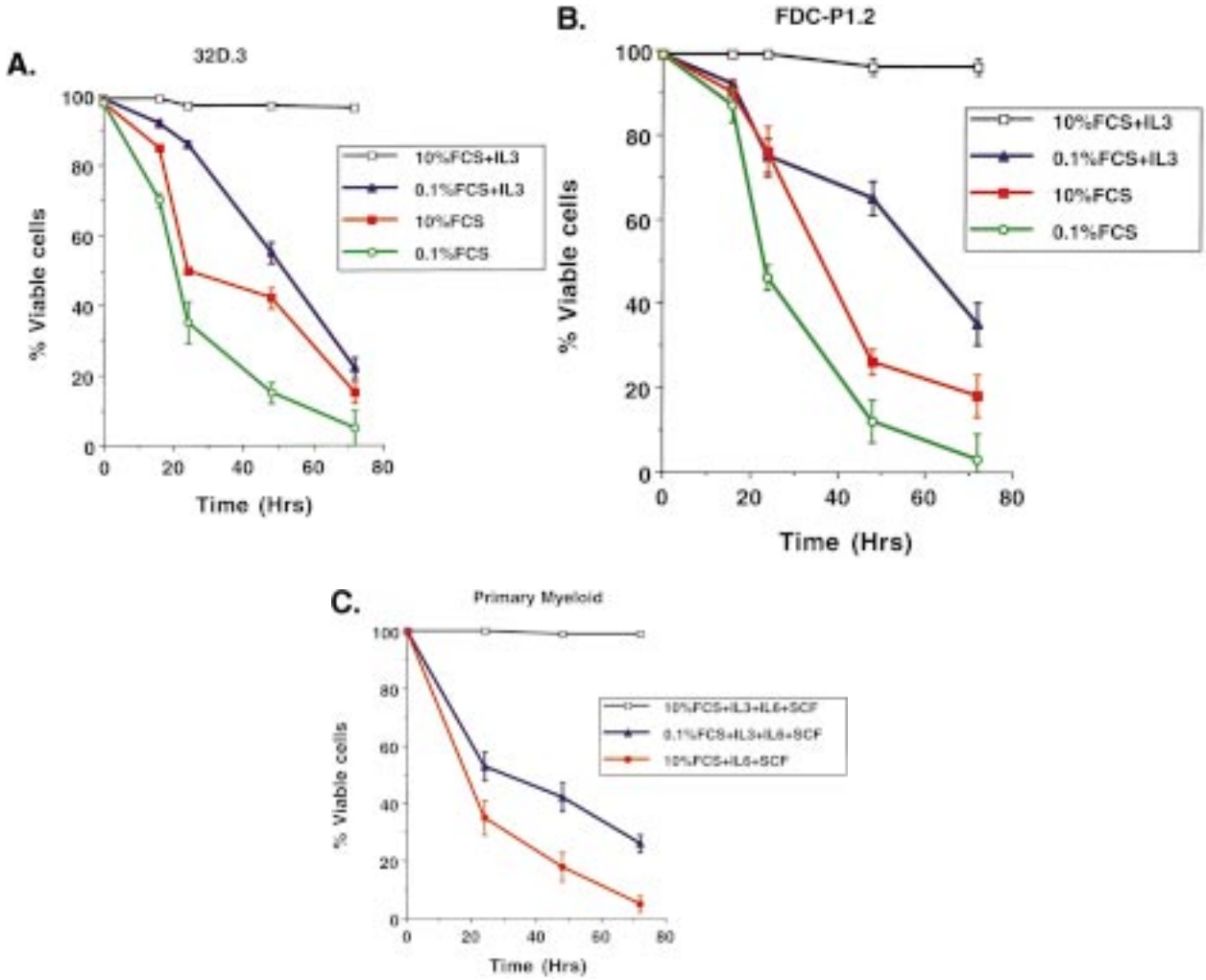
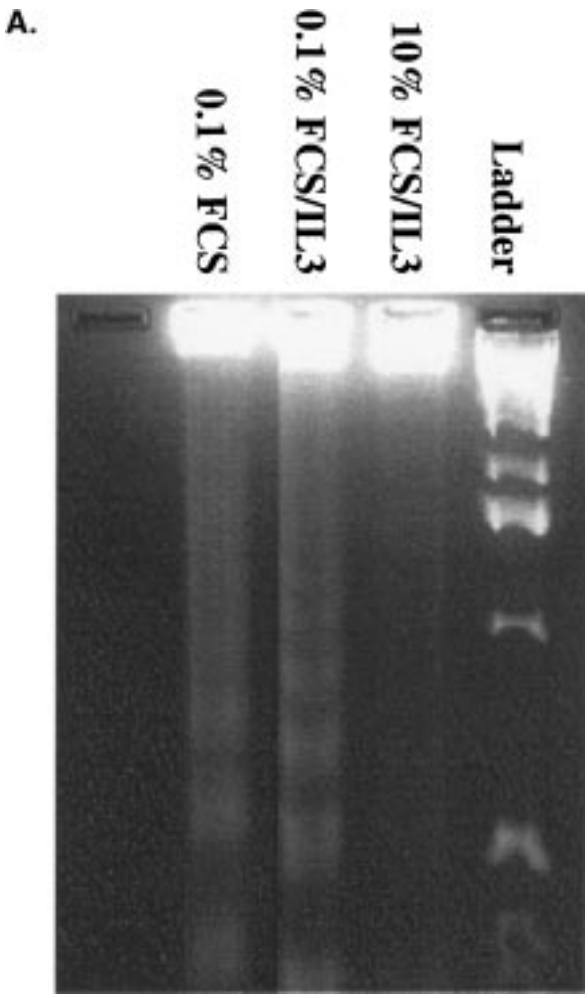


Fig. 1. Serum deprivation induces cell death of primary and established myeloid progenitor cells. 32D.3 (**A**) or FDC-PI.2 (**B**) cells, cultured in IL-3 and 10% FCS, were washed twice in PBS and then incubated (5×10^5 cells/ml) for the designated intervals in the medium indicated. Viability was determined by trypan blue dye exclusion. Results shown are the mean of eight independent experiments +/− standard deviation. (**C**) Primary myeloid progenitor cells were isolated from culture of day E15.5 fetal liver cells in RPMI-1640 medium supplemented with IL-3, IL-6, SCF and 10% FCS. Cells (5×10^5 cells/ml) were washed twice in PBS and then cultured in the indicated medium. At the intervals noted, viability was determined by trypan blue dye exclusion. Results shown are the mean of three separate experiments +/− standard deviation.

associated with apoptosis, including membrane blebbing and nuclear condensation followed by fragmentation into micronuclei (Fig. 2B). An early event following the induction of apoptosis is the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. This exposes PS, which can be detected with Annexin V-FITC by FACS analysis [Bossy-Wetzel and Green, 2000]. Indeed, there were marked increases in Annexin-V positive 32D.3 cells when they were cultured in low serum IL-3 medium, whereas cells cultured in complete medium were essentially Annexin-V negative (Fig. 2C). The cleavage of genomic DNA into oligonucleosomal fragments is caused by the caspase-3-dependent cleavage of DFF45/ICAD, an inhibitor of the DNA endonuclease DFF40/CAD [Liu et al., 1998; Liu et al., 1999]. Immunoblot analyses confirmed that the apoptosis caused by serum withdrawal was indeed associated with the proteolytic cleavage of both caspases-3 and -9 (Fig. 2D and data not shown). Therefore, myeloid cells deprived of serum undergo many of the hallmarks that are associated with apoptosis seen when myeloid cells are deprived of IL-3 [Askew et al., 1991; Kinoshita et al., 1995; Dai et al., 1999].

IGF-1 Suppresses Myeloid Cell Apoptosis Caused by IL-3, but not Serum, Withdrawal

Several years ago, Evan et al. [1992] proposed that the induction of apoptosis in serum-deprived fibroblasts could be due to either nutrient deprivation or to the absence of cytokines. Subsequent studies pointed to the actions of insulin-like growth factor-1 (IGF-1) and platelet derived growth factor (PDGF) as the principle components present in serum that block fibroblast cell death when they are deprived of serum, or when they are engineered to overexpress the c-Myc oncoprotein [Harrington et al., 1994]. 32D.3 and FDC-P1.2 cells express receptors for IGF-1 but not PDGF [JLC, unpublished], and we therefore assessed whether IGF-1 was the serum factor required to promote myeloid cell survival. Cells were deprived of either IL-3 or serum (0.1% FCS) and supplemented with IGF-1 (100 ng/ml). The addition of IGF-1 effectively delayed the death of both 32D.3 and FDC-P1 cells cultured in IL-3-free medium supplemented with 10% FCS (Fig. 3A and B). In contrast, the addition of IGF-1 to cells cultured in IL-3 and 0.1% FCS failed to delay their rates of cell death (Fig. 3A and B). Again, as judged by typical changes in morphology, cell deaths seen in low serum cultures were apoptotic. Suppression of apoptosis in serum deprived 32D.3 and FDC-P1 myeloid cells must therefore involve other "factors".



Iron is the Serum Factor Required for Myeloid Cell Survival

Over a decade ago it was recognized that cell proliferation in tissue culture required the presence of transferrin [Bottenstein, 1986]. Transferrin is an essential transporter of iron and iron-bound transferrin has high affinity for the transferrin receptor. With the exception of mature erythrocytes, transferrin receptors are probably expressed on all cells with high numbers found on rapidly dividing normal cells [Ponka et al., 1998]. Following binding of the transferrin-iron complex to this receptor, endocytosis occurs and iron is released within the cell [Aisen, 1994; Richardson and Ponka, 1997; Ponka et al., 1998], where it is required for

Fig. 2. Serum withdrawal induces apoptosis of myeloid cells. **A:** Serum deprivation results in cleavage of genomic DNA. Exponentially growing 32D.3 cells cultured in IL-3 and 10% FCS were washed twice in PBS and then cultured in the indicated medium. Genomic DNA from 1×10^6 cells was prepared after 48 h and analyzed by agarose gel electrophoresis as described [Askew et al., 1991]. Note the typical DNA ladder in cells deprived of serum or IL-3. **B:** Serum deprivation induces morphological changes typical of apoptotic cells. Exponentially growing 32D.3 cells, and cells cultured in low (0.1% FCS) serum medium for 24 h, were analyzed for morphology by staining cytospin preparations with Wright-Geimsa. Photomicrographs of a field of typical cells are shown. Apoptotic cells are indicated by arrows. **C:** 32D.3 cells in low serum have typical alterations in their cell membrane. An early event during apoptosis is the flipping of phosphatidylserine from the inner to the outer leaflet of the plasma membrane, and PS can be detected by flow cytometry using FITC conjugated Annexin-V [Bossy-Wetzel and Green, 2000]. Results shown are cells cultured in complete medium (top panel) versus cells cultured in low serum medium (bottom panel). The percentages of apoptotic cells are given. Results shown are representative of four separate experiments. **D:** Caspase-3 is proteolytically cleaved in 32D.3 cells deprived of serum. Cells were cultured, as indicated, for 24 h, extracts prepared and 100 μg of cell extract analyzed for caspase-3 by immunoblot analyses. Results shown are representative of two separate experiments. Analyses of FDC-P1.2 and primary myeloid cells deprived of serum showed similar results for Annexin-V binding and/or typical changes in morphology (data not shown).

B.

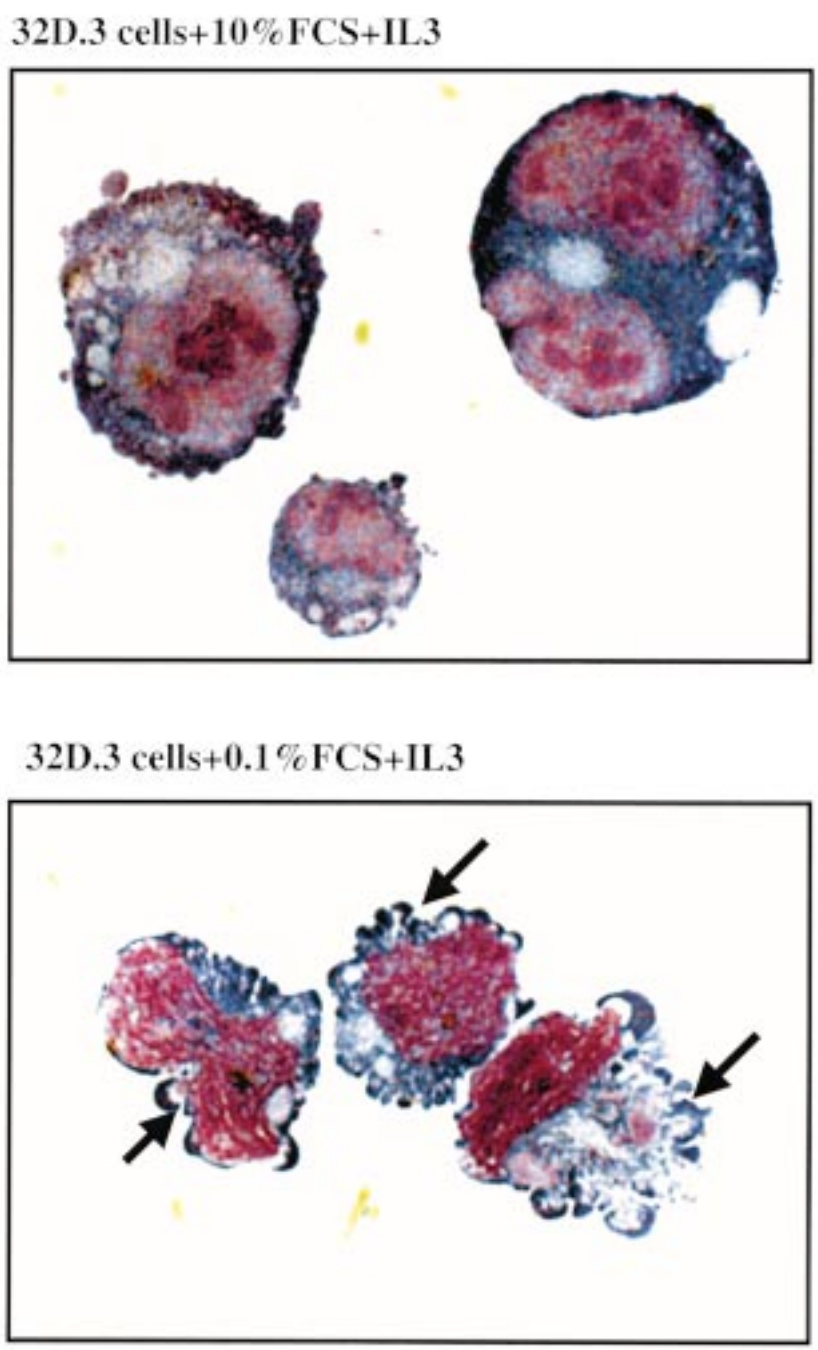


Fig. 2. (*Continued*)

many cellular functions, including energy metabolism, cellular respiration and cell proliferation [Boldt, 1999]. We, therefore, hypothesized that requirement for FCS simply reflected serum as an essential nutrient source of iron. In particular, cells deprived of iron are unable to proceed into the S-phase of the cell cycle, and this is thought to be due to inhibition of ribonucleotide reductase, an iron-containing enzyme required for the production of deoxyribonucleotides for DNA synthesis [Lederman et al., 1984; Alcain et al., 1991].

The direct measurement of iron levels in 0.1% FCS medium was, as expected, two orders of magnitude lower that the levels present in 10% FCS (data not shown). We, therefore, initially

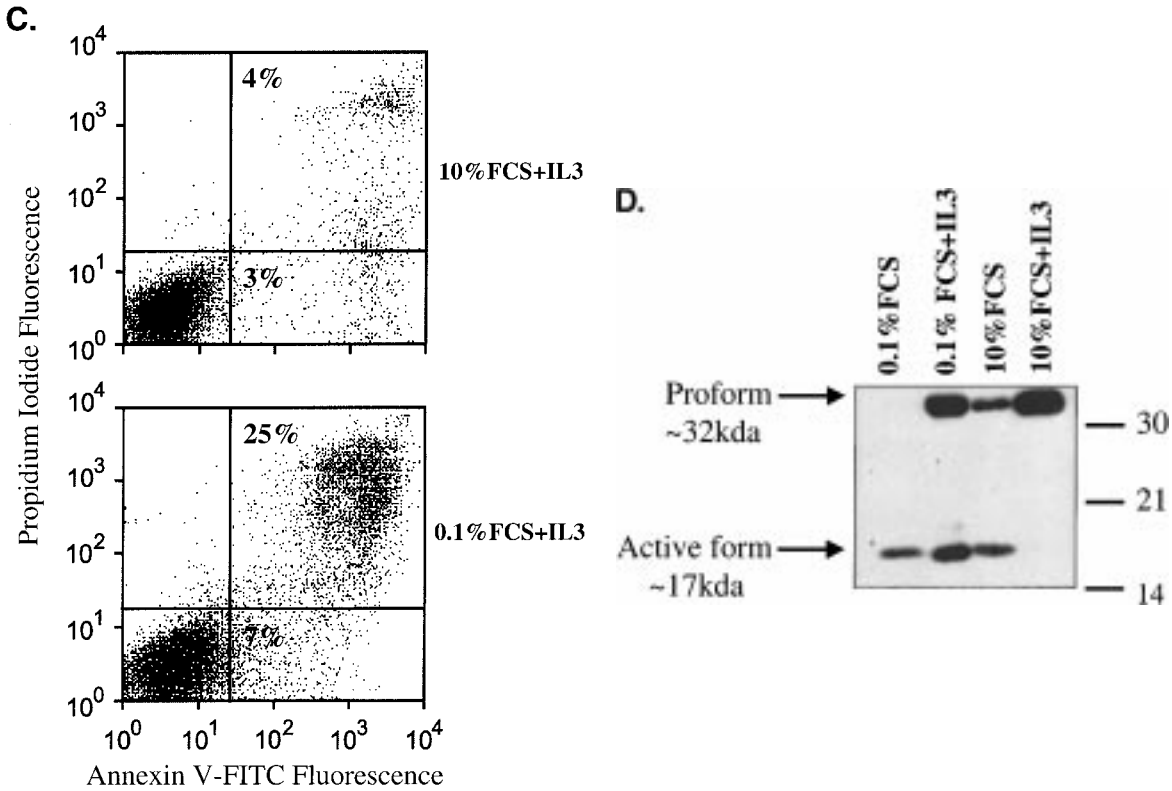


Fig. 2. (*Continued*)

tested the effects of both iron-bound and iron-free transferrin on the viability of myeloid cells deprived of serum, but cultured in IL-3. Notably addition of iron-bound transferrin, but not iron-free transferrin, effectively blocked apoptosis when myeloid cells were deprived of serum (Fig. 4A). Furthermore, the addition of ferric ammonium citrate (FAC, 100 μM) to the medium totally blocked apoptosis of myeloid cells cultured in IL-3 but deprived of serum (Fig. 4B). Finally, to mimic the loss of iron pools in serum depleted media, we also cultured 32D.3 and primary myeloid cells with the ferric-specific iron chelator, Desferrioxamine (DFO).

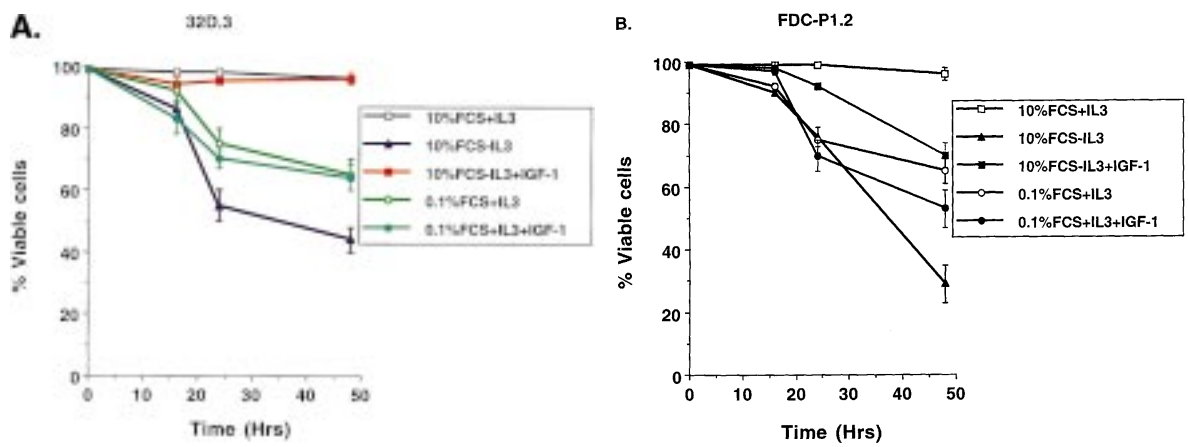


Fig. 3. IGF-1 suppresses apoptosis caused by IL-3, but not serum, withdrawal. **A:** 32D.3 and FDC-P1.2 (**B**) cells were cultured in the indicated media and viability was assessed by trypan blue dye exclusion. Results shown are the mean of six experiments performed. Note that IGF-1 effectively delayed death of myeloid cells deprived of IL-3, but failed to suppress the death of myeloid cells deprived of serum.

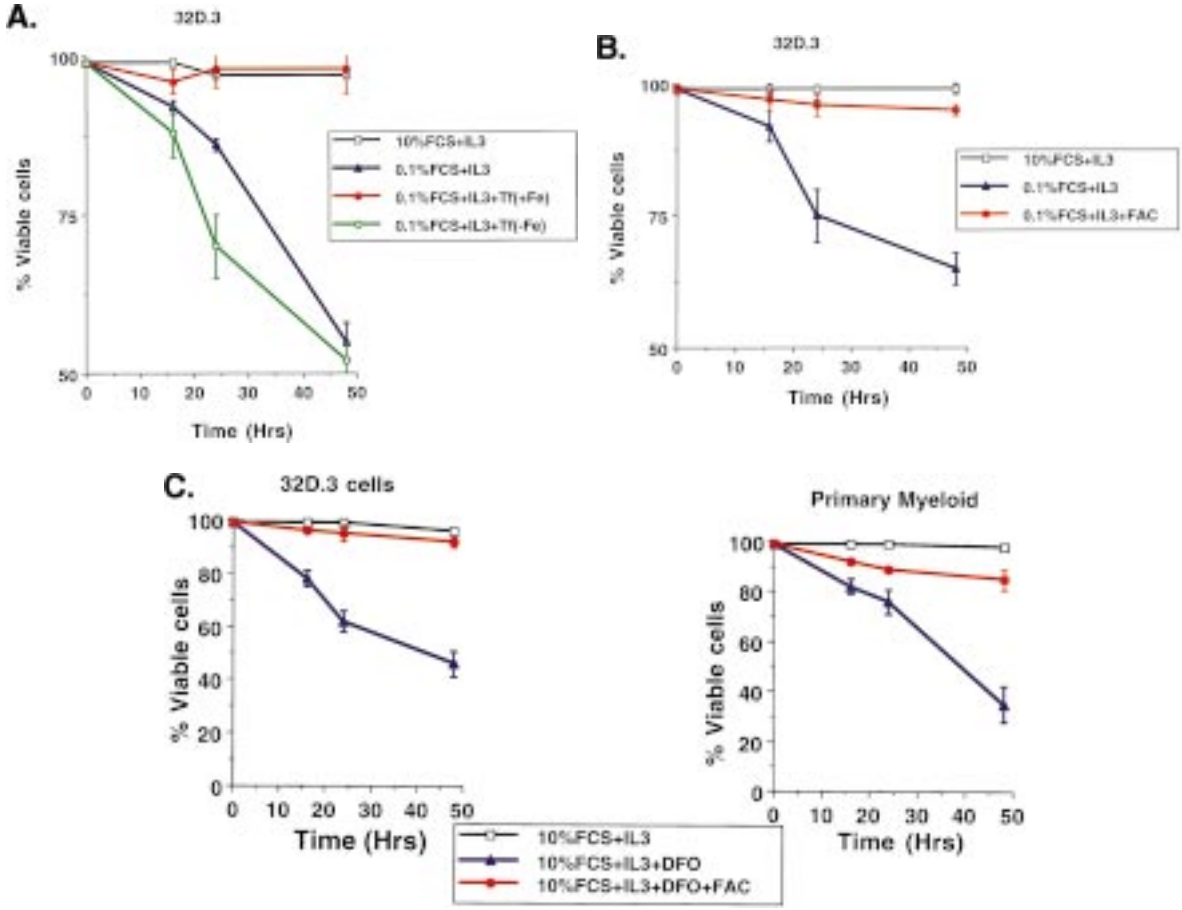


Fig. 4. Iron is the serum component required for myeloid cell survival. **A**: Iron-bound, but not iron-free, transferrin promotes myeloid cell survival in low serum medium. Exponentially growing cultures of 32D.3 cells were washed in PBS and cultured in the indicated media. Viability of cells was assessed by trypan blue dye exclusion. **B**: Ferric ammonium citrate (100 μM) promotes survival of myeloid cells in low serum medium. Exponentially growing 32D.3 cells were cultured in the indicated medium and viability was assessed by trypan blue exclusion. **C**: Direct depletion of iron compromises myeloid cell survival. Cells were cultured in IL-3 + 10% FCS medium and then treated with Desferrioxamine (100 μM) +/− 100 μM FAC. At the indicated intervals viable cell number was determined by trypan blue dye exclusion. Experiments shown in (A) and (B) are the mean of four experiments +/− the standard deviation and those shown in (C) are the mean of three experiments +/− the standard deviation.

Depletion of iron using this chelator has been shown to have apoptotic effects on many cell types, including the leukemia-derived K562 and HL60 cell lines [Hileti et al., 1995]. DFO induced rapid apoptosis of both 32D.3 and fetal liver-derived primary myeloid cells, and these cell deaths were effectively blocked by the concomitant addition of FAC (Fig. 4C). Thus iron is the serum component that is required to support myeloid cell survival ex vivo.

Apoptosis Induced by Iron Deprivation is Impaired by Bcl-2 but not by Caspase Inhibition

The withdrawal of serum led to cleavage of both caspase-9 and caspase-3, suggesting that the apoptosis observed following iron deprivation was caspase-dependent. To address this issue we pre-treated cells with the cell permeable and broad-spectrum inhibitor of all caspases, zVAD-fmk [Slee et al., 1996]. Treatment of 32D.3 deprived of only IL-3 effectively delayed their rate of death [data not shown, and see Dai et al., 1999]. In contrast, z-VAD-fmk failed to delay the rate of death of 32D.3 cells cultured in IL-3 and low serum, and in fact rates of death were somewhat accelerated (Fig. 5A). Thus, although caspase activation occurs following serum withdrawal, a caspase-independent pathway also contributes to these cell deaths.

Members of the Bcl-2 family regulate apoptosis by modulating the function of key organelles

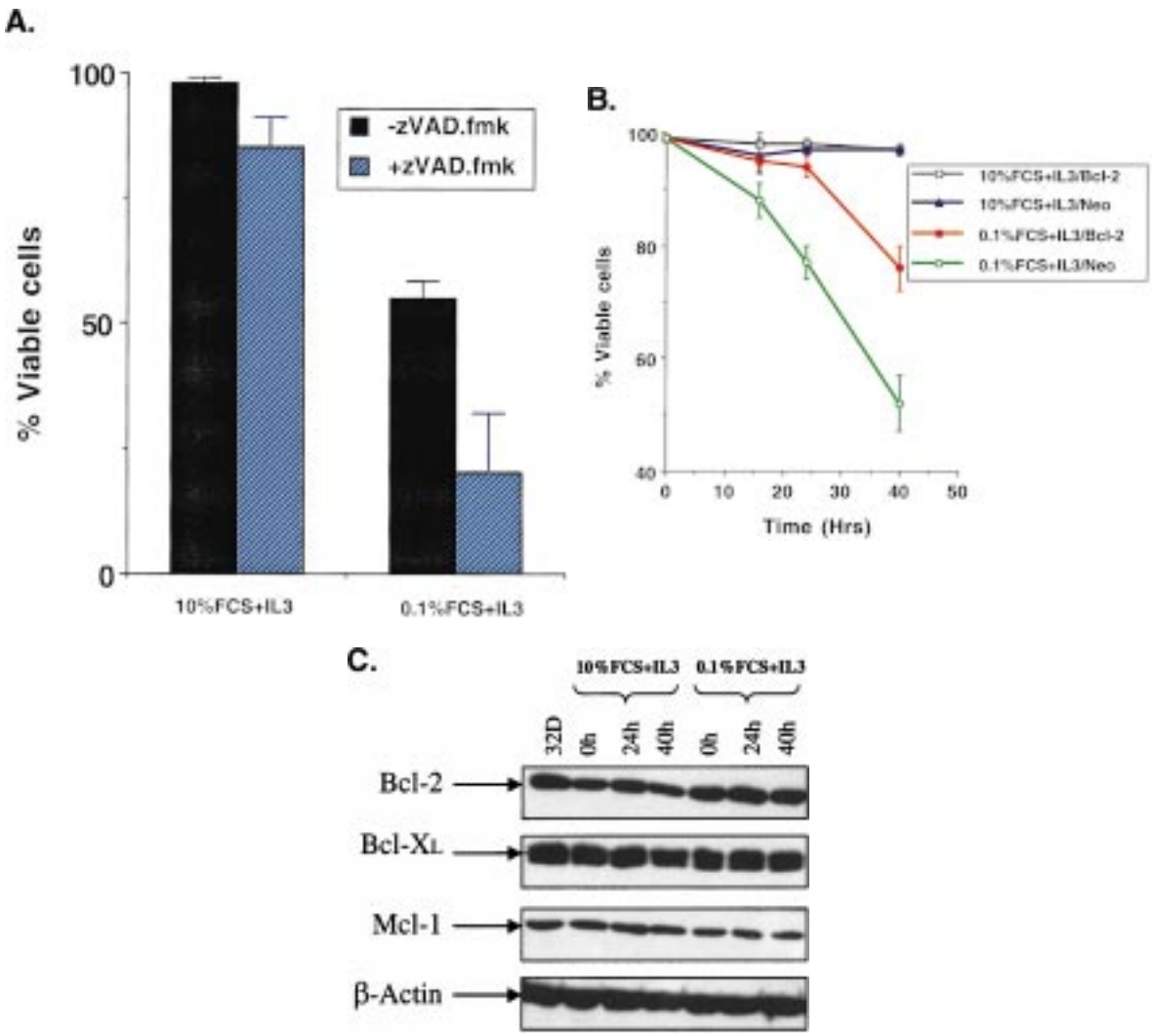


Fig. 5. Overexpression of Bcl-2, but not caspase inhibition, promotes myeloid cell survival in low serum medium. **A**: Caspase inhibition fails to block serum deprivation induced cell death. Exponentially growing 32D.3 cells were pre-treated with 400 μM zVAD-fmk for 1 h, washed and then cultured in the indicated medium +/− zVAD-fmk. The numbers of viable cells at 48 h of culture are shown. Results shown are the mean of five separate experiments +/− the standard deviation and similar results were obtained using FDC-P1.2 cells. Not shown is that this concentration of zVAD-fmk impairs 32D.3 cell apoptosis when cells are deprived of IL-3 [Dai et al., 1999]. **B**: Bcl-2 overexpressing 32D.3 cells are more resistant to serum deprivation induced cell death. Clones overexpressing Bcl-2 [Nip et al., 1997] and vector-only clones generated in parallel, were cultured in the indicated medium and the number of viable cells determined by trypan blue dye exclusion. Results shown are the mean of three experiments +/− standard deviation. **C:** Serum deprivation does not alter the expression of the anti-apoptotic proteins Bcl-2, Bcl-X_L, or Mcl-1. Cells were cultured in the media indicated and extracts prepared and analyzed by immunoblots with antibodies specific for Bcl-2, Bcl-X_L, Mcl-1 and Actin. Results shown are representative of three separate experiments and similar results were obtained in FDC-P1 and primary myeloid cells (data not shown). In addition, serum deprivation failed to significantly alter levels of the pro-apoptotic proteins Bax, Bad and Bak (data not shown).

such as mitochondria. Damage to mitochondria is a hallmark of apoptosis and results in the activation of both caspase-dependent and—ndependent cell death [Henkart and Grinstein, 1996; Mancini et al., 1998; Bortner and Cidlowski, 1999; Bossy-Wetzel and Green, 1999]. Bcl-2 functions as an anti-apoptotic protein by virtue of its localization to the outer membrane of mitochondria and thus prevents both of these pathways [Adachi et al., 1998; Qi and Sit, 2000]. Therefore, we tested whether Bcl-2 overexpression could protect cells from

apoptosis following serum withdrawal in 32D cells. 32D.3-derived clones that overexpress Bcl-2 were compared to vector-only neo-resistant clones generated in parallel [Nip et al., 1997]. As expected, Bcl-2 overexpressing 32D.3 cells displayed a marked resistance to death when they were deprived of IL-3, relative to rates of apoptosis of vector-only controls (data not shown). Bcl-2 overexpression also protected cells, albeit less completely, when cultured in IL-3 and 0.1% FCS or when cultured in complete medium but treated with DFO (Fig. 5B and data not shown).

The partial nature of the effects of Bcl-2 protection could formally be due to effects of iron loss on the expression of other Bcl-2 family members. In particular, the expression of the anti-apoptotic Bcl-2 family member Bcl-X_L is selectively dependent upon cytokines and is required for progenitor cell survival [Motoyama et al., 1995; Packham et al., 1998]. We, therefore, examined whether deprivation of serum led to alterations in the expression of Bcl-2 family members by immunoblot analyses. None of the anti-apoptotic family members tested (Bcl-2, Bcl-X_L, Mcl-1) showed appreciable changes following the withdrawal of serum (Fig. 5C), and 32D.3 clones engineered to overexpress Bcl-X_L showed a similar resistance to serum deprivation-induced cell death (data not shown).

Mitochondria are Damaged Following Serum Withdrawal From Myeloid Cells

The partially protective effects of Bcl-2 overexpression, but not caspase inhibition, following serum withdrawal, suggested that damage to mitochondria could be involved in cell deaths caused by iron depletion. In mitochondria iron is required for electron transfer reactions, including oxidative phosphorylation and the Haber-Weiss and Fenton reactions, which are involved in the generation of hydroxyl radicals from hydrogen peroxide [Pierre and Fontecave, 1999]. Initially we checked for changes in levels of reactive oxygen species (ROS) as alterations in mitochondrial function are sometimes associated with these changes [Cai and Jones, 1999; Chandel and Schumacker, 2000]. However, no changes in the steady state levels or rates of generation of ROS were observed (negative data not shown). To assess whether serum deprivation was associated with change in mitochondrial membrane potential we used the fluorescent probe JC1 [Bortner and Cidlowski, 1999], which, unlike other dyes, is specific for measuring changes in the mitochondria membrane. JC1 forms either J-aggregates or monomers depending on the state of the mitochondrial potential. The high mitochondrial membrane potential of normal cells loaded with JC1 allows for the formation of J-aggregates, detected by a peak in fluorescence at 585 nm. As the mitochondrial membrane potential is lost, these aggregates dissipate into monomers, which are detected as a shift in fluorescence from 585 to 530 nm by flow cytometry [Bortner and Cidlowski, 1999]. Following the depletion of serum from 32D.3 cells cultured in IL-3 there was a significant loss in the signal at 585 nm and a corresponding increase in signal at 530 nm, indicating loss of mitochondrial membrane potential (Fig. 6). Similarly, 32.3 cells cultured in both IL-3 and serum yet treated with DFO also displayed a loss in ψA_m (Fig. 6). Therefore, apoptosis induced by serum and iron withdrawal is associated with damage to mitochondria.

Apoptosis and Mitochondrial Damage Following Iron Loss is Due to the Depletion of ATP

In most cells, the major source of energy for cellular metabolic processes is derived from oxidative phosphorylation and the generation of ATP, and these processes are dependent upon

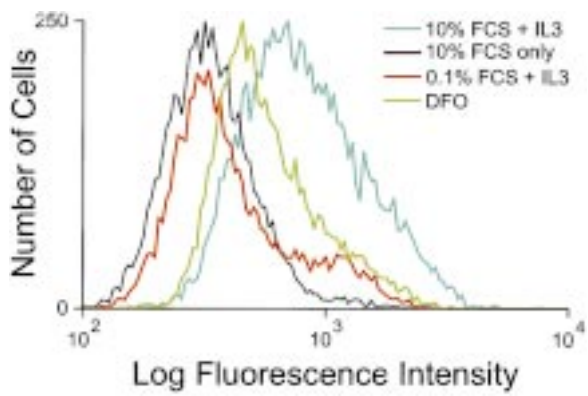


Fig. 6. Serum deprivation results in the loss of mitochondrial membrane potential. 32D.3 cells were incubated in the indicated medium for 24 h. The mitochondrial membrane potential dye, JC1 (50 μM) was then added for 30min at 37°C. Cells were then washed twice in PBS and resuspended in 1ml of PBS prior to analysis by flow cytometry. Histograms indicating mean cell fluorescence are representative of four independent experiments. Key for the histograms is as follows: Blue, IL-3 + 10%FCS; black, 10% FCS; red, IL-3 + 0.1%FCS; green, IL-3 +10% FCS + DFO (100 μM).

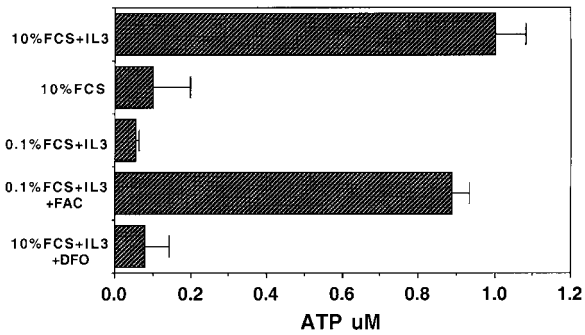


Fig. 7. Serum and iron deprivation in myeloid cells leads to marked reductions in intracellular ATP levels. 32D.3 cells were incubated in the indicated medium for 24 h and diluted to a concentration of 10^5 cells/ml in their appropriate medium. Samples in a volume of 50 μL were assayed with 50 μL luciferase reagent. The light signal was integrated for 10 s after a delay of 2 s. Histograms indicating mean ATP bioluminescence +/− standard deviations are shown for four independent experiments.

iron [Ha and Snyder, 1999; Rathmell et al., 2000]. The maintenance of the proper ratio of ATP:ADP is essential to maintain mitochondrial membrane potential, and thus depletion of ATP leads to apoptosis [Vander Heiden et al., 1999]. To investigate whether serum withdrawal or the depletion of iron was associated with alterations in ATP levels, we directly measured intracellular ATP levels. As expected from previous studies [Whetton et al., 1988; Vander Heiden et al., 1999], levels of ATP were significantly diminished when 32D.3 cells were deprived of IL-3 (Fig. 7). However, levels of ATP were also markedly reduced when cells were cultured in IL-3 and low serum, or when they were cultured in complete medium containing DFO (Fig. 7). Furthermore, the addition of FAC to cells cultured in medium containing IL-3 but low serum effectively reversed ATP depletion (Fig. 7). Therefore, serum withdrawal or iron depletion leads to marked reductions in ATP levels, to mitochondrial damage and apoptosis, and all three are effectively reversed by simply supplementing the cells with iron.

DISCUSSION

Serum deprivation induces apoptosis in many cell model systems. Serum components required for survival in fibroblasts and endothelial cells have been identified as IGF-1, PDGF and/or endothelin-1 [Harrington et al., 1994; Shichiri et al., 1997]. In particular, in fibroblasts IGF-1 is a potent survival signal but a poor mitogen, and is very effective at suppressing Myc-induced apoptosis [Harrington et al., 1994]. However, neither PDGF nor IGF-1 alone, nor prolonged treatment with the broad-spectrum caspase inhibitor zVAD-fmk, is capable of suppressing Myc-induced apoptosis during prolonged periods of serum starvation [McCarthy et al., 1997]. Here we have shown that serum deprivation induces both caspase-dependent and caspase-independent pathways that lead to apoptosis of myeloid progenitors, and have identified iron as the critical serum component necessary and sufficient to prevent apoptosis.

Iron added either in the form of transferrin and ferric ammonium citrate was sufficient to block the apoptosis of myeloid cells cultured in serum depleted media. Iron deficiency or excessive iron levels and the induction of apoptosis are well documented [Fukuchi et al., 1994; Hileti et al., 1995; Kovar et al., 1997; Kyriakou et al., 1998; Yuan, 1999; Rakba et al., 2000], yet the mechanisms by which these cell deaths occur have been controversial. For example, excessive levels of iron have been proposed to induced oxidative stress, as iron is known to promote monoamine oxidation, and to catalyze the conversion of H_2O_2 into the highly reactive $OH^{\bullet}$ [Velez-Pardo et al., 1997]. In contrast, iron deprivation in murine lymphoma cells has been attributed to the activation of p53, and/or to decreases in Bcl-2 expression [Kovar et al., 1997]. None of these scenarios apply to the system described herein, as serum withdrawal or direct depletion of iron had no effect on ROS levels, and did not result in the activation of p53 or changes in the expression of Bcl-2 family members (Fig. 5 and KM, unpublished results). However, loss of iron and apoptosis is most closely associated with the impaired function of the iron-containing enzymes in the respiratory chain required to generate ATP, and to subsequent damage to mitochondria.

Exit of cytochrome *c* from mitochondria into the cytosol has been demonstrated to be a critical initiator of caspase-dependent cell death pathways. Once released, cytochrome *c* binds the CED-4 homolog Apaf-1, thereby triggering activation of caspase-9 [Jiang and Wang, 2000], which then cleaves and activates caspase-3. Activated caspase-3 then targets several proteins required for cellular integrity, including PARP, the nuclear lamins [Cohen, 1997] and the inhibitor of the DNA endonuclease DFF45/

ICAD [Liu et al., 1999]. As judged by the processing of caspase-9 and caspase-3, and by the appearance of the classic DNA ladder, this pathway is clearly initiated by serum (i.e., iron) withdrawal. However, blocking caspase activity with the broad-spectrum caspase inhibitor zVAD-fmk did not prevent cell death. Presumably this reflects the failure of zVAD-fmk to prevent mitochondrial-dependent changes that are associated with serum and/or iron depletion and apoptosis.

The intimate involvement of mitochondria in apoptosis is now well established [Kroemer, 1999], and the alterations in mitochondrial function are a very plausible explanation for the induction of apoptosis observed following serum and/or iron depletion in primary and established myeloid progenitor cells. Mitochondrial dysfunction in this scenario, as in most apoptotic responses, includes a loss in the mitochondrial membrane potential. The loss of membrane potential appears to result from the failure of the adenine nucleotide translocator (ANT)/voltage-dependent anion channel (VDAC) complex to maintain proper exchange of ATP and ADP [Vander Heiden et al., 1999]. It is well established that the withdrawal of required cytokines leads to a rapid reduction in ATP levels [Garland and Halestrap, 1997; Vander Heiden et al., 1999], and presumably this is due to the requirement for cytokines for the expression of Bcl-X_L [Packham et al., 1998], which appears to sustain the function of the VDAC complex [Shimizu et al., 1999]. Depriving myeloid cells of serum has no effect on the expression of Bcl-2 family members, but the end result of incubating myeloid cells in a serum-deprived environment is essentially the same in terms of maintenance of ATP levels, and demonstrates that a balance of cytokine signals and serum components (here iron) is required to sustain ATP levels (Fig. 7). A drop in mitochondrial ATP levels seen following serum or iron depletion should thus lead to marked alterations in the ATP/ADP exchange, mitochondrial inner membrane hyperpolarization and matrix swelling. Collectively these changes would ultimately compromise the function of the organelle.

One current thought to selectively induce the death of rapidly proliferating leukemia cells is to target cytokine pathways that are usually required for their survival. The results presented here suggest that another potential target for leukemia therapy is simply plasma sources of iron, which are likely required for the survival of any rapidly proliferating hematopoietic cells.

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