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Prevention of apoptosis in J2E erythroid cells by erythropoietin: involvement of JAK2 but not MAP kinases

David Chappell[1,2], Peta A. Tilbrook[1], Thomas Bittorf[3], Shane M. Colley[1], Geoffrey T. Meyer[4] and S. Peter Klinken[1,5]

[1] Department of Biochemistry, Royal Perth Hospital, GPO Box X2213, Perth WA 6001, Australia
[2] The University of Western Australia,
[3] Institute of Medical Biochemistry University of Rostock, Germany
[4] Department of Anatomy and Human Biology, The University of Western Australia, Nedlands WA 6009, Australia
[5] corresponding author: S. Peter Klinken tel: (9) 224 2426, fax: (9) 224 2491

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Abstract

The J2E erythroid cell line, transformed by retroviral v-*raf*/v-*myc* oncogenes, proliferates and differentiates in response to erythropoietin. Here we show that J2E cells undergo apoptosis rapidly after serum withdrawal and that only erythropoietin of seven growth factors tested, could protect the cells from death. The role of JAK2 and MAP kinases in transmitting viability signals initiated by erythropoietin was examined in these cells. Despite constituitive raf kinase activity, phosphorylation of MAP kinases fell after serum withdrawal. However, an antisense oligonucleotide strategy revealed that JAK2, but not the MAP kinases, was involved in transmitting signals to maintain the viability of J2E cells. Several cell cycle proteins and transcription factors were also studied; although c-jun rose sharply during apoptosis, erythropoietin could not suppress this increase. It was concluded that erythropoietin-induced protection from apoptosis involved JAK2, but not MAP kinases or c-jun.

Keywords: erythropoietin, apoptosis, JAK2, MAP kinases, c-jun

Abbreviations: epo, erythropoietin; MEL, murine erythroleukemia; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; MAP kinases, mitogen activated protein kinases; JAK2, Janus kinase 2; SCF, Stem cell factor; IL-3, interleukin 3; GM-CSF, granulocyte macrophage colony stimulating factor; IGF-1, insulin-like growth factor 1; PCNA, proliferating cell nuclear antigen; G-CSF, granulocyte colony stimulating factor.

Introduction

Erythropoiesis is regulated by the glycoprotein hormone erythropoietin (epo), which acts by binding to specific receptors on erythroid precursor cells, thereby promoting growth and differentiation into mature red blood cells (Krantz, 1991; Jelkmann, 1992; Koury and Bondurant, 1992). The action of epo has been studied in primary cell cultures and transformed cell lines, and the signal transduction mechanism of the hormone is gradually being elucidated (Koury and Bondurant, 1992; Wojchowski and He, 1993; Ihle, 1995). Various laboratories have reported that the processes of proliferation and differentiation in erythroid cells can be separated (Noguchi *et al*, 1988; Sytkowski *et al*, 1980, Liboi *et al*, 1993; Busfield and Klinken, 1992), which suggests that they are mediated by different pathways. It has also been demonstrated that epo is involved in preventing the death of erythroid precursors (Koury and Bondurant, 1988, 1990; Spivak *et al*, 1991). Studies on Friend virus-infected erythroid cells and the epo-dependent HCD-57 line have shown that epo retards DNA breakdown and maintains cell viability (Koury and Bondurant, 1988, 1990; Spivak *et al*, 1991). Our recent studies have indicated that distinct epo-induced signalling pathways may exist in cells which lead to cell division, maturation and maintenance of viability (Tilbrook *et al*, 1996a). In addition, IL-2, IL-3 and GM–CSF transmit signals that promote cell survival, independent of initiating cell division (Okuda *et al*, 1994; Inhorn *et al*, 1995; Kinoshita *et al*, 1995; Boise *et al*, 1995).

Apoptosis is the term used to describe physiological cell death which may be initiated either by external signals, or by the lack of viability factors, and appears to be programmed by the cell (Kerr *et al*, 1972; Wyllie *et al*, 1980; Raff, 1992). One of the characteristic features of cells undergoing apoptosis is nuclear condensation and fragmentation. These membrane-bound apoptotic bodies are clearly visible by light and electron microscopy. Additionally, DNA in apoptotic cells is degraded by endogenous endonucleases and typical nucleosomal banding patterns can be observed by gel electrophoresis (Koury and Bondurant, 1990; Williams *et al*, 1990; Spivak *et al*, 1991).

In this report, we describe the susceptibility of the erythroid J2E cell line to apoptosis. This clonal population of cells was immortalized at the proerythroblast stage of development by infection with the J2 virus containing the v-*raf* and v-*myc* oncogenes (Klinken *et al*, 1988). The cells do not require epo for growth in culture, but respond to the hormone by proliferating and terminally differentiating (Klinken *et al*, 1988; Busfield and Klinken, 1992; Busfield *et al*, 1993a,b,c, 1995a,b; Callus *et al*, 1995; Tilbrook *et al*, 1996a,b). We show here that the J2E cells become committed to apoptosis 4–6 h after withdrawal of fetal calf serum (FCS), and only epo, of seven growth factors tested, was able to inhibit cell death. Experiments with intracellular signalling molecules revealed that Janus kinase 2 (JAK2) plays an important role in epo signalling for cell viability, whereas mitogen activated protein (MAP) kinases do not appear to be involved in this process.

Results

J2E cells undergo apoptosis after serum withdrawal

As most cell lines require growth and viability factors present in FCS for survival, we investigated the effect of removing serum from J2E cultures. Figure 1a shows that the viability of J2E cells was dependent on the concentration of FCS – below 5% FCS the viability of J2E cells fell dramatically. This decrease was time dependent, and could be reversed by re-introduction of FCS up to 4 h after serum deprivation (Figure 1b). Thereafter, fewer cells could be rescued and commitment to cell death appeared irrevocable. Significantly, DNA synthesis ceased within 3 h of FCS removal (data not shown).

To determine if the cells were undergoing programmed cell death, their morphology was examined after serum withdrawal. Cells were collected and stained with May-Grunwald-Geimsa and two DNA-binding flurochromes (Figure 2). Unlike normal J2E cells in culture, the serum-starved cells appeared smaller and contained fragmented nuclei (Figure 2a). Fluorescence microscopy using acridine orange and H33258 revealed that the highly condensed fragments did indeed contain DNA (Figure 2b and c). These data were confirmed by the strong correlation between the number of cells with degraded DNA, as identified by Apotag staining, and cells unable to exclude eosin (r=0.96).

To investigate this phenomenon in greater detail, electron microscopy was employed to view the cells at higher magnification. Figure 3 shows some normal J2E cells with large nuclei and diffuse chromatin structure; organelles such as mitochondria, endoplasmic reticulum and ribosomes are clearly visible. In contrast, other cells in these cultures displayed nuclear condensation and fragmentation, decreased cell volume and condensed membrane-bound organelles. These features are characteristic of cells undergoing apoptosis (Kerr *et al*, 1972; Wyllie *et al*, 1980; Raff, 1992).

Another feature commonly associated with programmed cell death is the nucleosomal banding pattern of degrading DNA. When DNA was extracted from J2E cells and separated by gel electrophoresis, nucleosomal banding associated with DNA degradation increased markedly with time (Figure 4). The breakdown of DNA could also be demonstrated by propidium iodide staining and FACS analysis as cells with less than diploid DNA content

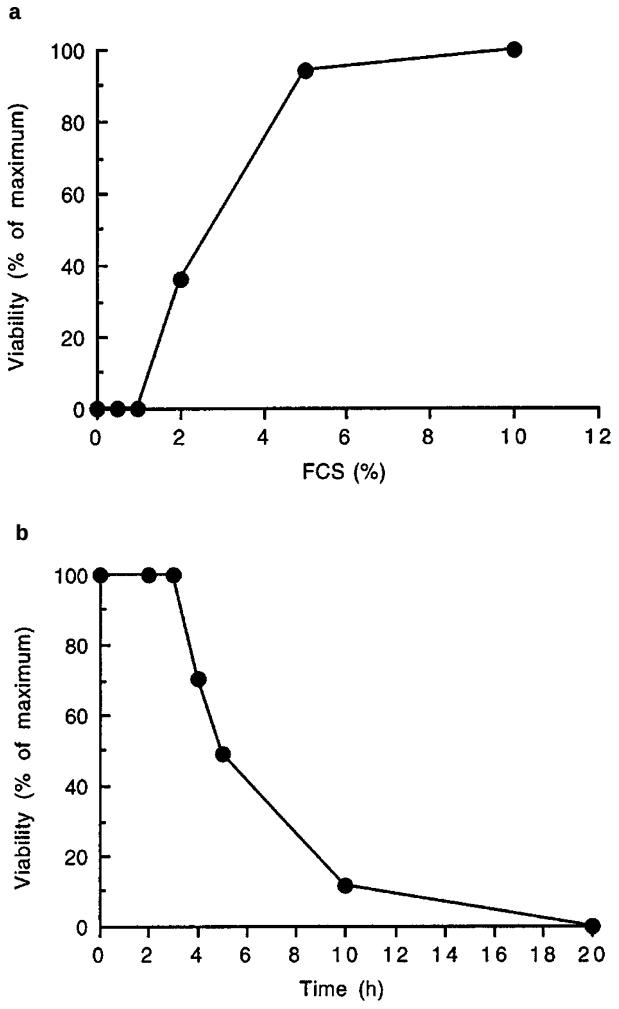

Figure 1 Serum withdrawal results in rapid death of J2E cells. (a) J2E cells were washed thoroughly and replated with different concentrations of FCS. The following day viability was assessed. (b) Cells were washed and cultured in media without FCS. At various time points FCS (10%) was re-introduced into the cultures and viability assessed after 24 h. These experiments are representative of three separate experiments.

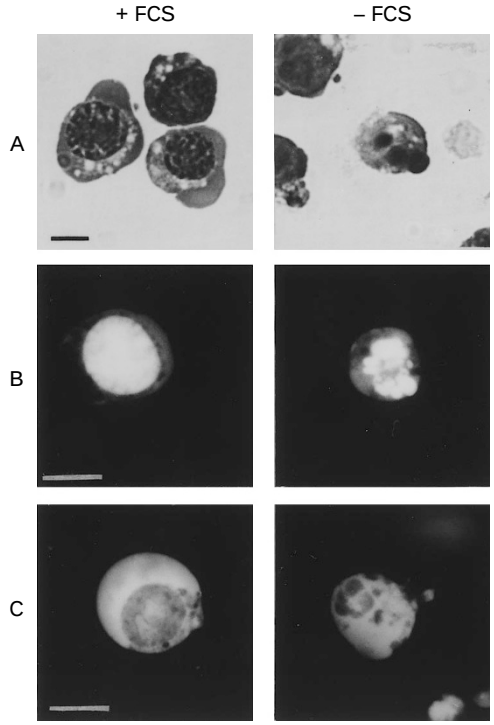

Figure 2 Morphological changes after serum withdrawal. J2E cells were grown with, or without, FCS for 24 h, then cytocentrifuged onto glass slides and stained with either May Grunwald Giemsa (A) bisbenzimide H33258 (B) or acridine orange (C).

accumulated (data not shown). These data are consistent with the notion that the cells were undergoing apoptosis, and confirmed that removal of FCS initiated programmed cell death in J2E cultures.

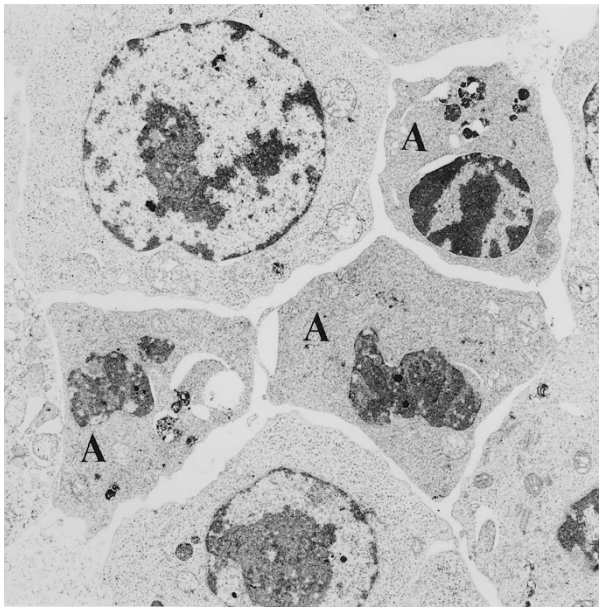

Figure 3 Ultrastructural alterations with serum withdrawal. Electron microscopy was used to determine the ultrastructure of J2E cells in cultures deprived of serum. A – denotes cells that are undergoing apoptosis.

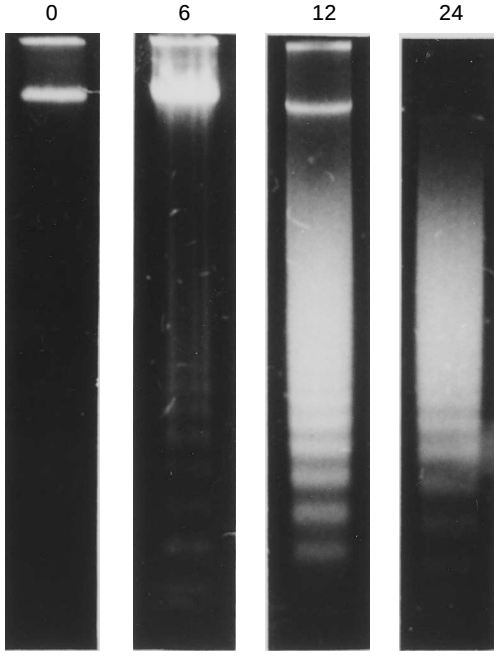

Figure 4 DNA degradation after serum withdrawal. J2E cultures were established without serum, then cells were collected at various times (0 – 24 h) for DNA extraction and separation on agarose gels.

J2E cells are more susceptible to apoptosis than MEL cells

The rate of cell death for J2E cells was compared with Friend-virus transformed murine erythroleukemic (MEL) cells because they are immortalized at similar stages of erythroid differentiation (Klinken *et al*, 1988; Dube *et al*, 1975). However, unlike the J2E line, MEL cells do not differentiate in response to epo and mature only with xenobiotic stimulation (Marks and Rifkind, 1978). Data presented in Table 1 show that the J2E cell died more rapidly following serum withdrawal than the MEL cells. Thus, the J2E cells appear to be inherently more sensitive to serum deprivation than a comparable erythroid cell line. In addition to dying when serum was removed, J2E cells also underwent apoptosis shortly after cultures became confluent (data not shown). In contrast, MEL cells grown in parallel were much more robust than J2E cells. One explanation for these observations is that J2E cells are susceptible to cell death whenever replication is impeded.

To determine if J2E cells would die when cell division was impeded, cultures were established at low cell density with ample FCS, then inhibited from proliferating by addition of aphidicolin, a drug which inhibits the cell cycle at the

Table 1 Epo support viability of J2E cells but not MEL cells

	Viability (% of control)			
	J2E		MEL	
Time (h)	– Epo	+Epo	– Epo	+Epo
0	100	100	100	100
3	91 ± 5	100 ± 1	100 ± 1	100 ± 3
6	72 ± 6	90 ± 3[a]	100 ± 2	100 ± 3
18	45 ± 5	71 ± 5[a]	76 ± 4	74 ± 5
24	21 ± 4	59 ± 6[b]	70 ± 4	67 ± 6

Cultures of J2E and MEL cells were washed thoroughly and grown in serum-free media. The effect of epo (5 U/ml) on the viability of cells was determined over 24 h. Each value is the mean of three experiments (± SD). [a]Significant increase versus – epo (P<0.01). [b]Significant increase versus – epo (P<0.001)

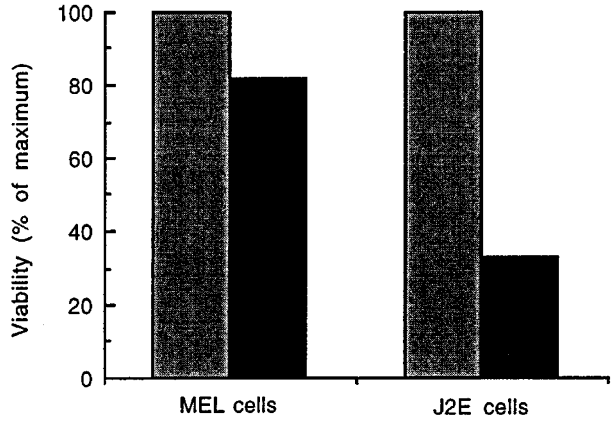

Figure 5 J2E cells die when cultured with aphidicolin. Cultures of J2E and MEL cells were established in 10% FCS with (black histogram) or without (stippled histogram) 2.5 mM aphidicolin, a cell cycle inhibitor. The following day viabilities were determined.

beginning of S phase. In the presence of aphidicolin the viability of J2E cells was reduced to 28% after 16 h (Figure 5). Analysis of the DNA content, together with cytocentrifuge preparations, clearly demonstrated an increase in DNA cleavage and the presence of apoptotic bodies (data not shown). Under identical conditions the viability of MEL cells decreased slightly to 82%. These results indicate that J2E cells are highly susceptible to apoptosis if they are unable to traverse the cell cycle.

Epo prevents apoptosis of J2E cells

It has been suggested that epo plays a crucial role in maintaining the viability of erythroid cells (Koury and Bondurant, 1988, 1990; Spivak *et al*, 1991). In addition, a number of other factors including stem cell factor (SCF), interleukin 3 (IL-3), granulocyte-macrophage colony stimulating factor (GM–CSF), insulin, insulin-like growth factor 1 (IGF-1) and growth hormone are thought to be involved in promoting the survival of immature erythroid cells. These factors were added to cultures depleted of FCS to determine whether they could support the viability of J2E cells. Figure 6a and Table 1 show that epo could maintain the viability of J2E cells in the absence of FCS, whereas the other factors provided no protection from apoptosis. Furthermore, epo was able to restrict DNA degradation in the absence of FCS (Figure 6b). From these data we concluded that epo could act as a viability factor for these immature erythroid cells. In contrast, epo could not protect MEL cells from dying in the absence of serum (Table 1). However, epo is not the molecule present in FCS which maintains the viability of J2E cells (Figure 1b) as no detectable epo is present in the serum (data not shown).

To determine if the concentration of epo required to maintain the survival of J2E cells was the same as that required for induction of cell division and differentiation, a dose response curve to the hormone was generated. Figure 7 shows that proliferation and maturation peaked between 0.1 and 0.3 U/ml epo, as we have described previously (Busfield and Klinken, 1992; Tilbrook *et al*, 1996b). Significantly, maintenance of viability by epo occurred at almost exactly the same concentration. This indicated that cell survival was concentration dependent, and did not differ from the amount of epo necessary for proliferation and differentiation.

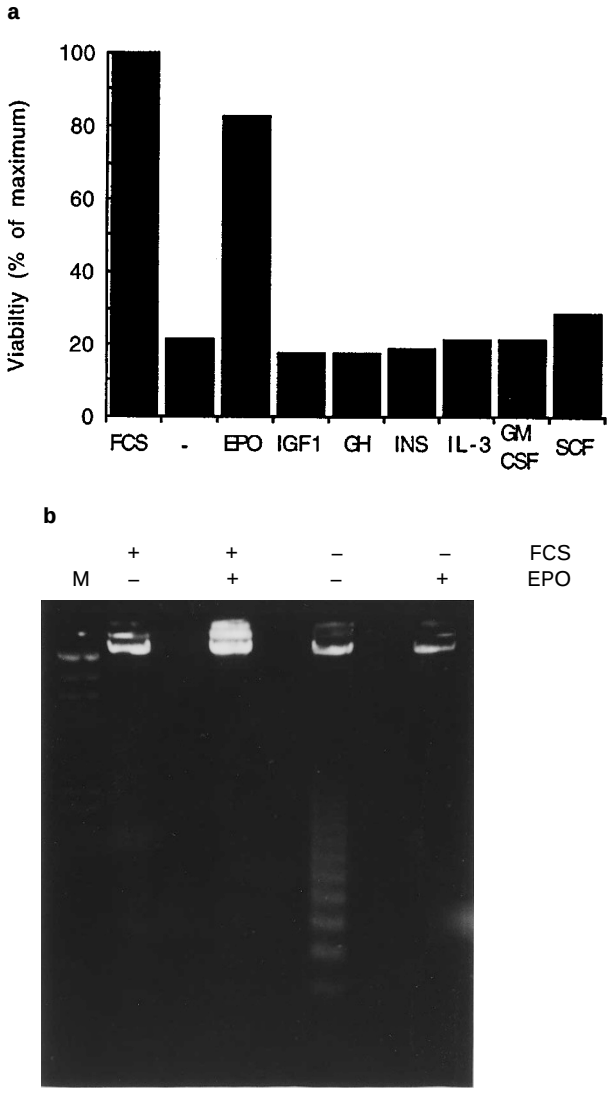

Figure 6 Epo protects J2E cells from apoptosis and DNA degradation. (a) J2E cells were washed thoroughly and replated in 10% FCS, no serum (–) or no serum plus 10 U/ml epo, IGF-1, growth hormone (GH), insulin (INS), IL-3, GM-CSF and SCF. Viabilities were determined 24 h later. (b) J2E cells were grown in the presence, or absence, of 10% FCS with, or without, 10 U/ml epo for 24 h before DNA was extracted and separated on agarose gels.

JAK2 is required for the epo survival signal

Immediately after binding its ligand the epo receptor dimerizes and the ancillary kinase JAK2 is activated, enabling signals to be transduced within the cell. Using antisense oligonucleotides we have shown elsewhere that JAK2 is essential, not

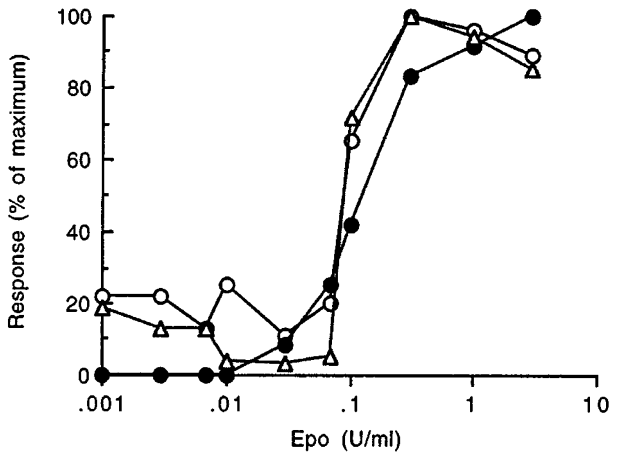

Figure 7 Viability dose response curve for J2E cells. J2E cells were grown in 10% FCS and exposed to a range of epo concentrations (0.001–10 U/ml) before [^{3}H]-thymidine incorporation (open triangles) was determined after 24 h, and benzidine staining for haemoglobin production (closed circles) was measured at 48 h. Simultaneously, cells were deprived of FCS and cultured with the same range of epo concentrations and viability (open circles) assessed after 24 h.

only for epo-induced proliferation, but also for differentiation (Tilbrook *et al*, 1996b). Here we tested the role of JAK2 in epo signalling for cell viability. Anti-sense oligonucleotides to JAK2 were added to J2E cultures and after 3 days JAK2 protein content was less than 10% of the original amount (Tilbrook *et al*, 1996b). Serum was then withdrawn from the cultures and viability assessed. Table 2 shows that by blocking JAK2, the ability of epo to promote cell survival was significantly suppressed. In contrast, a control oligonucleotide had no effect on viability. These data demonstrate that JAK2 plays an important role in epo-initiated signal transduction pathways which prevent cell death.

Epo does not maintain viability via the MAP kinase pathway

As epo activates the ras/MAP kinase signal transduction pathway in J2E cells (Tilbrook *et al*, 1996a), the effects of serum deprivation on raf and MAP kinases were investigated. Raf kinase was constitutively activated in J2E cells and the level of activity was maintained after FCS removal from culture (Figure 8). This result was not surprising as J2E cells were transformed by the v-*raf*/v-*myc* containing J2 virus (Klinken *et al*, 1988). Immunoblotting revealed that p42 and p44 MAP kinases were highly phosphorylated during normal cell growth (Figure 9), due in part to the elevated raf activity. However, upon serum withdrawal the phosphorylation status of the MAP kinases declined appreciably, despite raf kinase activity being maintained (Figures 8 and 9). As reintroduction of FCS activated the MAP kinases (Figure 9), it appears that FCS, and not just an activated raf gene, is needed to maintain MAP kinase activity.

We have shown elsewhere that MAP kinase activity is linked to epo-induced differentiation (Tilbrook *et al*, 1996a), and others have demonstrated an association with the proliferative effects of epo (Miura *et al*, 1994; Todokoro *et al*, 1994; Bittorf *et al*, 1994; Gobert *et al*, 1995). As MAP kinase phosphorylation decreased markedly after serum withdrawal, we speculated that it may also be involved in viability signalling. To this end the anti-sense oligonucleotide strategy employed by Sale *et al* (1995) was used to determine the role of p42 and p44 MAP kinases in epo-induced survival. Table 3 shows that, unlike JAK2, inhibition of the MAP kinases made no difference to the maintenance of viability supported by epo. In these experiments, MAP kinase protein content was reduced by more than 70% and differentiation was suppressed accordingly. These data indicate that the MAP kinase pathway is not involved in regulating cell survival and are consistent with a role in differentiation.

Table 2 Anti-sense JAK2 oligonucleotides suppress epo-supported viability

Treatment	Viability (%)	Increase over control (%)
Control	8±4	0
Random oligo	6±3	–25
Anti-sense oligo	8±3	0
Epo	31±6	287
Epo±random oligo	35±6	337
Epo±anti-sense oligo	16±5[a]	100

J2E cells were cultured with oligonucleotides for 72 h to reduce the level of JAK2 protein by >90%. They were then washed thoroughly and deprived of FCS. Epo and oligonucleotides were added as indicated and viabilities determined after 48 h. Each value represents the mean of three experiments (±SD). [a]Significant decrease versus epo alone (P<0.05) and epo±random oligo (P<0.02)

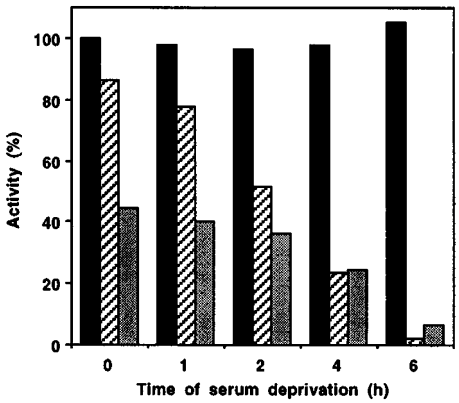

Figure 8 Activity of raf and MAP kinases after serum withdrawal. J2E cells were deprived of serum and the activity of raf (black histogram) determined over 6 h. The phosphorylation status of p42 (hatched histogram) and p44 (stippled histogram) MAP kinases was followed for the same time period.

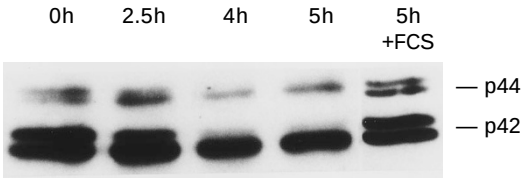

Figure 9 Dephosphorylation and reactivation of MAP kinases. Immunoblot showing the decrease in the phosphorylated (upper band) form of p42 and p44 MAP kinases over several hours after serum deprivation. FCS was re-introduced after 5 h culture without serum.

Table 3 Anti-sense MAP kinase oligonucleotides inhibit differentiation but do not affect epo-supported viability

Treatment	Viability (%)	Increase over control (%)	Benzidine positive cells (%)
Control	6±2	0	9±2
Random oligo	6±2	0	12±2
Anti-sense oligo	7±3	17	9±3
Epo	18±4	200	28±2
Epo+random oligo	21±5	250	22±3
Epo+anti-sense oligo	21±4	250	11±3[a]

J2E cells were cultured with oligonucleotides for 72 h and the levels of p42 and p44 MAP kinases fell by 84 and 72% respectively (v-raf was used as the loading control). They were then washed thoroughly and deprived of FCS. Epo and oligonucleotides were added as indicated and viabilities determined after 48 h. Parallel experiments to determine the effect on differentiation (as measured by benzidine staining) were conducted in the presence of FCS, 24 h after addition of epo. Each value is the mean of three experiments (±SD). [a]Significant decrease versus epo alone (P<0.01) and epo+random oligo (P<0.02)

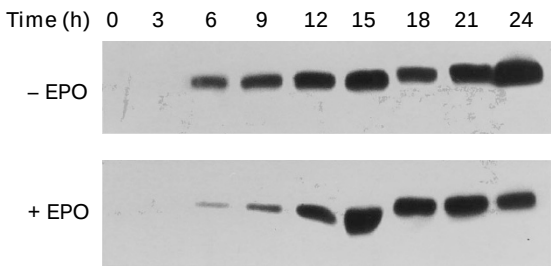

Figure 10 c-jun increases during apoptosis of J2E cells. (A) Cells were deprived of serum for 0–24 h and protein extracted for immunoblotting with anti-c-jun antibodies at various time points. (B) Cells were serum starved but cultured with 10 U/ml epo for 24 h before protein extracts were immunoblotted with anti-c-jun antibodies.

c-jun increases during apoptosis

Changes to a number of cell cycle proteins and transcription factors molecules have been associated with apoptosis. The following molecules were investigated to determine if they changed during programmed cell death of J2E cells and whether epo would reverse these effects: cdc 2 (p34), cdk 2, cdk 4, cdk 5, proliferating cell nuclear antigen (PCNA), cyclin A, cyclin B1, cyclins D1-D3, cdc 25, fos, jun and myc. Most of the proteins did not alter after withdrawal of serum. However, cyclin B1 showed a transient increase which was not influenced by epo, and cyclin D3 underwent a brief elevation upon epo stimulation. In contrast, c-jun protein content rose appreciably within cells deprived of FCS (Figure 10). Similar increases in c-jun were observed when J2E cultures were overgrown and underwent apoptosis (data not shown). Increases in c-jun have been associated with the genesis of cell death in a number of cell systems (Colotta *et al*, 1992; Estus *et al*, 1994; Goldstone and Lavin, 1994). While epo could reduce death of J2E cells, it did not suppress the rise in c-jun (Figure 10). To determine if c-jun was involved in the apoptotic process, anti-sense oligonucleotides to the transcription factor were added to J2E cultures. However, inhibiting c-jun had no effect on apoptosis or the maintenance of viability promoted by epo (data not shown). We concluded that the rise in c-jun may not necessarily be responsible for cell death in J2E cells.

Discussion

J2E cells die rapidly when serum is withdrawn, bearing the hallmarks of apoptosis (Kerr *et al*, 1972; Wyllie *et al*, 1980; Raff, 1992). Microscopic analyses revealed nuclear condensation and fragmentation, reduced cell volume and condensed membrane-bound organelles. Nuclear disruption was associated with extensive DNA breakdown as evidenced by the increased number of cells with less than diploid DNA content, and the emergence of oligonucleosomal banding patterns. Commitment to cell death occurred 4 h after FCS withdrawal and significant loss in viability was seen thereafter. Similar effects have been observed in numerous haemopoietic systems after growth factor depletion e.g. when epo is withdrawn from Friend virus-infected erythroblasts and HCD-57 cells (Koury and Bondurant 1988, 1990; Spivak *et al*, 1991), and when IL-3, GM–CSF or granulocyte colony stimulating-factor (G–CSF) is removed from myelomonocytic FDCP cells (Williams *et al*, 1990).

Epo was able to protect J2E cells from apoptosis caused by serum depletion – viability of the cells was maintained and DNA breakdown reduced substantially. Several other factors were unable to prevent the inexorable progression to death. These results support the proposition that epo has the capacity to promote survival of erythroid precursor cells (Koury and Bondurant, 1988, 1990; Spivak *et al*, 1991). However, Friend virus-infected erythroblasts are heterogenous in their susceptibility to apoptosis, and DNA breakdown is inhibited over a wide range of epo concentrations (Kelley *et al*, 1993), whereas the clonal J2E line responds to epo over the narrow dose response range that also initiates cellular maturation (Busfield and Klinken, 1992). Interestingly, apoptosis of erythroblasts deprived of epo can occur at any stage of the cell cycle (Kelley *et al*, 1994).

JAK2 appears to be intimately involved in the survival signal transmitted by epo. Suppression of JAK2 with anti-sense olignucleotides not only prevented proliferation and differentiation of J2E cells (Tilbrook *et al*, 1996b), but also restricted the maintenance of viability by epo (Table 2). This result is compatible with the data of Zhuang *et al* (1995), who noted that JAK2 was involved in epo signalling for maintaining viability in myeloid FDCP-1 cells transfected with epo receptor and JAK2 constructs. Our studies extend their observations to show the involvement of JAK2 with epo-induced viability in an erythroid cell line. Both studies suggest that phosphorylation of the receptor via JAK2 may play an important role in viability signalling, or alternatively, JAK2 has some direct role in supporting survival. It is noteworthy that JAK2 can be activated in a mutant clone of J2E cells (J2E-NR) which does not proliferate or differentiate in response to epo, but does remain alive when epo is present (Tilbrook *et al*, 1996a). The role of STATs in JAK2 signalling in erythroid cells is unclear at present. There have been numerous reports on epo activating STATs (Damen *et al*, 1995; Penta and Sawyer, 1995; Quelle *et al*, 1996; Ohashi *et al*, 1995; Gouilleux *et al*, 1995; Tilbrook *et al*, 1996b) but whether they participate in proliferation, differentiation or viability remains to be determined.

The commitment to programmed cell death in J2E cells also occurred whenever the cells' capacity to divide was restricted. Attainment of confluence, exposure to aphidicolin and withdrawal of FCS were all associated with reduced cell division and subsequent apoptosis. This susceptibility of J2E cells to apoptosis may be linked with dysregulation of the *myc* gene, as the cells contain an aberrant v-*myc* in the J2 virus (Klinken *et al*, 1988). Constitutive expression of c-*myc* in fibroblasts and IL-3, or epo-, dependent myeloid cells has been shown to promote cell death when proliferation is blocked (Evan *et al*, 1992; Shi *et al*, 1992; Askew *et al*, 1991). It has been postulated that c-*myc* induces mitosis when activated in conjunction with another growth signal; in the absence of the secondary signal, *myc*-mediated cell death occurs (Harrington *et al*, 1994). Clearly there are factors present in FCS which provide additional

signals for proliferation of J2E cells – if these are removed, or cell division is inhibited by other means, the J2E cells die. However, in contrast with the data presented here, Askew *et al* (1993) observed that epo was unable to prevent apoptosis in 32D myeloid cells inappropriately expressing c-*myc*.

The J2E erythroid cells were far more susceptible to apoptosis than MEL cells which are immortalized at a similar stage of erythroid development (Marks and Rifkind, 1978). The difference in viability between these cell lines is consistent with aberrant *myc* expression in J2E, but not MEL cells. Significantly, J2E cells could be protected from cell death by epo, whereas MEL cells were not. This inability could reflect binding of the viral gp55 protein to the epo receptor in MEL cells (Li *et al*, 1990), or result from the severe truncation to the cytoplasmic domain of the receptor that we have observed recently (Bittorf *et al*, 1996). Therefore, in addition to being unable to differentiate or proliferate in response to epo, MEL cells are incapable of transducing any epo-initiated viability signals.

J2E cells also constitutively express the v-*raf* oncogene and it has been suggested that raf may suppress apoptosis (Cleveland *et al*, 1994), possibly via a link with bcl2 (Wang *et al*, 1994). In this case *raf* appears to provide little protection from rapid cell death – perhaps the effect of *myc* on apoptosis is dominant over *raf*? As raf is upstream of MAP kinase in the phosphorylation cascade, it was not surprising to observe that the MAP kinases were highly phosphorylated in J2E cells. However, with serum deprivation raf kinase activity remained unaltered, while MAP kinase activation fell precipitously. There appear to be two explanations for this phenomenon: (i) v-*raf* is not entirely responsible for the phosphorylation status of MAP kinases in J2E cells and serum factors also contribute to its activation, (ii) a phosphatase (perhaps a MAP kinase specific phosphatase) is stimulated with FCS depletion. Although MAP kinases could be partially re-activated by FCS, serum factors are also mitogenic and it is conceivable that the phosphorylation of MAP kinases is related to intracellular signalling for proliferation, rather than for maintaining viability. Two pieces of evidence support the concept that the MAP kinase pathway is associated with proliferation and differentiation, but not survival. First, J2E-NR cells remain alive in the presence of the epo and MAP kinases are not activated (Tilbrook *et al*, 1996a). Second, down-regulation of MAP kinases with anti-sense oligonucleotides had no effect on epo-stimulated viability of J2E cells (Table 3). The data agree with the observation that v-*abl* suppression of apoptosis is independent of MAP kinase activation (Owen-Lynch *et al*, 1995).

Materials and Methods

Cell culture

J2E cells (Klinken *et al*, 1988) and MEL cell line, F4N (Dube *et al*, 1975) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. Recombinant growth factors used in these studies were: 10 U/ml human epo (Eprex; Janssen-Cilag, Sydney, Australia), 100 U/ml SCF (a gift from Dr P Steinlein, Institute for Molecular Pathology, Vienna, Austria), 100 U/ml IL-3 and GM–CSF (gifts from Dr Nicos Nicola, Walter and Eliza Hall Institute, Melbourne, Australia), 100 U/ml insulin (Commonwealth Serum Laboratories, Melbourne, Australia), 100 U/ml IGF-1 and 100 U/ml growth hormone (gifts from Dr G Werther, Royal Children's Hospital, Melbourne, Australia). Aphidicolin (Sigma, St Louis, Mo, USA) was used at a final concentration of 2.5 mM.

Cytocentrifuge preparations and electron microscopy

Cells were cytocentrifuged at 300 rpm for 5 min onto glass slides using a cytospin 2 (Shandon, Cheshire, UK) and fixed with methanol. May-Grunwald-Geimsa stain (BDH, Poole, England), bisbenzimide H33258 (Calbiochem-Novabiochem, San Diego, CA, USA) or acridine orange (Sigma, St Louis, MO, USA) were placed on the slides for 5 min, washed in Sorenson's buffer (66 mM Na_2HPO_4, 66 mM KH_2PO_4) and then viewed with either a Zeiss light microscope or a Leitz Ortholux 2 fluorescence microscope and photographed with a Leitz Orthomat 2 camera. Cell viability was determined by exclusion of 0.4% eosin as viewed on a haemocytometer (Klinken *et al*, 1988; Busfield and Klinken, 1992), while differentiation was quantitated by benzidine staining of haemoglobin producing cells (Cooper *et al*, 1974). Apotag was purchased from ONCOR (Gaithersberg, MD, USA). For ultrastructural analyses cells were fixed in 2.5% paraformaldehyde/2.5% glutaraldehyde, sectioned, stained with uranylacerate and viewed under a Philips 410 electron microscope as described previously (Busfield *et al*, 1993c).

Cell cycle analysis and [^{3}H]-thymidine incorporation

Cells were prepared for cycle analysis by adding 0.25% Triton X-100 and 250 mM propidium iodide to 10^6 cells. The stained nuclei were then analyzed on a FACScan II unit (Becton Dickinson, Mountain View, CA, USA) and the proportion of cells in each phase of the cell cycle calculated (Busfield and Klinken, 1992). To measure DNA synthesis 0.5 μCi [^{3}H]-thymidine (25 mCi/mmol [methyl-^{3}H]-thymidine; Amersham, Bucks, UK) was added to 5×10^4 cells for 6 h. DNA was then collected onto glass fibre filters (Whatman, Kent, UK) using a microharvester (Skatron, Lier, Norway) and incorporation of labelled nucleotide measured by scintillation counting in a Beckman LS 6800 counter (Busfield and Klinken, 1992).

Data analysis

DNA integrity was assessed by a modification of the method of Strange *et al* (1992) as used by us previously (Tilbrook *et al*, 1996a). Cells were pelleted, lysed with 6 M guanidine hydrochloride (BRL Life Technologies, MD, USA) and nucleic acids precipitated with ethanol. The pellet was then resuspended in 10 mM Tris, 10 mM EDTA, 10 mM NaCl, 0.5% SDS. 0.2 μg/ml proteinase K and incubated at 50°C overnight. After phenol/chloroform extraction and ethanol precipitation the pellet was resuspended in 10 mM Tris, 1 mM EDTA 20 mg/ml RNase A (Sigma) and incubated at 37°C for 1 h. The DNA content was determined by staining with bisbenzimide H 33258 (Calbiochem-Novabiochem), according to the method of Labraco and Paigen (1980), before 7.5 μg of each sample was separated through a 1% agarose gel.

Protein analysis

J2E cells were washed three times in DMEM and resuspended at $2-5\times10^5$ cells/ml DMEM. For MAP kinases analysis, cells were incubated for up to 5 h before re-addition of 10% FCS for 30 min. For transcription factor and cell cycle protein analyses, cells were incubated for up to 24 h in the presence or absence of epo (5 U/ml). Cells were lysed and proteins analysed by immunoblotting with anti-MAP kinase (Erk 1), jun, fos, myc, cdc 2(p34), cdk2, cdk4, cdk5, PCNA, cyclins A_1, B_1, D_1, D_2, D_3, and cdc 25 antibodies (Santa Cruz Biotechnology, CA, USA) as described previously (Busfield *et al*, 1995a,b; Callus *et al*, 1995; Tilbrook *et al*, 1996a,b).

Raf activity

J2E cells were washed three times in DMEM, resuspended at 2×10^6/ml and incubated for up to 6 h in DMEM without serum. Cells were lysed and 1 mg protein immuno-precipitated with anti-raf antibody (Santa Cruz Biotechnology) as described previously (Busfield *et al*, 1995a; Tilbrook *et al*, 1996a,b). The immunoprecipitates were washed in kinase buffer (25 mM HEPES pH 7.4, 25 mM β-glycerol phosphate, 1 mM DTT, 10 mM $MgCl_2$), resuspended in 100 μl kinase buffer with 20 μCi [^{32}P]γATP (Amersham), 0.1 mM ATP and either 20 μg Syntide (Santa Cruz Biotechnology) or 200 μg myelin basic protein (Sigma) for 20 min at 30°C. The reaction was terminated by addition of $1\times$ Laemelli buffer, the samples were then separated on an 8–25% gradient gel, which was later dried and exposed to X-ray film. Incorporation of [^{32}P]γATP into the substrate was quantitated densitometrically.

Anti-sense oligonucleotide protocols

Phosphorothioate modified oligonucleotides were purchased from Macromolecular Resources (CO, USA) and Biognostik (Göttingen, Germany). Anti-sense oligo-nucleotides were generated to JAK2 (5′-GCT TGT GAG AAA GC-3′), p42 and p44 MAP kinases (5′-GCC GCC GCC GCC GCC AT-3′) and c-jun (5′-CGT TTC CAT CTT TGC AGT-3′) as described previously (Tilbrook *et al*, 1996b; Sale *et al*, 1995; Soprano *et al*, 1992). The random/scrambled oligonucleotides were 5′-GTC CCT ATA CGA AC-3′ (Tilbrook *et al*, 1996b), 5′-CGC GCG CTC GCG CAC CC-3′ (Sale *et al*, 1995) and 5′-CGT GTC AAT AAT GGC AGT-3′ (Soprano *et al*, 1992). J2E cells were established at 5×10^4 cells/ml and incubated with 2–10 μM oligonucleotide for three days; cultures were diluted to 5×10^4 cells/ml daily and the oligonucleotide replenished. Cells were then washed three times in DMEM, replated at $3-5\times10^5$ cells/ml with 2–10 μM oligonucleotide and epo (5 U/ml). Cultures were maintained for a further 24–48 h and the oligonucleotide was replenished after 24 h. Protein levels were analysed by Western blots and quantitated by densitometry. Statistical significance was demonstrated by students t-test where a P value of <0.05 was considered significant.

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