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c-Myc-induced apoptosis in fibroblasts is inhibited by specific cytokines

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We have investigated the mechanism by which deregulated expression of c-Myc induces death by apoptosis in serum-deprived fibroblasts. We demonstrate that Myc-induced apoptosis in low serum is inhibited by a restricted group of cytokines, principally the insulin-like growth factors and PDGF. Cytokine-mediated protection from apoptosis is not linked to the cytokines' abilities to promote growth. Protection from apoptosis is evident in the post-commitment (mitogen-independent) S/G_2/M phases of the cell cycle and also in cells that are profoundly blocked in cell cycle progression by drugs. Moreover, IGF-I inhibition of apoptosis occurs in the absence of protein synthesis, and so does not require immediate early gene expression. We conclude that c-Myc-induced apoptosis does not result from a conflict of growth signals but appears to be a normal physiological aspect of c-Myc function whose execution is regulated by the availability of survival factors. We discuss the possible implications of these findings for models of mammalian cell growth *in vivo*.
Key words: apoptosis/cytokines/c-Myc/serum deprivation

Introduction

The c-*myc* gene encodes a short-lived basic helix-loop-helix/leucine zipper (bHLH-LZ) nuclear phosphoprotein, c-Myc, which binds DNA in a sequence-specific manner when dimerized with the heterologous protein Max (reviewed in Evan and Littlewood, 1993). In fibroblasts, c-Myc expression is continuously and tightly regulated by mitogen availability (Dean *et al.*, 1986; Waters *et al.*, 1991). Following mitogenic stimulation, quiescent fibroblasts show rapid induction of c-Myc, which peaks at 3–5 h and then falls to an intermediate level that is maintained for as long as mitogens remain present. Removal of mitogens from fibroblasts causes the rapid, synchronous and cell-cycle independent down-regulation of both c-*myc* mRNA and protein (Dean *et al.*, 1986; Waters *et al.*, 1991) and is accompanied by eventual growth arrest in the G_1 phase of the cell cycle. Inhibition of c-Myc function, either by inhibition of expression with antisense oligonucleotides (Heikkila *et al.*, 1987; Coffey *et al.*, 1988; Prochownik *et al.*, 1988; Wickstrom *et al.*, 1989) or via the dominant negative effects of Max over-expression (Amati *et al.*, 1993a) effectively blocks cell proliferation. Moreover, ectopic expression of c-Myc alone is sufficient to drive growth factor-deprived fibroblasts into cycle and keep them there (Eilers *et al.*, 1989, 1991; Evan *et al.*, 1992). Thus, c-Myc appears to be both necessary and, at least in fibroblasts, sufficient for continuous cell proliferation.

Recently, we described the surprising observation that, although deregulated c-Myc expression induces proliferation in serum-deprived fibroblasts, cell numbers do not necessarily increase because substantial cell death occurs by apoptosis (Evan *et al.*, 1992). Induction of apoptosis by c-Myc was greatest in cells expressing high levels of the protein but was nonetheless clearly apparent in cells expressing 'normal' levels of c-Myc, i.e. levels found in untransformed proliferating fibroblasts. Analogous Myc-dependent apoptosis was also reported in haematopoietic cells upon deprivation of growth factors (Askew *et al.*, 1991), suggesting that it may be a general phenomenon.

The observation that c-Myc induces apoptosis in serum-deprived cells raises two important questions. First, is it representative of a normal physiological process or does it merely reflect the fact that 'inappropriate' or 'unbalanced' activation of potent growth potentiators like c-Myc generates a lethal conflict in growth signals? Second, what is the molecular mechanism by which c-Myc-induced apoptosis occurs? This latter is important to determine, irrespective of the relevance of Myc-induced apoptosis to normal cells, because deregulated and elevated expression of c-Myc is so ubiquitous in tumours and may thus constitute an inbuilt limitation to the progression of tumours.

Mutagenesis studies show that regions of the c-Myc protein necessary for induction of apoptosis precisely overlap with regions necessary for co-transformation (Evan *et al.*, 1992) and include the N-terminal transactivation domain (Kato *et al.*, 1990) and the C-terminal bHLH-LZ region involved in sequence-specific DNA binding and dimerization with Max (Blackwell *et al.*, 1990; Blackwood and Eisenman, 1991; Fisher *et al.*, 1991; Prendergast and Ziff, 1991; Prendergast *et al.*, 1991; Amati *et al.*, 1992, 1993a; Kato *et al.*, 1992). Moreover, using mutants of c-Myc and Max with reciprocally exchanged dimerization specificity, such that the c-Myc mutant (MycEG) can no longer interact with wild-type Max but can dimerize with the corresponding MaxEG mutant (Amati *et al.*, 1993a), we have recently demonstrated that Max is absolutely required for induction of apoptosis by c-Myc in fibroblasts (Amati *et al.*, 1993b). These data strongly argue that c-Myc induces apoptosis through its action as a transcription factor—presumably by modulation of appropriate target genes.

Evidence also suggests that the implementation by c-Myc of the transcriptional programme required for

apoptosis is not restricted to situations in which a conflict in growth signals arises but is latent in cells that exhibit no overt signs of death, such as those growing in high serum. Addition of cycloheximide to viable Rat-1/c-Myc cells growing in high serum rapidly induces c-Myc-dependent apoptosis (Evan *et al.*, 1992). As cycloheximide is a potent inhibitor of protein synthesis, this implies that the molecular machinery required for apoptosis must already exist within overtly healthy cells as a consequence of their having expressed c-Myc. Thus, c-Myc is involved in establishing the potential for apoptosis rather than in its actual execution.

In this paper, we have addressed the question of why serum deprivation induces apoptosis in fibroblasts that constitutively express c-Myc. We show that apoptosis induced by serum deprivation in fibroblasts is suppressed by certain serum cytokines, specifically the insulin-like growth factors and PDGF. This indicates that apoptosis is not a mere consequence of nutritional privation but due to cytokine deprivation. Other tested mitogenic growth factors confer no protection from cell death. We further show that cytokine-mediated inhibition of apoptosis is not restricted to death induced by serum deprivation but is also manifest in cells in which apoptosis is triggered by the action of cytotoxic drugs. Moreover, cytokine-mediated protection is also evident during post-commitment phases of the cell cycle when growth factors are not required for cell cycle progression. Thus, suppression of apoptosis by cytokines is not necessarily linked to their action as factors that promote cell cycle progression. These results suggest that c-Myc-induced apoptosis in low serum arises not from a conflict in growth signals but because the cells are deprived of cytokines required to suppress a default apoptotic programme implemented by c-Myc. If correct, this conclusion has widespread implications for our understanding of mammalian cell growth control.

Results

Myc-induced apoptosis of fibroblasts in low serum is blocked by specific cytokines

In principle, induction of apoptosis in serum-deprived Rat-1/Myc fibroblasts could be due either to nutrient deprivation (i.e. privation) or to absence of specific cytokines that are necessary for survival of cells expressing c-Myc. To discriminate between these two possibilities, we examined the abilities of a range of individual cytokines to block Myc-induced apoptosis in serum-deprived Rat-1 fibroblasts. Rat-1 cells constitutively expressing the conditional c-Myc chimera, Myc-ER, were grown in medium containing 10% fetal calf serum (FCS) and then transferred to serum-free medium. In such conditions, neither cell proliferation nor apoptosis occurs although both are rapidly induced upon activation of c-Myc with either β-oestradiol (Evan *et al.*, 1992) or 4-hydroxytamoxifen (not shown). Specific cytokines were added to

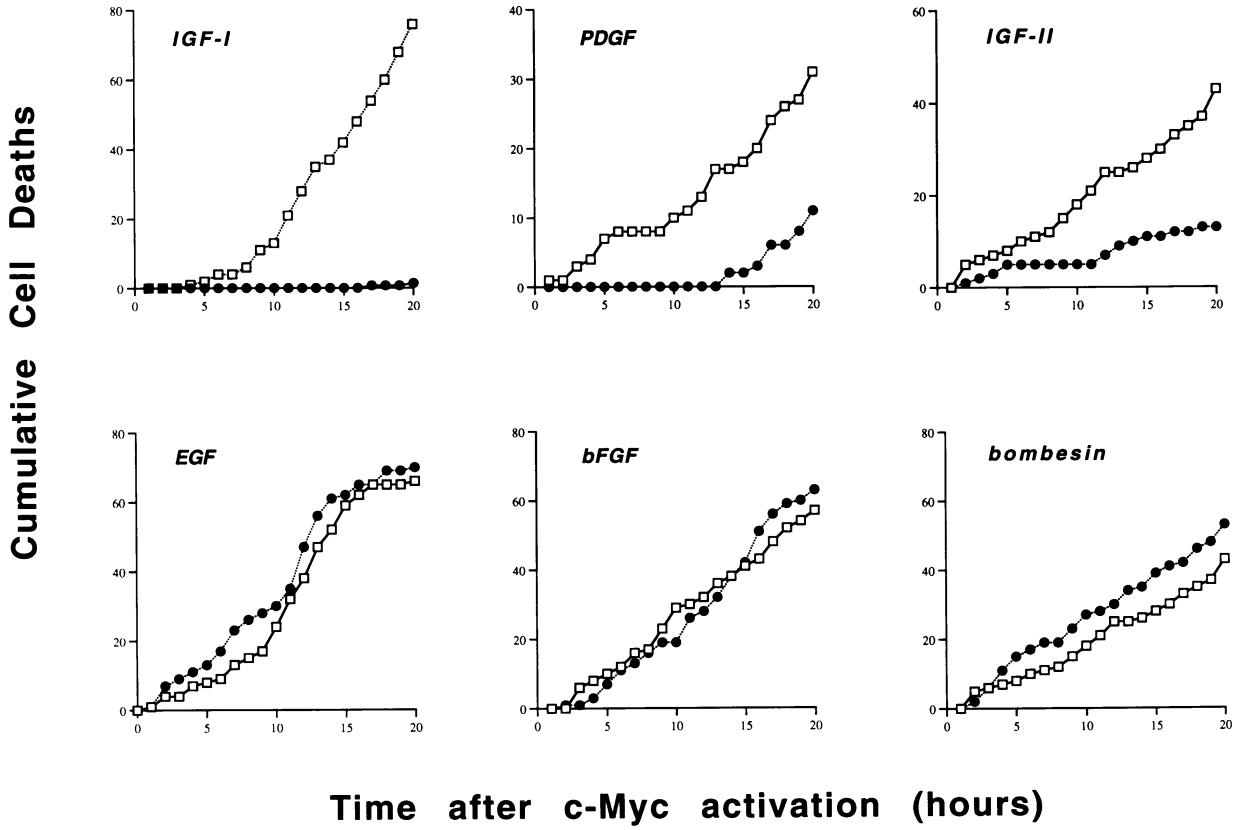

Fig. 1. Effects of various growth factors upon c-Myc-induced apoptosis in serum-deprived Rat 1/c-Myc-ER fibroblasts. Subconfluent Rat 1/c-Myc-ER fibroblasts were deprived of serum for 48 h and c-Myc was then activated by the addition of 2 μM β-oestradiol in the presence (black circles) or absence (white squares) of IGF-1 (100 ng/ml), PDGF (10 ng/ml), IGF II (10 ng/ml), EGF (10 ng/ml), bFGF (100 ng/ml) or bombesin (10 ng/ml), as indicated. The cells were then monitored for 20 h by time-lapse video microscopy. Apoptotic cell deaths were scored as described and cumulative deaths were plotted against time. For each experiment, 100 randomly selected cells within the visual field were examined and images were acquired at a rate of 12 frames per hour.

the serum-deprived Rat-1/c-Myc-ER cells, c-Myc was activated by addition of β-oestradiol, and apoptosis monitored and quantified by time-lapse video microscopy, as described (Evan *et al.*, 1992; Fanidi *et al.*, 1992). Replacement of serum with a cytokine-free serum substitute (CSPR-2, Sigma) that provides the same essential nutrients as serum failed to block apoptosis, suggesting that c-Myc-induced apoptosis does not result from nutritional privation (data not shown; see also Evan *et al.*, 1992). However, addition of any one of the cytokines IGF-I, IGF-II, insulin, PDGF AB or PDGF BB significantly suppressed apoptosis in the absence of any other exogenous cytokines or nutrients. Data for PDGF AB, IGF-I and IGF-II are shown in Figure 1. In contrast, EGF, basic FGF (bFGF), acidic FGF, interleukin-1 and bombesin failed to inhibit apoptosis. Results for EGF, bFGF and bombesin are also shown in Figure 1.

To verify that Rat-1/Myc-ER cells express receptors for all the cytokines tested, we assayed induction of c-Fos and Egr-1/Zif268 proteins in Rat-1/Myc-ER cells following addition of each cytokine. Both c-Fos and Egr-1/Zif268/NGFIA proteins are expressed as an immediate early response in various cell types following a range of extracellular stimuli. Rat-1/Myc-ER cells were grown to ~30% confluence in multi-well chamber slides and then deprived of serum for 48 h. Without changing the medium, individual growth factors were then added to the culture and 60 min later the cells were fixed and examined for c-Fos or Egr-1 protein expression by immunofluorescence. All tested cytokines induced the rapid and transient expression of both c-Fos and Egr-1/Zif268/NGFIA nuclear proteins (Figure 2), demonstrating the presence both of appropriate receptors and of intact downstream signal transduction pathways for each cytokine.

We next investigated the effective concentration ranges at which each cytokine was effective at blocking apoptosis in Rat-1/c-Myc-ER fibroblasts. The cytokines IGF-I, PDGF AB, insulin, EGF, bFGF and bombesin were each tested for anti-apoptotic activity over at least a 10^4-fold concentration range which bracketed that at which each cytokine exerts detectable mitogenic effects. Cell viability was determined using the MTT assay which assesses mitochondrial integrity (Mosmant, 1983). The anti-apoptotic effects of IGF-I, PDGF and insulin were titratable and evident at levels similar to those at which each cytokine is mitogenic (10–100 ng/ml, 1–10 ng/ml and 1–5 μg/ml respectively). In contrast, EGF, bFGF and bombesin were all ineffective at inhibiting apoptosis even at levels 10-fold higher than those commonly used mitogenically (i.e. 100, 1000 and 100 ng/ml respectively) (Figure 3). The effective anti-apoptotic concentration ranges of IGF-I and PDGF AB are consistent with notional physiological

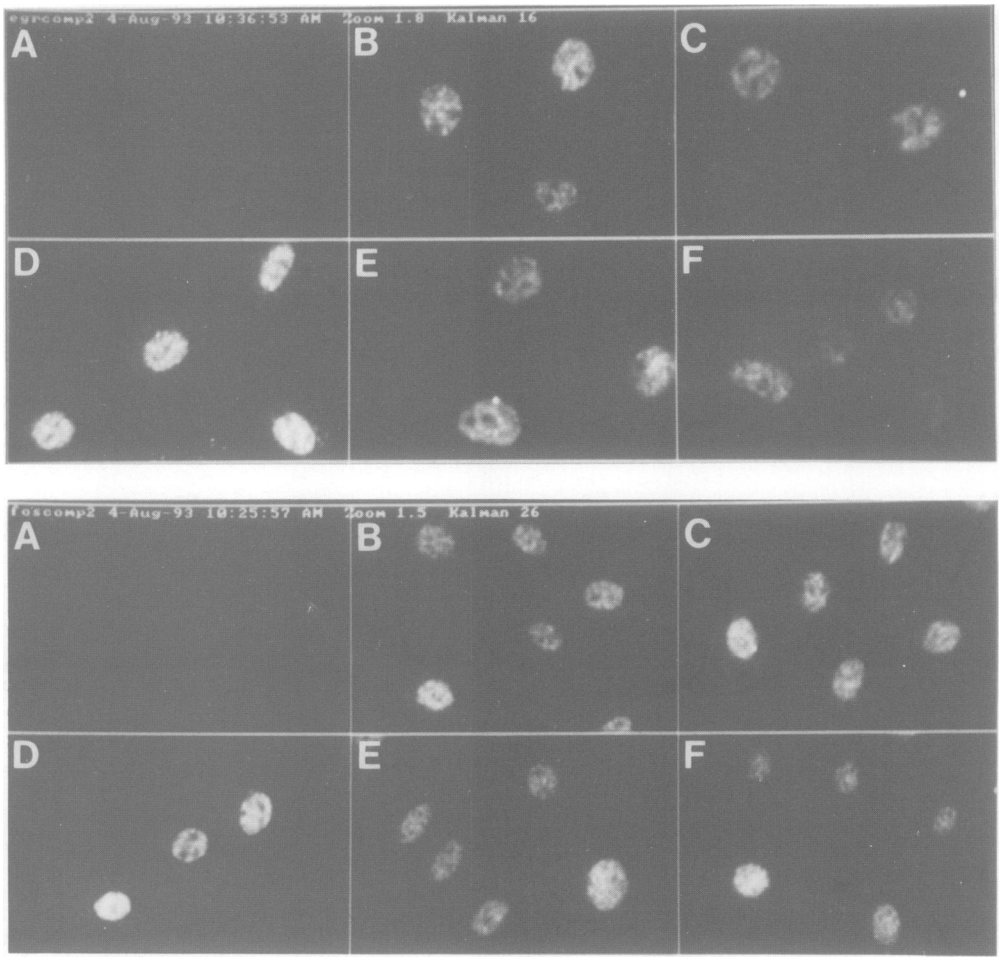

Fig. 2. Induction of c-Fos and Egr-1 proteins by cytokines. Subconfluent Rat-1 cells were deprived of serum for 48 h. Growth factors were then added to the media as follows: (**A**) none, (**B**) PDGF (10 ng/ml), (**C**) IGF-1 (100 ng/ml), (**D**) bombesin (10 ng/ml), (**E**) EGF (10 ng/ml) and (**F**) FGF (100 ng/ml). After a further hour, the cells were fixed and stained for Egr-1 (top panel) or Fos (bottom panel) proteins using appropriate antibodies.

concentrations in so far as they are present at around these levels in media containing 10% FCS: arguably, the relevance of the cytokine environment provided by FCS to the environs of a normal somatic cell in *vivo* is debatable. In contrast, insulin is anti-apoptotic only at unphysiologically high concentrations (>5 μg/ml), pre-

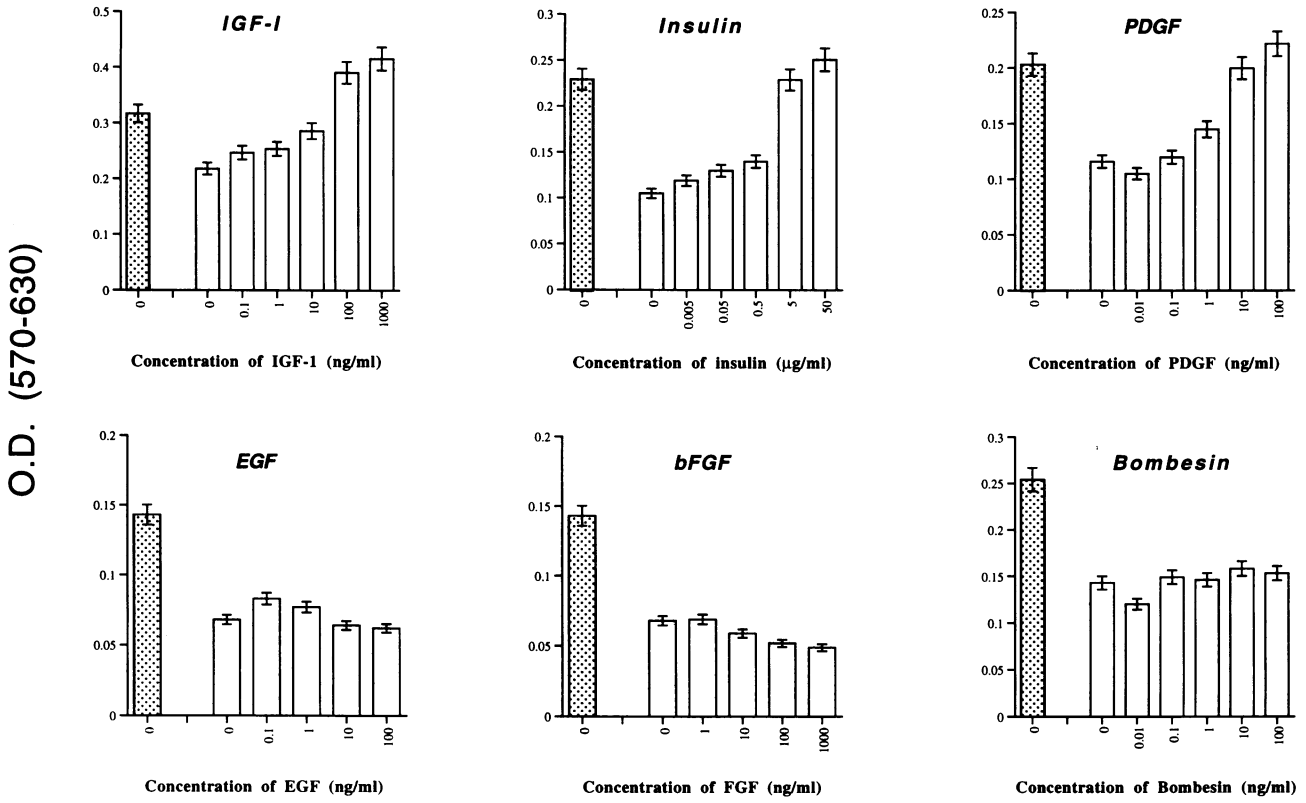

Fig. 3. Titration of effective concentrations of growth factors upon c-Myc-induced apoptosis in serum-deprived Rat-1/ c-Myc-ER cells. Subconfluent Rat 1/c-Myc-ER fibroblasts were deprived of serum for 48 h and c-Myc was then activated by the addition of β-oestradiol to 2 μM in the presence of various concentrations of growth factors as indicated (open bars). In a parallel culture, β-oestradiol was omitted (stippled bars). Cell viability was determined using the MTT assay (see Materials and methods) 48 h later.

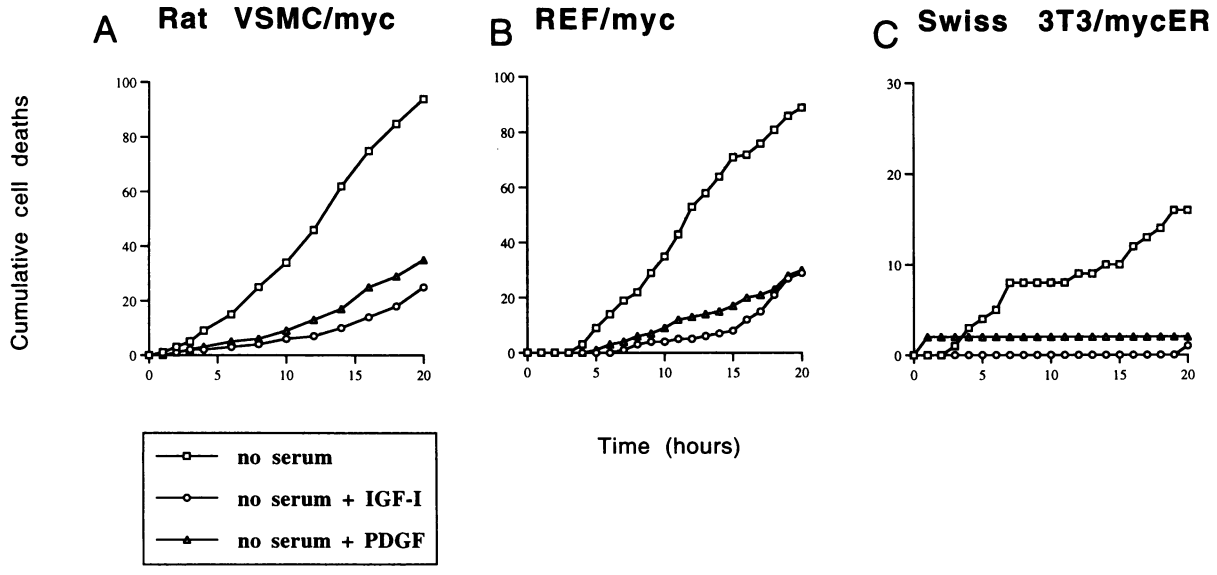

Fig. 4. Inhibition of c-Myc-induced apoptosis by IGF-1 and PDGF in various mesenchymal cells. (**A**) Subconfluent rat VSMC/c-Myc cells were deprived of serum in the presence or absence of IGF-1 or PDGF. The cells were then monitored by time-lapse video microscopy. Apoptotic cell deaths were scored as described and cumulative deaths are shown plotted against time. Each field contained 100 cells at start and images were taken at a rate of 12 frames per hour. (**B**) Subconfluent REF/c-Myc cells were treated and monitored as for (A). (**C**) Subconfluent Swiss 3T3/c-Myc-ER were deprived of serum for 48 h, c-Myc was then activated by the addition of 2 μM β-oestradiol in the presence or absence of IGF-1 or PDGF. After c-Myc activation, cells were monitored by time-lapse video microscopy as described for (A).

sumably reflecting its role as a low affinity agonist for the IGF-I receptor that is empirically added to cell culture systems to promote viability.

To be sure that the anti-apoptotic effects we observed were not merely idiosyncrasies of the Rat-1 cell line, we tested the anti-apoptotic effects of IGF-I and PDGF on other mesenchymal cells (Figure 4). Either secondary rat embryo fibroblasts or primary rat vascular smooth muscle cells that constitutively express c-Myc were deprived of serum. In both cell types, as with Rat-1 cells, serum deprivation triggers apoptosis. Addition of either IGF-I or PDGF AB substantially suppressed apoptosis in both cases. Similarly, Swiss 3T3 cells that constitutively express the c-Myc-ER chimera die in low serum upon activation of c-Myc with β-oestradiol. Again, both IGF-I and PDGF AB were potent inhibitors of apoptosis. With all three cell types examined, EGF, bFGF and bombesin had no anti-apoptotic activity (data not shown). Thus, the anti-apoptotic effects of IGF-I and PDGF appear to be general for rodent cells of mesenchymal origin.

Induction of apoptosis by c-Myc and its inhibition by IGF1 or PDGF is not restricted to pre-commitment G_1 cells

Rat-1 fibroblasts are typical of non-transformed fibroblasts *in vitro* in their requirement for growth factors only during the G_1 pre-commitment phase of the cell cycle (Pardee, 1989). After the late G_1 commitment point, their proliferation becomes mitogen-independent and, even in the absence of serum, S phase cells will complete their cycle and divide on schedule. To demonstrate this, serum-deprived G_0 Rat-1 cells were stimulated with 10% FCS in the presence of 2 mM thymidine for 16 h, after which time almost all of the cells (>92%; data not shown) are arrested at the G_1/S interface. The thymidine block was then removed and 100 randomly picked cells allowed to proceed to mitosis under observation, either in the presence or absence of serum. The mean time taken to enter mitosis was essentially identical in the presence (9.2 ± 2.2 h) or absence (9.4 ± 2.4 h) of serum and, in both cases, >85% of cells divided. Thus, Rat-1 cells are independent of serum growth factors for S/G_2/M progression.

To investigate how closely linked with growth were the induction of apoptosis by c-Myc and its inhibition by IGF-I, we examined the consequences of c-Myc expression during serum deprivation of post-commitment S/G_2/M Rat-1 cells. We reasoned that if c-Myc induces apoptosis in serum-deprived Rat-1 cells because of a conflict between the growth-promoting activity of c-Myc and the growth-inhibitory effect of low serum, then c-Myc should induce apoptosis in serum-deprived Rat-1 cells only during periods of the cell cycle when the cells require serum growth factors for proliferation. That is, during the pre-commitment G_1 phase of the cell cycle. By the same argument, if the ability of IGF-I to suppress apoptosis is dependent upon its mitogenic activity, IGF-I should only suppress c-Myc-induced apoptosis during the same pre-commitment period. In contrast, if c-Myc continuously activates an apoptotic pathway, and cytokines like IGF-I act to suppress it (Evan and Littlewood, 1993), the actions of both should be independent of the cell cycle. Accordingly, we directly tested whether c-Myc induces apoptosis during S/G_2 and whether IGF-I can inhibit such apoptosis.

Asynchronous exponentially proliferating Rat-1/c-Myc-ER cells growing in 10% FCS were pulse-labelled with BrdU for 60 min, the cells then transferred to serum-free medium and c-Myc-ER activated by addition of β-oestradiol to the culture medium. After a further hour, the cells were fixed, permeabilized and stained with propidium iodide, to allow morphological identification of apoptotic cells, and with anti-BrdU antibodies to identify S-phase cells. At least 2000 cells were scored in each experiment. As the G_2/M period of Rat-1 cells is at least 3 h (data not shown), 2 h is too short a period for any BrdU-labelled S-phase cell to exit S, traverse G_2 and M and re-enter G_1. Thus, any BrdU-positive cell must be in the post-commitment S/G_2/M period of the cell cycle.

After 1 h in serum-free medium, 1.63% of all cells exhibited pycnotic nuclei and a cell morphology indicative of apoptosis. In contrast, no apoptotic cells were visible in cultures maintained in serum. At the end of the experiment, 37% of all cells scored positive for BrdU incorporation and were thus either in S-phase or within 2 h of leaving S-phase and, therefore, post-commitment. 1.60% of all BrdU-positive cells were apoptotic—essentially the same proportion of cells as in the BrdU-negative population. An example of a BrdU-positive apoptotic cell is shown in Figure 5. Addition of either IGF-I or PDGF AB during the 1 h period of serum-deprivation completely prevented any detectable apoptosis in either BrdU-negative or BrdU-positive populations (data not shown). Thus, c-Myc can induce apoptosis in post-commitment serum-deprived fibroblasts and this apoptosis is inhibited by the anti-apoptotic cytokines IGF-I and PDGF.

Although it remains formally possible that the BrdU incorporation we observe in apoptotic cells is a consequence of repair rather than DNA synthesis, we believe this is unlikely for several reasons. First, there is no *a priori* reason why substantial repair should be occurring in otherwise normal log-phase cells growing in high serum during the BrdU labelling period. Second, the extent of

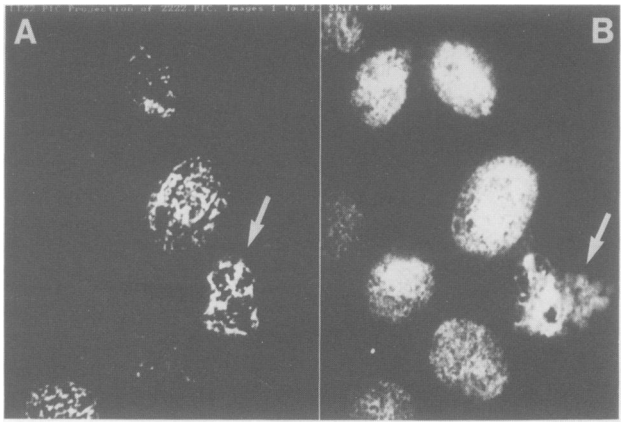

Fig. 5. c-Myc induces apoptosis in serum-deprived Rat-1/c-Myc ER cells in the post-commitment phase of the cell cycle. Asynchronous log phase Rat-1/c-Myc ER cells were metabolically labelled with BrdU for 1 h. BrdU was then washed away, the cells deprived of serum and c-Myc activated by the addition of 2 μM β-oestradiol to the medium. After a further hour, cells were fixed and stained for (**A**) BrdU incorporation and (**B**) DNA with propidium iodide. An example is shown of a BrdU-positive apoptotic cell.

BrdU incorporation within the nuclei of BrdU-positive apoptotic cells is substantial and consistent with that observed in normal S-phase cells. Third, the proportion of apoptotic cells in BrdU-negative (pre-commitment) and BrdU-positive (post-commitment) populations is the same, arguing that apoptosis does occur without incorporation of BrdU. We therefore conclude that c-Myc induces apoptosis in factor-deprived post-commitment cells and that this apoptosis is inhibited by IGF-I or PDGF at a time when neither cytokine is required for cell cycle progression.

Inhibition of drug-induced apoptosis by cytokines

c-Myc induces apoptosis in Rat-1 fibroblasts exposed to either the DNA synthesis inhibitor thymidine or the topoisomerase II-inhibitor etoposide. These drugs arrest cells in S and S/G_2 respectively (Evan *et al.*, 1992; Fanidi *et al.*, 1992), both subsequent to the late G_1 commitment point and therefore at points in the cell cycle when cells are no longer dependent upon growth factors for cell cycle progression. We were therefore interested to determine whether the presence of IGF-I or PDGF influenced such drug-induced cell death.

Identical cultures of subconfluent log-phase Rat-1/c-Myc-ER cells were grown for 20 h in medium containing 10% FCS and either 2 mM thymidine or 0.1 μM etoposide. Effective growth arrest was ascertained by flow cytometry and time-lapse video microscopy (data not shown). In order to exclude any effects resulting from cytokines present in serum, the medium was then replaced for 1 h with serum-free medium containing the appropriate drug. Although serum-deprivation is itself sufficient to trigger c-Myc-dependent apoptosis, both drugs markedly increased the onset of cell death over and above this basal rate (not shown; see also Evan *et al.*, 1992). Either IGF-I or PDGF was then added to the growth medium as appropriate and c-Myc activated by addition of β-oestradiol. The cells were then monitored for apoptosis and division by time-lapse video microscopy. In cultures where c-Myc was not activated (β-oestradiol omitted), apoptosis was minimal (not shown; see also Evan *et al.*, 1992). In contrast, activation of c-Myc in the absence of any cytokines triggered the onset of significant apoptosis. Addition of IGF-I significantly delayed and suppressed apoptosis (Figure 6). PDGF also afforded protection from apoptosis although it was less effective than IGF-I (data not shown). No cell division was observed in drug-treated cultures, demonstrating that neither IGF-I nor PDGF can relieve drug-induced cytostasis (data not shown).

Thus, IGF-I and PDGF both suppress Myc-dependent apoptosis in post-commitment drug-treated fibroblasts. Moreover, in this experiment, the presence of each drug was maintained throughout with the result that the affected cells remained arrested and unable to traverse their cell cycle. The protection afforded by IGF-I and PDGF under such severe conditions attests to their anti-apoptotic efficacy and underscores the notion that their anti-apoptotic effects are unlinked to their growth-promoting properties.

IGF-1-suppression of apoptosis does not require new protein synthesis

IGF-I and PDGF are both inducers of immediate early genes in quiescent or serum-deprived fibroblasts (Baserga, 1992), raising the possibility that either might exert its anti-apoptotic effects by modulation of specific genes. As discussed above, the inhibitor of protein synthesis cycloheximide is a potent inducer of apoptosis in Rat-1 fibroblasts that express c-Myc protein (Evan *et al.*, 1992) over and above that triggered by serum deprivation alone (Figure 7). To explore whether or not IGF-I- or PDGF-mediated protection from apoptosis requires new protein synthesis, we tested the ability of IGF-I and PDGF AB to inhibit cycloheximide-induced apoptosis in Rat-1/Myc cells. Identical subconfluent log-phase Rat-1/Myc cells

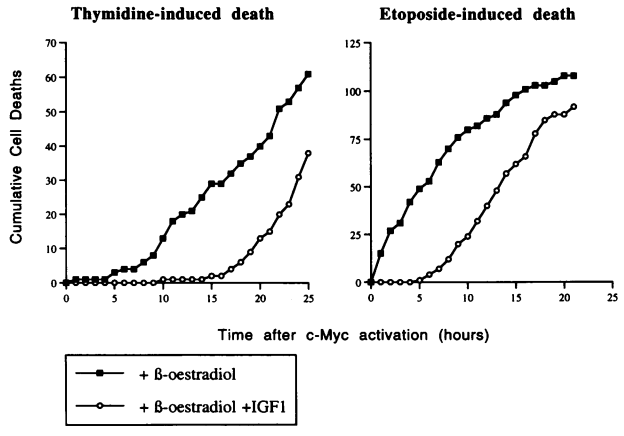

Fig. 6. IGF-1 inhibits c-Myc induced apoptosis in drug-treated Rat-1/c-Myc-ER cells. Asynchronous subconfluent log-phase Rat-1/c-Myc-ER cells were treated with either 2 mM thymidine or 0.1 μM etoposide for 24 h in the presence of 10% FCS. The medium was then replaced for 1 h with serum-free medium containing each drug and then c-Myc was activated by the addition of 2 μM β-oestradiol in the presence (white circles) or absence (black squares) of IGF-1.

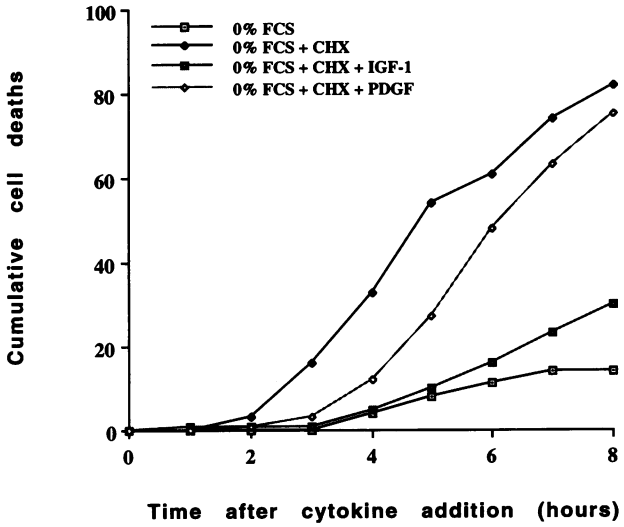

Fig. 7. IGF-1 inhibits apoptosis in Rat-1/c-Myc-ER cells in the presence of cycloheximide. Subconfluent Rat 1/c-Myc-ER cells were treated with 50 μg/ml cycloheximide in the absence of serum for 1 h and IGF-1 or PDGF AB were then added. A parallel culture was deprived of serum in the absence of cycloheximide. The cells were then monitored by time-lapse video microscopy. Apoptotic cell deaths were scored as described and cumulative deaths were plotted against time. In each experiment, 100 randomly selected cells within the visual field were examined and images were acquired at a rate of 12 frames per hour.

growing in 10% FCS were transferred into medium containing 0% FCS for 1 h. Cycloheximide was then added to a final concentration of 50 μg/ml. After a further hour, during which all detectable protein synthesis ceased (data not shown), either IGF-I or PDGF was added as indicated and 100 randomly picked cells were monitored for apoptosis by time-lapse video microscopy. The results (Figure 7) show that IGF-I affords substantial protection from cycloheximide-induced apoptosis whereas PDGF is only weakly protective. We conclude that the inhibition of apoptosis mediated by IGF-I is manifest in the absence of new protein synthesis or gene expression.

Discussion

Induction of apoptosis by c-Myc protein

We had previously demonstrated that expression of c-Myc induces apoptosis in serum-deprived rodent fibroblasts (Evan *et al.*, 1992). In this paper we have confirmed the generality of this phenomenon using various primary and immortalized fibroblasts and vascular smooth muscle cells. In order to control effectively the activity of c-Myc, we have made use of c-Myc-ER fusion proteins whose activity is dependent upon the presence of exogenous β-oestradiol. Recently, however, potential problems have emerged with the use of ER fusion proteins when studying transcription factors, principally because the ER domain utilized possesses its own transactivation activity. Thus, any biological effects observed with c-Myc-ER could be due to the targeting of this contaminating ER transactivation domain to DNA by the attached c-Myc moiety. However, in the case of induction of proliferation and apoptosis by c-Myc-ER, there are several reasons why this is very unlikely to be the case. First, cells expressing wild-type c-Myc protein exhibit identical phenotypes to c-Myc-ER cells treated with β-oestradiol: namely, inability to arrest growth in low serum and concomitant induction of apoptosis. Second, the c-Myc mutant Δ106–143, which lacks a critical N-terminal c-Myc transactivation domain (Kato *et al.*, 1990) required for co-transformation (Stone *et al.*, 1987) and induction of apoptosis (Evan *et al.*, 1992), is totally inactive in inducing apoptosis when fused to ER (Evan *et al.*, 1992). Third, apoptosis in serum-deprived Rat-1/c-Myc-ER cells is triggered by 4-hydroxytamoxifen (unpublished data of E.Harrington, A.Fanidi and G.I.Evan). The ER fragment used in construction of the c-Myc-ER chimeras has a binding site for TAF-2 only, the TAF-1 site is absent. The TAF-2 transactivating function is not induced by 4-hydroxytamoxifen (Berry *et al.*, 1990). Thus, we conclude that the induction of apoptosis in serum-deprived fibroblasts expressing c-Myc-ER is a function of c-Myc rather than a contaminating activity arising from the ER domain.

Two simple models may be invoked to explain the induction of apoptosis by c-Myc in serum-deprived fibroblasts. In the first, apoptosis is a consequence of some catastrophic imbalance in proliferative pathways—in effect, a conflict of growth signals. Thus, cells that ectopically express c-Myc undergo apoptosis in low serum because of a conflict between the 'GO' signal afforded by c-Myc and the 'STOP' signal arising from serum deprivation. Put another way, serum mitogens act as obligate auxiliary 'GO' signals that are needed to relieve this conflict in growth signals. In this 'conflict' model, induction of apoptosis is clearly not a normal function of c-Myc but a pathological consequence of its inappropriate or unbalanced expression. This 'imbalance' or 'conflict' scenario is an intuitively plausible explanation and has been widely invoked to explain c-Myc-induced apoptosis (e.g. Touchette, 1992; Cohen, 1993).

Recently, we proposed an alternative hypothesis to explain the induction of apoptosis by c-Myc (Evan and Littlewood, 1993). In this 'dual signal' model, the implementation of a genetic programme for apoptosis is not confined to instances of 'conflict' but is a normal and *obligate* function of c-Myc. Thus, an apoptotic programme is implemented whenever c-Myc is expressed—for example, in response to mitogenic stimulation—and ensuing survival of the cell is dependent upon the availability of 'survival factors' that suppress implementation of the apoptotic programme. According to this 'dual signal' model, cells expressing c-Myc die in low serum not because of a conflict of growth signals but because the cells are deprived of 'survival factors' that mitigate the underlying apoptotic programme implemented by c-Myc. Alternatively, cell survival might be mediated by expression of anti-apoptotic proteins such as Bcl-2 (Bissonnette *et al.*, 1992; Fanidi *et al.*, 1992; Wagner *et al.*, 1993) or adenovirus E1B (Rao *et al.*, 1992; White *et al.*, 1992), neither of which needs, in this model, to have any growth-promoting activity.

c-Myc-induced apoptosis in mesenchymal cells is blocked by specific cytokines

Data from mutagenesis studies, together with its obligate requirement for Max, all strongly argue that c-Myc induces apoptosis transcriptionally. Moreover, the observation that cycloheximide induces apoptosis in c-Myc-expressing cells indicates that the apoptotic programme does not require new protein synthesis to be manifest and must therefore pre-exist in otherwise healthy cells. This argument effectively excludes the possibility that c-Myc implements its transcriptional apoptotic programme only in response to some nascent intracellular conflict. Instead, it seems that c-Myc must continuously implement the potential for apoptosis but that the actual execution depends upon other factors—for example, the availability of serum.

Accordingly, we began by determining if defined components of serum can modulate c-Myc-dependent apoptosis in fibroblasts. We examined the anti-apoptotic effects of a range of cytokines, all present at detectable levels in FCS and all able to induce immediate early gene expression. Of those cytokines tested, PDGF and the insulin-like growth factors exerted significant anti-apoptotic effects at concentrations similar to those present in FCS. In contrast, insulin was anti-apoptotic only at unphysiologically high levels (~50 μg/ml), consistent with the notion that it exerts its survival-potentiating effects *in vitro* by acting as a low affinity agonist of IGF-I. No other tested cytokine exhibited any detectable anti-apoptotic activity, even if present at very high concentrations. This is despite the fact that EGF, bFGF and bombesin, all induce expression of immediate early proteins and are mitogenic for both Swiss 3T3 (Rozengurt, 1986) and Rat-1 cells (Thies *et al.*, 1989 and unpublished data).

Although PDGF and IGFs are both very effective at suppressing c-Myc-induced apoptosis, neither factor alone is sufficient to suppress apoptosis in c-Myc-expressing cells during prolonged periods of serum starvation. This may reflect the fact that combinations of serum cytokines, some not yet identified, are required for maximal protection from apoptosis or that exogenously added cytokines become rapidly exhausted from serum-free media both via receptor uptake and by non-specific adsorption to tissue culture plastic. We are currently investigating both of these possibilities.

In the 'conflict of growth signals' model, IGFs and PDGF block apoptosis because they provide ancillary growth signals that are required for viable integration of the cell's growth programme. In contrast, the 'dual signal' hypothesis postulates that PDGF and IGFs prevent cell death by directly modulating the cell death pathway, irrespective of growth status of the cell. Thus, the 'dual signal' model makes a unique and strong prediction that the abilities of survival factors to inhibit apoptosis are not dependent upon the competence of those factors to promote cell cycle progression. With this distinction in mind, we have attempted to evaluate the 'conflict' and 'dual signal' hypotheses in two ways. First, by asking whether the induction of apoptosis by c-Myc in fibroblasts deprived of growth factors is restricted to periods of the cell cycle during which fibroblasts require growth factors for proliferation. Second, by asking whether anti-apoptotic cytokines inhibit c-Myc-induced cell death under conditions where they cannot promote cell growth.

A preliminary indication that the mitogenic and survival-potentiating properties of serum cytokines might be unlinked arose from examination of which cytokines are most effective in each function. IGF-I alone is poorly mitogenic in fibroblasts (Baserga, 1992; unpublished data of G.I.Evan), yet at any tested concentration appears the most effective anti-apoptotic cytokine. PDGF is both potent mitogen and survival factor. However, EGF, bFGF and bombesin are all potent fibroblast mitogens (Rozengurt, 1986) yet possess no anti-apoptotic activity at any concentration. Thus, there is no obvious correlation between mitogenic and anti-apoptotic activity.

Fibroblasts are dependent on growth factors only during the pre-commitment G_1 phase of the cell cycle (Larsson *et al.*, 1985; Zetterberg and Larsson, 1985; Pardee, 1989): post-commitment $S/G_2/M$ fibroblasts complete their cycle on schedule in the absence of serum (see above). We reasoned that if c-Myc triggers apoptosis in serum-deprived cells because of a conflict in growth signals required for cell cycle progression, activation of c-Myc in serum-deprived S/G_2 fibroblasts should not induce cell death until the cells re-enter G_1 and recover their requirement for mitogens. In contrast, if c-Myc implements a default apoptotic programme that is antagonized by anti-apoptotic cytokines, as proposed in the 'dual signal' model, then neither induction of apoptosis by c-Myc nor its inhibition by IGF-I or PDGF should be cell cycle-dependent. Consistent with the dual signal model, activation of c-Myc in post-commitment S/G_2 cells induces the immediate onset of apoptosis in post-commitment cells, and this apoptosis is inhibited by IGF-I and PDGF at a time when neither cytokine is required for cell cycle progression.

Cells that are profoundly blocked in post-commitment parts of the cell cycle with either thymidine (S) or etoposide (S/G_2) are induced to undergo apoptosis upon activation of c-Myc (Evan *et al.*, 1992; Fanidi *et al.*, 1992). There are two simple explanations for such drug-induced apoptosis. In the first, drug-induced apoptosis once again arises from a conflict, in this case between the growth-promoting activity of c-Myc and the cytostasis imposed by the drug. In this case, the presence of additional growth-promoting agents such as IGF-I or PDGF might reasonably be expected to aggravate the conflict and increase apoptosis. The second explanation holds that the induction of apoptosis by drugs like thymidine and etoposide arises not from cytostasis *per se* but because both induce DNA damage that triggers cell death. In the 'dual signal' model, c-Myc expression, by implementing the potential for apoptosis, might be expected to increase sensitivity to triggering of apoptosis by drugs. Equally, such apoptosis would be mitigated by the presence of anti-apoptotic cytokines. This latter explanation is most consistent with our observation that both IGF-I and PDGF exert anti-apoptotic activity in Myc-fibroblasts exposed to thymidine or etoposide. Similar induction of resistance to drug toxicity has been demonstrated in irradiated bone marrow cells by the haematopoietic survival factor interleukin-3 (Collins *et al.*, 1992). A discrete anti-apoptotic activity for PDGF and IGF-I, unlinked to mitogenesis, is entirely consistent with the activities of both cytokines as survival factors in many terminally differentiated and non-proliferating cell types (reviewed in Raff *et al.*, 1993).

IGF-I is an inducer of an immediate early genetic response in fibroblasts. Substantial evidence argues that such genetic responses are necessary for any mitogenic or differentiation-dependent effects of the cytokine (Baserga, 1992). Our observation that IGF-I can delay and suppress induction of apoptosis in the presence of cycloheximide, and hence without *de novo* protein synthesis, demonstrates that the survival signalling pathway provoked by IGF-I must, at least in part, be discrete from those other pathways. In contrast, the anti-apoptotic effect of PDGF in the presence of cycloheximide is much less profound and appears to require *de novo* expression of genes or proteins to be manifest. We also investigated any effects of cytokines on Bcl-2 within fibroblasts, under conditions of protection from c-Myc-induced apoptosis. We saw no evidence of change either in the level of Bcl-2 protein or in Bcl-2 post-translational modification as assessed by mobility in SDS–polyacrylamide gels (data not shown). This suggests that neither IGF-I nor PDGF acts through Bcl-2. However, recent awareness of the complexity of the Bcl-2 family (Boise *et al.*, 1993; Kozopas *et al.*, 1993; Lin *et al.*, 1993; Oltvai *et al.*, 1993) means that a more comprehensive survey of expression of Bcl-2 family members is required before a role for these proteins in cytokine-mediated protection in fibroblasts can be excluded.

In summary, our data show that the effect of IGF-I in suppressing c-Myc-induced apoptosis in fibroblasts is unlinked to the growth status of the cells or to the particular situation that is triggering cell death, i.e. factor-deprivation or cytotoxic drug. We believe these data strongly favour the 'dual signal' hypothesis in which c-Myc induces apoptosis not because of a conflict in growth

signals but because c-Myc transcriptionally activates a potential apoptotic pathway whose execution is then critically dependent upon the balance of factors that either suppress it, such as IGF-I, or trigger it, such as genotoxic agents. Clearly, these factors and the way they are counterposed may vary considerably between cell types.

Implications of the 'dual signal' hypothesis for control of cell growth and the suppression of carcinogenesis

If true, the 'dual signal' hypothesis has important implications for the growth of cells both *in vitro* and *in vivo* because it necessarily couples the pathways of proliferation with those of programmed cell death. The hypothesis implies that a proliferating somatic cell *in vivo* survives only so long as it is in receipt of appropriate survival factors. Provided, as seems likely (Raff *et al.*, 1993), a substantial level of apoptosis can be tolerated in tissues, limited availability of survival cytokines in the soma would act as a potent homeostatic mechanism to curtail inappropriate cell expansion.

Although coupling of the contradictory pathways of cell proliferation and cell death appears paradoxical, the 'dual signal' hypothesis incorporates a potent mechanism for the suppression of carcinogenesis (Evan *et al.*, 1992; Evan and Littlewood, 1993), a major risk sustained by physically large, long-lived multicellular organisms. The coupling of the mitogenic and apoptotic pathways means that any lesion that activates the mitogenic pathway will prove lethal should the affected cell and its progeny outgrow the paracrine environment enabling their survival. Only in the event that the affected cell rapidly sustains a compensating mutation suppressing cell death will that clone survive. The chances of such simultaneous double mutation are so unlikely as to make the cancer cell extremely rare. Carcinogenesis may therefore not arise by the sequential accumulation of individual mutations, each of which confers some growth advantage on the affected cell. Instead, certain lesions, for example *myc* gene deregulation, may be obligatory for carcinogenic progression yet intrinsically unstable, so curtailing further carcinogenic progression.

In mammalian cell culture, typical criteria of success are minimal cell death and maximal cell growth. However, if the real situation for proliferating cells in somatic tissues is to exist on a 'knife-edge' between survival and death, then the empirical addition of esoteric supplements to *in vitro* culture media may have less to do with physiology and more to do with aesthetics. In addition, *in vitro* growth of cells in media containing high levels of anti-apoptotic cytokines suppresses cell suicide arising from genetic damage, in a manner analogous to that of Bcl-2 (Fanidi *et al.*, 1992; Miyashita and Reed, 1992). Thus, cell culture conditions that optimize survival will necessarily promote survival of damaged cells and thereby increase the net mutation rate of cells *in vitro*. By further analogy with Bcl-2 (Reed *et al.*, 1990; Fanidi *et al.*, 1992), our data suggest that mutations that specifically activate survival signalling pathways in cells may also contribute to carcinogenesis without directly affecting either growth rates of cells or their morphology. Such mutations may be of critical importance in the development of both neoplasia and drug resistance.

Materials and methods

Cell culture and cell lines

Details of Rat-1 fibroblast cell lines expressing various c-Myc mutants and c-Myc-ER chimeras have already been described (Penn *et al.*, 1990a,b; Evan *et al.*, 1992; Fanidi *et al.*, 1992). Rat vascular smooth muscle cells (VSMCs) were isolated from thoracic aortic explants of 6 week old Sprague–Dawley rats. VSMCs were identified by their typical hill and valley morphology in culture and their characteristic immunocytochemical staining for α-smooth muscle actin. Cells at passage 5 were used for experiments and retrovirus vector infections. VSMCs, Swiss 3T3 and secondary rat embryo fibroblasts expressing various c-Myc mutants and c-Myc-ER chimeras were isolated by selection and cloning following infection with appropriate retrovirus vectors, essentially as described for the Rat-1 derivatives (Penn *et al.*, 1990a,b; Evan *et al.*, 1992; Fanidi *et al.*, 1992). Usually, individual clones of each cell type were used in experiments shown. However, all experiments were carried out on several independent clones and also on initial pooled populations of c-Myc-transfected cells, with essentially identical results. Average levels of expression of c-Myc protein in each cell type were determined by ELISA (Moore *et al.*, 1987) and semi-quantitative immunoblotting. All control cells and cells expressing transfected c-*myc* genes were normally maintained in Dulbecco's modified E4 medium supplemented with 10% FCS and, where appropriate, under constant selection by 1 mg/ml Geneticin. Cells were passaged by standard trypsinization and seeded directly onto tissue culture plastic. c-Myc-ER and Δ106–143 c-Myc-ER-expressing clones were maintained in phenol red-free Dulbecco's E4 medium supplemented with 10% charcoal-dextran stripped FCS and 1 mg/ml Geneticin. Myc was functionally activated in c-Myc-ER-expressing cells by the addition to the culture medium of either β-oestradiol to a final concentration of 2 μM (Eilers *et al.*, 1989, 1991; Evan *et al.*, 1992; Fanidi *et al.*, 1992) or 4-hydroxytamoxifen to a final concentration of 100 nM.

Basic and acidic FGF, PDGF AB and BB, EGF, angiotensin II, IGF-I and IGF-II were all obtained from Sigma, insulin from Gibco. Bombesin was a gift from Dr H.Rozengurt (ICRF).

Antibodies and immunological techniques

Antibodies specific for c-Myc (Evan *et al.*, 1992; Littlewood *et al.*, 1992), c-Fos (Hunt *et al.*, 1987) and Egr-1 (Waters *et al.*, 1990) proteins have been described previously. Expression of α-smooth muscle actin was detected using a monoclonal antibody (Sigma). Rabbit antibodies specific for rodent Bcl-2 protein were prepared by immunizing animals with synthetic peptides GAAPTPGIFSFQPE and VHREMAARTSPLR-PLV, corresponding respectively to residues 41–54 and 61–76 of mouse Bcl-2α, conjugated to keyhole limpet haemocyanin. Immunocytochemical and immunoblotting analyses were carried out as previously described (Waters *et al.*, 1990, 1991).

Biochemical and analytical techniques

Time-lapse video microscopy was carried out using Olympus inverted microscopes and phase contrast images acquired with monochrome CCD video cameras (Sony). Individually time-stamped images were stored sequentially on BetaCam broadcast quality (700+ lines) video recorders (Sony). Data acquisition was regulated by dedicated time-lapse controllers (EOS, Wales). Video images were down-loaded into S-VHS format for analysis on an S-VHS recorder (Mitsubishi). Time-lapse cine microscopy was carried out as previously described (Evan *et al.*, 1992; Fanidi *et al.*, 1992). Cell division events were scored at the time at which septa formed between two daughter cells. Apoptotic cell death events were scored midway between the last appearance of normality and the point at which the cell became fully detached and fragmented, an interval of typically 30–60 min (Fanidi *et al.*, 1992). Apoptotic cells were also identified by direct fluorescence microscopic examination of fixed, detergent-permeabilized cells stained with propidium iodide. Under these conditions, apoptotic cells exhibit a characteristic vesicularized appearance, usually associated with condensed and pycnotic nuclei. Viability of cell populations was determined by their ability to convert soluble 3-(4,5-dimethyl thiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) into an insoluble blue-black formazan product through the action of mitochondrial dehydrogenase (Mosmant, 1983). Fibroblasts were seeded in Labtec 96-well plates at 3000 cells per well in 10% FCS. The next day, the growth medium was replaced with serum-free medium. After 48 h, c-Myc was activated by addition of 2 μM β-oestradiol to the growth medium in the presence or absence of various cytokines. 48 h later, 10 μl MTT (Sigma) from a stock of 5 mg/ml in PBS was added to each well and the plates incubated at 37°C for 4 h. 100 μl of 10%

SDS/10 mM HCl was then added to each well, the plates left overnight at 37°C. The OD_{570nm}–OD_{630nm} of each well was then determined using a Vmax Microtitre Plate Reader (Molecular Devices). Blank wells contained growth medium plus MTT but no cells. Percent cell survival was defined by the formula [(experimental−blank)/(control−blank)] × 100.

BrdU incorporation into cell nuclei was determined immunocytochemically using a monoclonal rat anti-BrdU antibody (Sera Lab) followed by FITC-conjugated rabbit anti-rat Ig (Dako) on acid-ethanol fixed cells. Cells were examined using a Bio-Rad MRC600 confocal microscope.

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