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Phil. Trans. R. Soc. Lond. B 1994 **345**, 269-275
doi: 10.1098/rstb.1994.0105

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Integrated control of cell proliferation and cell death by the c-*myc* oncogene

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SUMMARY

Regulation of multicellular architecture involves a dynamic equilibrium between cell proliferation, differentiation with consequent growth arrest, and cell death. Apoptosis is one particular form of active cell death that is extremely rapid and characterized by auto-destruction of chromatin, cellular blebbing and condensation, and vesicularization of internal components.

The c-*myc* proto-oncogene encodes an essential component of the cell's proliferative machinery and its deregulated expression is implicated in most neoplasms. Intriguingly, c-*myc* can also act as a potent inducer of apoptosis. Myc-induced apoptosis occurs only in cells deprived of growth factors or forcibly arrested with cytostatic drugs. Myc-induced apoptosis is dependent upon the level at which it is expressed and deletion mapping shows that regions of c-Myc required for apoptosis overlap with regions necessary for co-transformation, autoregulation, inhibition of differentiation, transcriptional activation and sequence-specific DNA binding. Moreover, induction of apoptosis by c-Myc requires association with c-Myc's heterologous partner, Max. All of this strongly implies that c-Myc drives apoptosis through a transcriptional mechanism: presumably by modulation of target genes.

Two simple models can be invoked to explain the induction of apoptosis by c-Myc. One holds that death arises from a conflict in growth signals which is generated by the inappropriate or unscheduled expression of c-Myc under conditions that would normally promote growth arrest. In this 'Conflict' model, induction of apoptosis is not a normal function of c-Myc but a pathological manifestation of its deregulation. It thus has significance only for models of carcinogenic progression in which *myc* genes are invariably disrupted. The other model holds that induction of apoptosis is a normal obligate function of c-Myc which is modulated by specific survival factors. Thus, every cell that enters the cycle invokes an obligate abort suicide pathway which must be continuously suppressed by signals from the immediate cellular environment for the proliferating cell to survive. Evidence will be presented supporting this second 'Dual Signal' model for cell growth and survival, and its widespread implications will be discussed.

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1. INTRODUCTION

In any population of unicellular organisms, mutants that acquire a proliferative advantage spontaneously overgrow their less vigorous siblings. One of the deepest and most abiding paradoxes of multicellularity is how such spontaneous outgrowth of faster-proliferating variants is suppressed while at the same time permitting substantial proliferation of component cells. In man, this problem is even more acute for three reasons: our substantial physical size, our longevity and the self-renewing nature of our tissues. Clearly, the larger an organism becomes, the greater the number of potential cellular targets for neoplastic mutations. Likewise, the longer an organism lives, the greater the chances of neoplasia occurring at some point in life. Finally, many of our tissues (notably epithelial and haematopoietic) undergo substantial proliferation throughout our lives, again increasing the possibility of neoplastic mutations' occurring during our lives. However, neoplasia is tightly suppressed in metazoans. We know this because, although cancer affects one in three individuals during their lives, cancer is a clonal disease that arises through expansion of a single affected cell. Thus, the successful cancer cell only ever arises in one in three persons, out of all the billions of proliferating cells within our bodies. The cancer cell is, therefore, extremely rare. This rarity is surprising because, in principal, any mutant cell that achieves some growth advantage over its fellows might be expected to undergo clonal expansion and thereby provide an increased target site for yet further carcinogenic mutations. Carcinogenic progression thus appears to be an inevitable consequence of natural selection within the soma, given enough

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mutations. The extreme rarity of the cancer in man must, therefore, imply the existence of powerful mechanisms to suppress neoplasia. Presumably, this is either by ensuring that rate of mutation in human cells is at an extremely low level or by the existence of mechanisms that eliminate potential tumour cells when they do arise. In this review, we suggest that an intrinsic anti-neoplastic mechanism exists in all cells and which works through the obligatory coupling of cell proliferative and cell suicide pathways.

2. MYC

c-*myc* encodes a short-lived sequence-specific DNA-binding protein whose expression is elevated or deregulated in virtually all tested tumours (Spencer & Groudine 1991). c-*myc* is one of several related *myc* genes present within the mammalian genome (DePinho *et al.* 1991). However, of this family only c-*myc* is expressed in fully differentiated 'adult' cells that retain proliferative capacity (e.g. epithelial, mesenchymal or lymphoid cells). The c-*myc* protein, c-Myc, is most probably a transcription factor. It possesses an N-terminal domain with a transcriptional modulatory domain that engages the basal transcription machinery activity (Kato *et al.* 1990; Amati *et al.* 1992; Kretzner *et al.* 1992) and may also interact with cell cycle regulatory components such as p107 (Gu *et al.* 1994), and a C-terminal DNA-binding/dimerization domain, akin to that present in many other transcription factors of the bHLHZ class, that mediates dimerization with the bHLHZ protein Max (reviewed in Evan & Littlewood 1993). However, to date, few c-Myc target genes have been defined and those that have offer little clue as to the biological function of c-Myc.

Expression of c-*myc* (or of another member of the *myc* gene family) appears necessary and, in some cases, sufficient for cell proliferation. Inhibition of c-*myc* expression with antisense c-*myc* oligonucleotides effectively blocks cell proliferation (Heikkila *et al.* 1987; Loke *et al.* 1988; Prochownik *et al.* 1988; Wickstrom *et al.* 1989; Bennett *et al.* 1993) and ectopic expression of c-*myc* is sufficient to drive quiescent fibroblasts into cycle (Eilers *et al.* 1989) and keep them there, even in the absence of mitogens or the presence of the anti-proliferative cytokine γ-interferon (Evan *et al.* 1992). Thus, c-Myc locks cells in a continuously proliferating state. Regions of the c-Myc protein required for both of these mitogenic effects are identical to those required for c-Myc to act as transcription factor (Evan *et al.* 1992). Thus, c-Myc most probably acts by regulating target genes that control entry into and exit from the cell cycle.

In vitro, deregulation of c-*myc* expression appears sufficient to generate continuously proliferating cells that can no longer respond to cues that would normally trigger their growth arrest, i.e. *de facto* tumour cells. This suggests that deregulation of a *single* gene, c-*myc*, is sufficient to convert a normal cell into tumour cell. However, this conclusion flies in the face of overwhelming evidence that neoplastic conversion is protracted and requires multiple mutations; one obvious manifestation of this being the extreme rarity of cancer already alluded to above. Thus, even though cells with deregulated c-*myc* expression exhibit unrestrained and unrestrainable proliferation *in vitro*, this cannot be sufficient for neoplastic transformation.

An explanation for why it is that cells with deregulated c-*myc* expression are not fully neoplastic comes from inspection of the growth rate of such cells under conditions where growth factors are limiting. In normal fibroblasts, c-*myc* expression is tightly dependent upon mitogen availability: in the absence of growth factors (i.e. low serum) c-*myc* is rapidly downregulated and the cells arrest in G1 (Dean *et al.* 1986; Waters *et al.* 1991). However, fibroblasts with deregulated c-*myc* expression continue to cycle in the absence of growth factors but their numbers do not necessarily increase because of substantial cell death. This cell death has all the features of apoptosis (figure 1); it is rapid (20–40 min), accompanied by cell surface blebbing, cell shrinkage and fragmentation, and cell DNA is cleaved into fragments of nucleosome length (Evan *et al.* 1992). Induction of apoptosis by c-Myc is greatest in cells expressing high levels of the protein, but is nonetheless clearly evident in cells expressing levels of c-Myc present in untransformed proliferating fibroblasts. Analogous Myc-dependent apoptosis has been reported also in growth factor-deprived haematopoietic cells (Askew *et al.* 1991), suggesting that induction of apoptosis by c-Myc may be a general phenomenon.

To determine by what molecular mechanism c-Myc triggers apoptosis in serum-deprived fibroblasts, we carried out site-directed mutagenesis on the c-Myc protein. We observed complete overlap of those regions required for c-Myc to function as an oncoprotein (promoting cell proliferation) (Stone *et al.* 1987) and those required to trigger apoptosis in low serum (Evan *et al.* 1992) (figure 2). The regions were the N-terminal transactivation domain, the sequence-specific DNA binding basic regions and the intact C-terminal helix-loop-helix-leucine zipper dimerization domain. The exact same regions are also required for c-Myc to function as a transcription factor. Thus, the activity of c-Myc as an inducer of apoptosis is genetically inseparable from its ability to promote the entirely contradictory function of cell growth, and both functions probably involve the specific modulation of c-Myc of target genes. All known transcription factors of the bHLHZ class require dimerization with a partner in order to bind DNA and exert their action. Therefore, to confirm further that c-Myc controls both mitogenic and apoptotic functions of c-Myc by a transcriptional mechanism, we investigated whether both of these functions required c-Myc to interact with its partner bHLHZ protein Max. Unfortunately, Max is ubiquitously expressed, making it impossible to ask directly if c-Myc can act in the absence of Max. We therefore made use of mutants of c-Myc and Max that have reciprocally exchanged dimerization specificities, such that the c-Myc mutant (MycEG) can no longer interact with wild-type Max but can dimerize with

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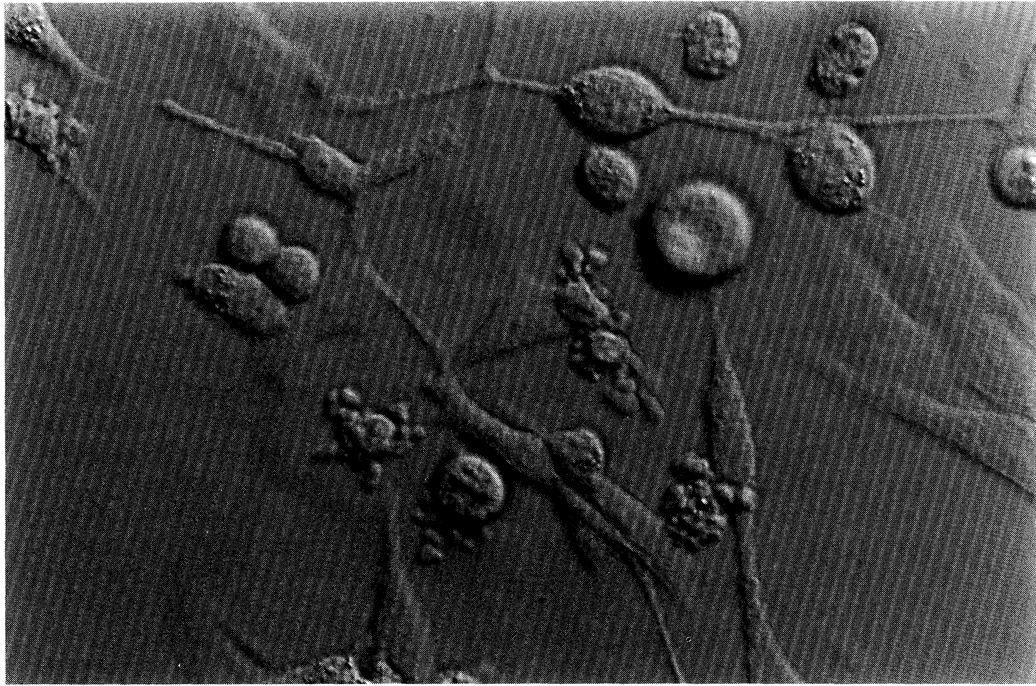

Figure 1. Apoptosis of Rat-1 fibroblasts constitutively expressing c-Myc in low serum. Rat-1 fibroblasts expressing human c-*myc* driven from a retrovirus LTR promoter were cultured in 0.5% foetal calf serum for 24 h. Cells were fixed in 3.5% paraformaldehyde in PBS and visualized by Nomarski Interference Optics.

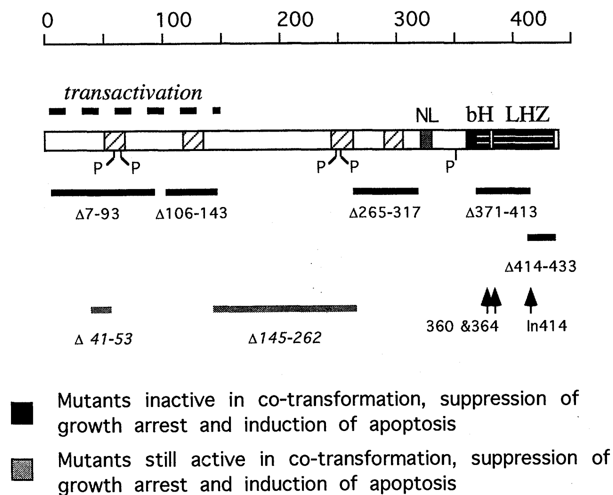

Figure 2. Mutagenesis study of the human c-Myc protein. Site directed mutants of human c-Myc were expressed in Rat-1 or Swiss 3T3 mouse fibroblasts and the cells assayed for co-transformation in association with activated H-*ras*, inability to arrest growth in low serum and for initiation of apoptosis in low serum. Δ mutants are deletions between the amino acid residues shown, *In* mutants are insertional mutations and the others are point mutations that disrupt the DNA binding domain. Regions of the c-Myc protein marked are the transactivation domain (N-terminal), known phosphorylation sites (P), the dominant nuclear localization signal (NL), the basic DNA-binding region (b) and the C-terminal helix-loop-helix-leucine zipper (HLHZ) dimerization domain.

the corresponding MaxEG mutant (Amati *et al.* 1993). By this strategy we have demonstrated that Max is absolutely required for induction of both cell proliferation and apoptosis by c-Myc. This finding significantly reinforces the notion that both proliferative and apoptotic functions of c-Myc are transcriptional.

3. THE BIOLOGICAL SIGNIFICANCE OF c-MYC-INDUCED APOPTOSIS

The observation that deregulated c-Myc expression triggers apoptosis marries well with a large amount of data indicating that genetic lesions that suppress apoptosis can synergize with c-Myc oncogenically. Transgenic animals whose lymphocytes express deregulated c-*myc* exhibit increased sensitivity to induction of apoptosis in lymphoid organs (Dyall & Cory 1988; Langdon *et al.* 1988; Neiman *et al.* 1991). The anti-apoptotic oncogene *bcl*-2 suppresses such c-Myc-induced apoptosis and synergizes with c-Myc to promote development of lymphomas (Vaux *et al.* 1988). Bcl-2 protein expression also blocks c-Myc induced apoptosis in fibroblasts in response to serum deprivation (Bissonnette *et al.* 1992; Fanidi *et al.* 1992; Wagner *et al.* 1993) or DNA damage (Fanidi *et al.* 1992). More generally, deregulation of c-*myc* is virtually ubiquitous in tumour cells, implying strong selection for c-*myc* activation during carcinogenesis. However, the observation that c-*myc* deregulation is also a potent trigger of apoptosis strongly suggests that anti-apoptotic lesions are likely to be common components of carcinogenesis.

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Deregulated expression of c-*myc* is, therefore, a potent trigger of apoptosis. But is there any evidence to suggest that normal c-*myc* expression might be involved in promoting apoptosis? Two simple models can be invoked to explain the induction of apoptosis by c-Myc following serum-deprivation (figure 3). The first (figure 3*a*) argues that cell death arises because of a conflict in signals between the growth promoting action of c-Myc and the growth inhibitory effect of growth factor deprivation. In this model, induction of apoptosis is a pathological consequence of 'inappropriate' c-Myc expression and is not a normal function of c-Myc but arises from a 'conflict of signals'. An alternative, if unorthodox, model for c-Myc-induced apoptosis (figure 3*b*) is to propose that induction of an apoptotic programme is a *bona fide* and obligate component of c-Myc action that necessarily accompanies proliferation, i.e. that proliferation and apoptosis are obligatorily coupled. In this model, successful (i.e. viable) cell proliferation requires two independent signals, one to trigger mitogenesis and the other to suppress the concomitant apoptotic programme. According to this 'Dual Signal' model,

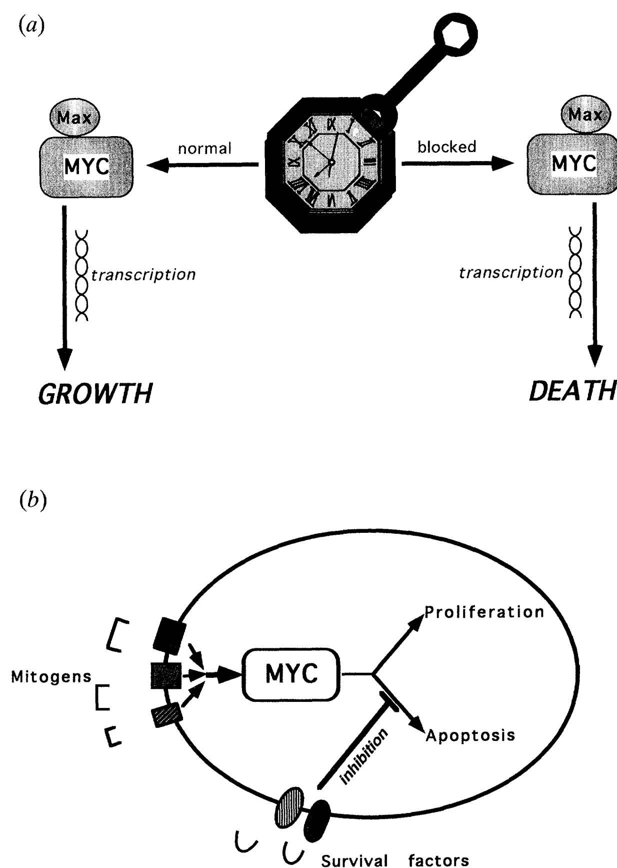

Figure 3. Alternative models to explain c-Myc-induced apoptosis in low serum. (*a*) Death arises because of a conflict of signals. Normally, c-Myc promotes a transcriptional programme leading to cell proliferation. However, in the absence of serum, this programme is interrupted and the resulting conflict directs c-Myc to activate an apoptotic programme. (*b*) 'Dual Signal' hypothesis. Induction of both proliferation and apoptosis are normal consequences of c-Myc expression. Cell fate is determined by ectopic factors that specifically modulate the apoptotic programme.

cells expressing c-Myc die in low serum not because of conflict in growth signals but because they are deprived of serum factors required to suppress the c-Myc-induced apoptotic programme. Induction of apoptosis by c-Myc is therefore a normal physiological aspect of c-Myc function.

As outlined above, c-Myc induces apoptosis almost certainly by a transcriptional mechanism. We reasoned that in the 'conflict' model c-Myc only induces its apoptotic transcriptional programme as a result of a conflict of signals. Accordingly, we sought to test the conflict model by blocking execution of the apoptotic programme with either cycloheximide or actinomycin D prior to establishing a conflict of signals (i.e. serum deprivation) (figure 3). Both cycloheximide or actinomycin D block dexamethasone-induced apoptosis in thymocytes which is therefore thought to require *de novo* protein synthesis. Surprisingly, we observed no inhibition of c-Myc-induced apoptosis by either cycloheximide or actinomycin D: indeed, merely adding either drug to otherwise fully viable cells expressing c-Myc in high serum triggered rapid apoptosis. The higher the level of c-Myc expression at the time either drug was added the more rapid and extreme was the apoptosis that occurred. The only conclusion possible from this experiment is that the c-Myc-induced apoptotic transcriptional programme already pre-existed, albeit in silent form, within these cells.

We conclude the following:

1. Induction of apoptosis by c-Myc involves a transcriptional programme (either activation of a pro-apoptotic programme or suppression of an anti-apoptotic programme).
2. The programme pre-exists in viable cells as a consequence of c-Myc expression but is silent in high serum.
3. The c-Myc-induced apoptotic programme becomes active in low serum.

Thus, it seems most likely that specific factors in serum act to suppress the activation of an apoptotic programme that is put in place by c-Myc. This idea is most consistent with the 'Dual Signal' model outlined above in which specific signals regulate the activity of an underlying c-Myc-induced apoptotic programme.

4. CYTOKINES THAT REGULATE c-MYC-INDUCED APOPTOSIS

Replacement of serum with a cytokine-free serum substitute which provides the same essential nutrients as serum does not suppress c-Myc-induced apoptosis in rat fibroblasts, indicating that cell death in low serum does not result from nutritional privation. Next, various cytokines were tested for their abilities to suppress c-Myc-induced apoptosis in serum-deprived cells. 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, whereas EGF, basic FGF, acidic FGF, Interleukin-1, TGFα, TGFβ and bombesin all failed to inhibit apoptosis (figure 4).

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Inability to suppress apoptosis is not due to lack of appropriate receptors and cognate downstream signal transduction pathways exist for each cytokine, as evidenced by the fact that all cytokines induced transient expression of the immediate early nuclear proteins c-Fos and Egr-1/Zif268/NGFIA. Moreover, EGF, bFGF and bombesin are all potent mitogens for fibroblasts, whereas IGF-I is only poorly mitogenic. Thus, there is no direct correlation between mitogenicity and ability to suppress c-Myc-induced apoptosis among tested cytokines. The anti-apoptotic effects of IGFs and PDGF are evident in all tested cells of mesenchymal origin: primary and immortalized fibroblasts of rodent or human origin and primary rat vascular smooth muscle cells. However, preliminary experiments indicate that other cytokines may fulfil analogous anti-apoptotic roles in other cell lineages.

In the 'conflict of growth signals' model, serum deprivation triggers apoptosis in cells expressing c-Myc because c-Myc alone is insufficient to promote a 'balanced' or 'integrated' mitogenic programme. Consequently, the cells enter a premature or 'unscheduled' cell cycle which results in their death. In this scenario, IGFs and PDGF block c-Myc-induced apoptosis by providing necessary auxiliary growth signals needed to integrate correctly the cell's growth programme. The abilities of IGF-I and PDGF to suppress c-Myc-induced apoptosis is therefore critically dependent upon the activity of both cytokines as mitogens promoting cell cycle progression. In contrast, the 'Dual Signal' hypothesis postulates that IGFs prevent cell death by directly modulating the cell death pathway, irrespective of growth status of the cell. The 'Dual Signal' model therefore makes a unique prediction that the abilities of survival factors to inhibit apoptosis will not be dependent upon the competence of those factors to promote cell cycle progression. To discriminate between the 'Conflict' and 'Dual Signal' hypotheses we therefore asked two key questions. First, can IGF-I suppress c-Myc-induced apoptosis under conditions where it cannot promote cell cycle progression; for example, when cells are profoundly blocked in cycle with cytostatic drugs. Second, does IGF-I suppress c-Myc-induced apoptosis under conditions where it is not required for cell cycle progression. To do this, we examined the effects of IGF-I in post-commitment S/G2 fibroblasts which are factor-independent for completion of their cell cycles.

Cells profoundly blocked in post-commitment parts of the cell cycle by either thymidine (S) or etoposide (S/G_2) undergo apoptosis upon activation of c-Myc (Evan *et al.* 1992; Fanidi *et al.* 1992; Harrington *et al.* 1994*a*). Neither IGF-I nor PDGF is able to relieve these potent blocks to cell cycle progression. We therefore asked whether IGF-I exerted any effect on apoptosis induced by either drug in fibroblasts expressing c-Myc. We observed both IGF-I and PDGF to exert significant anti-apoptotic activity in Myc-fibroblasts exposed to thymidine or etoposide. Thus, both cytokines can suppress apoptosis under

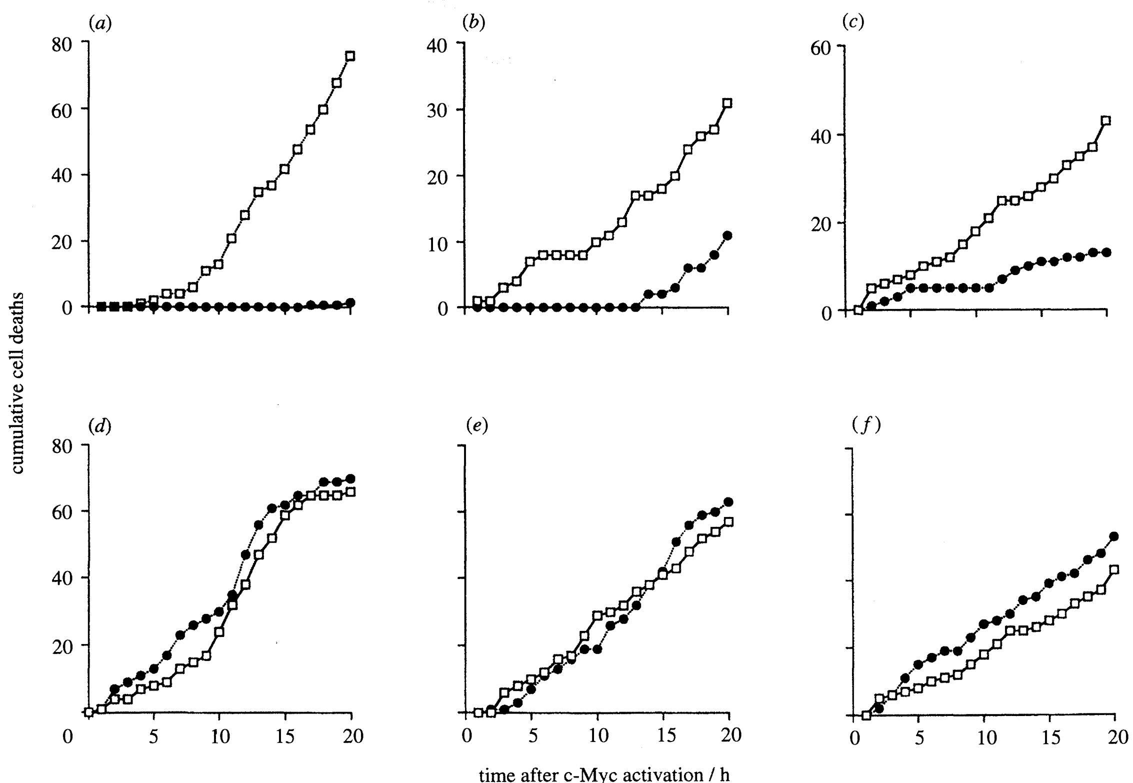

Figure 4. Effects of cytokines on c-Myc-induced apoptosis in fibroblasts. (*a*) IGF-I; (*b*) PDGF; (*c*) IGF-II; (*d*) EGF; (*e*) bFGF; and (*f*) bombesin. One hundred Rat-1 fibroblasts constitutively expressing c-*myc* were cultured in low serum for 20 h in the presence (open squares) or absence (filled circles) of the labelled cytokines (Harrington *et al.* 1994). Apoptotic cell deaths were monitored by time-lapse videomicroscopy. Cumulative cell deaths were plotted against time in hours.

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conditions where they are unable to promote cell proliferation.

Fibroblasts are dependent on growth factors only during the pre-commitment G_1 phase of the cell cycle (Zetterberg & Larsson 1985; Pardee 1989): post-commitment $S/G_2/M$ fibroblasts complete their cycle on schedule in the absence of serum and thus do not require any growth factors. We therefore asked whether IGF-I could suppress c-Myc-induced apoptosis in these post-commitment fibroblasts, a time in the cell cycle when the cytokine has no identifiable role in promoting cell proliferation. We observed that activation of c-Myc in serum-deprived post-commitment S/G2 cells induces the immediate onset of apoptosis and this apoptosis is inhibited by either IGF-I or PDGF. Thus, anti-apoptotic cytokines suppress apoptosis in situations where they exert no detectable mitogenic effect.

Finally, we are able to demonstrate that the anti-apoptotic effects of IGF-I are evident even in cells treated with cycloheximide or actinomycin D, which inhibit protein and RNA synthesis respectively. Thus, the anti-apoptotic effect of IGF-I does not require *de novo* gene expression or protein synthesis. This is in clear contrast to the mitogenic programme implemented by IGF-I, which does require *de novo* expression of a panoply of immediate-early growth response genes such as c-*fos*, c-*jun* and *erg*-1. Interestingly, PDGF does not exert any measurable anti-apoptotic effect in the presence of cycloheximide or actinomycin D, indicating that its protective effects do require expression of new gene products. Preliminary data suggest that PDGF may act by inducing IGF-I expression in cells.

In summary, therefore, we can clearly discriminate between the role of IGF-I and PDGF as mitogens and their roles as anti-apoptoptic factors.

5. SIGNIFICANCE OF THE 'DUAL SIGNAL' MODEL FOR CONTROL OF CELL GROWTH

We have demonstrated that c-Myc activates a transcriptional programme within cells, one outcome of which is to establish the capacity for apoptotic cell suicide. This programme is always present in cells that express c-Myc and, because c-Myc appears essential for cell proliferation, is thus always present in proliferating cells. In order to survive entry into cycle, this apoptotic programme must be continuously forestalled by the action of anti-apoptotic cytokines (or, perhaps, anti-apoptotic activities such as that provided by the oncogene *bcl*-2).

The obligatory coupling through c-Myc of the two contradictory programmes of cell proliferation and cell death appears paradoxical. However, the dependence of proliferating cells upon two independent signals, one for proliferation and one for survival, provides a powerful innate mechanism to suppress carcinogenesis (Evan *et al.* 1992; Evan & Littlewood 1993; Harrington *et al.* 1994*b*) which, as discussed above, is a major risk sustained by physically large, long-lived multicellular organisms such as man. The coupling of mitogenic and apoptotic pathways means that any growth-promoting lesion (e.g. activated oncogene) will be lethal for the affected cell and its progeny as soon as they outgrow their supply of paracrine survival factors. Suppression of neoplastic transformation thus becomes hardwired into the way metazoan cells proliferate. Only the rapid acquisition of a compensating mutation that suppresses cell death (e.g. deregulation of *bcl*-2 or perhaps autocrine activation of the IGF-I signalling pathway) will enable survival of the affected clone. The chance of such simultaneous double mutations occurring are so small that cancer becomes exceedingly rare. Clearly, the model predicts that anti-apoptotic lesions will be common, if not ubiquitous, in tumour cells. Identification and characterization of such lesions, and the molecular basis for their action, should provide an exciting and effective set of novel targets for future therapeutic intervention in cancer.

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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

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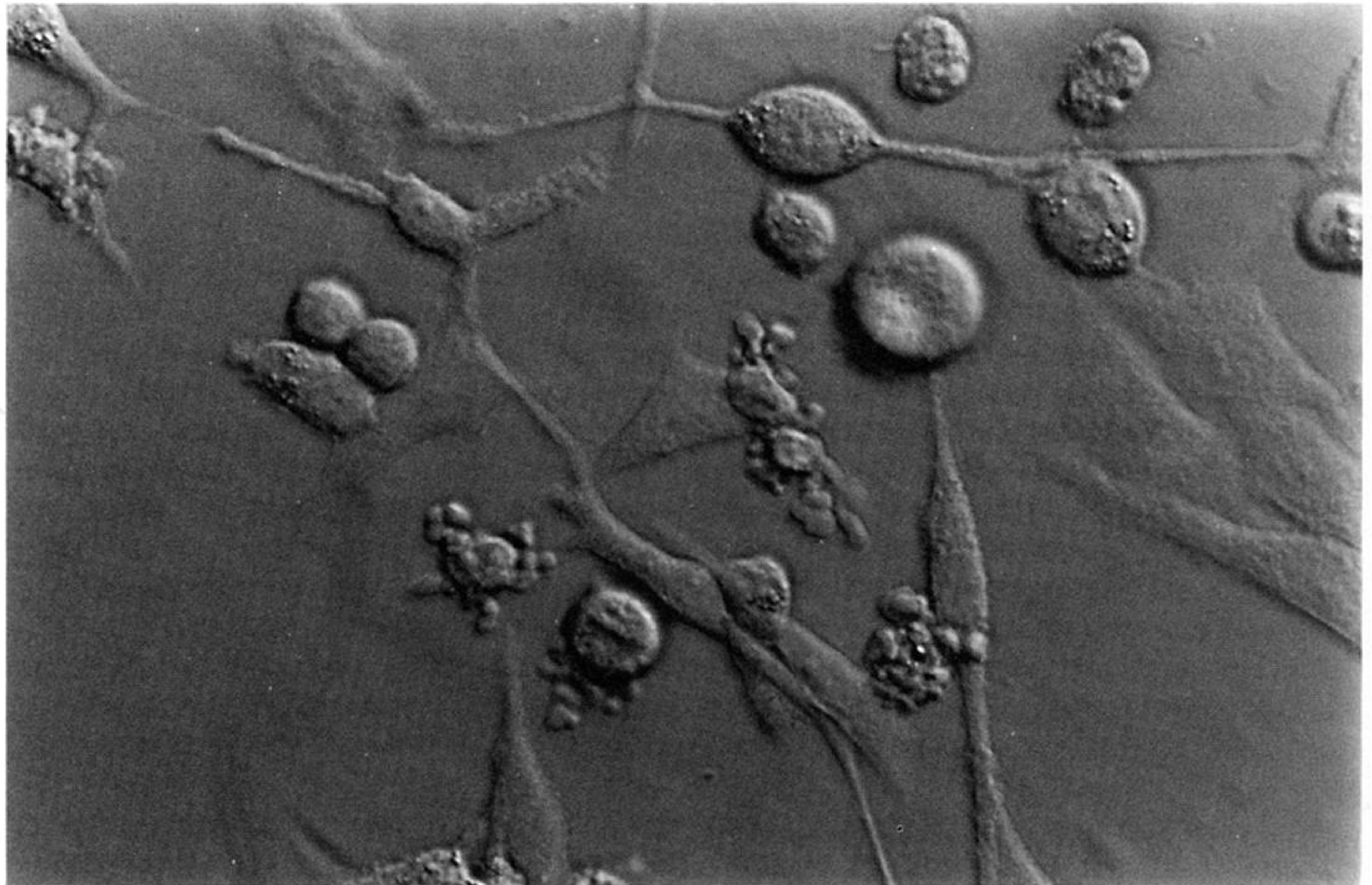

gure 1. Apoptosis of Rat-1 fibroblasts constitutively expressing c-Myc in low serum. Rat-1 fibroblasts expres- ıg human c-*myc* driven from a retrovirus LTR promoter were cultured in 0.5% foetal calf serum for 24 h. Cells ere fixed in 3.5% paraformaldehyde in PBS and visualized by Nomarski Interference Optics.