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Growth factors and apoptosis in development. The role of insulin like growth factor I and TGFß1 in regulating cell growth and cell death in a human teratocarcinoma derived cell line

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ABSTRACT The balance between different cell populations in the developing organism is controlled by regulating the rates of multiplication, differentiation or death of its constituent cells. The human teratocarcinoma derived cell line Tera 2, which in several aspects mirrors early embryonic cells, can be induced to undergo programmed cell death (apoptosis) by depriving cell cultures of serum. This study demonstrates that this process can be reversed by replacing serum with physiological concentrations of insulin like growth factor I (IGF I) . As a result, IGF I enhances the rate of Tera 2 cell proliferation in serum free medium. In contrast, Transforming Growth Factor ß1 did not exert any effect on growth or apoptosis in Tera 2 cells. The results indicate that one effect of growth factors on pluripotential cells is to regulate the balance between cell proliferation and cell death.

KEY WORDS: *teratoma, IGF I, TGFβ1, apoptosis, cell proliferation*

Introduction

Apoptosis or programmed cell death is now recognized as an important means of eliminating cells from the organism (White, 1993; Bates *et al.*, 1994; Fisher, 1994; McConkey and Orrenius, 1994; Oltika and Korsmeyer, 1994; Osborne and Schwartz, 1994). It has been frequently described as a major process in physiological tissue turnover. But apoptosis also plays an important role in embryonic development, an action which has been best defined in the developing nematode *Caenorhabditis elegans* where molecules essential for the induction of cell death, its regulation and the engulfment of dead cells have been defined in genetic terms (Hengartner and Horvitz, 1994). In this nematode, 131 out of 1090 somatic cells die in every organism. Mutation analysis has revealed the existence of 14 genes called *ced* genes (cell death abnormal) that act in concert to allow the correct cells to commit suicide at the right time point. The *ced*-9 gene, which is a negative regulator of cell death, bears a striking homology to a mammalian oncogene bcl-2. Recent data suggest that bcl-2 is only one member of a family of related genes (Lam *et al.*, 1994; Nunez and Clarke, 1994). An intracellular partner of bcl-2, Bax, has been identified and further studies have indicated that Bax regulates the anti-apoptotic effects of bcl-2. Another *ced* gene which rather drives cells into apoptosis (*ced*-3) has significant homology to another previously identified vertebrate gene, interleukin 1ß-converting enzyme (ICE) (Wang *et al.*, 1994). Interestingly, overproduction of ICE results in neuronal cell death in a fashion comparable to that of ectopic expression of *ced* -3 in *C. elegans* (Rubin *et al.*, 1994).

The first characterization of an actively regulated mechanism of cell death in mammalian cells demonstrated that glucocorticoid treated lymphocytes entered a programme of suicidal death (reviewed by Collins and Lopez-Rivas 1993). In addition lymphocytes have been shown to be sensitive to ionizing radiation. Whatever the cause, during apoptosis, cells shrink and were until recently believed to degrade their chromatin to oligonucleosome length fragments before loss of plasma membrane integrity. The cell remnants are recognized and rapidly phagocytosed by neighboring cells in the tissue. This efficient elimination of cell debris probably avoids any inflammatory response of the immune system to the dying cells, as DNA and other nuclear components are highly immunogenic and antibodies to such molecules are detected in autoimmune disease. This work has led to the identification of the p53 gene as a central regulator of apoptosis (Prives and Manfredi, 1993; Cuelles *et al.*, 1994). In mice lacking functional copies of the p53 tumor suppressor

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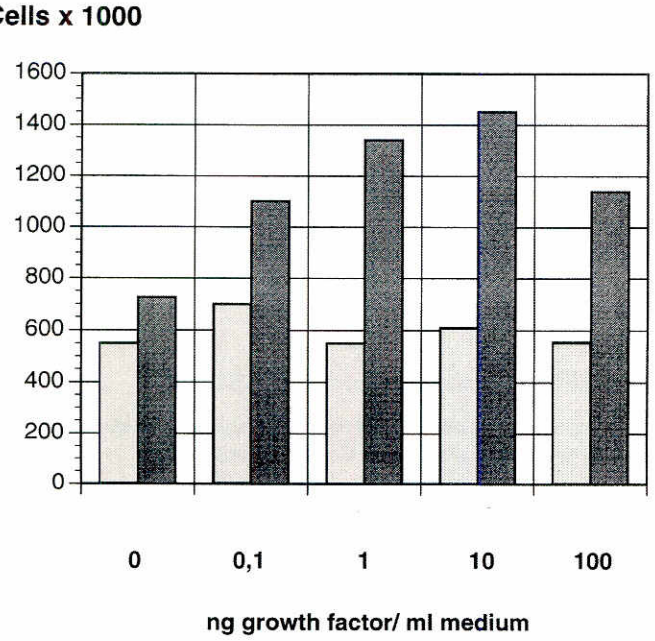

Fig. 1. Effect of growth factors on the proliferation of Tera 2 cells in serum free medium. *Cells were seeded onto 35 mm Petri dishes in 10% serum. After 24 h the serum was removed, the cells rinsed and exposed to fresh serum free medium containing transferrin and TGFß1 (black bars) or IGF I (grey bars) at concentrations indicated in the abscissa. After 24 h the cells were trypsinized off the dishes and counted in a Coulter counter. The experiment was repeated 3 times and the cells counted in triplicate.*

genes, thymocytes cannot undergo irradiation induced apoptosis. However thymocytes from such mice undergo apoptosis in the expected fashion in response to glucocorticoids or signals through the T-cell receptor. Taken together these data suggest that genes involved in one cell death pathway may not necessarily play an essential role in other pathways.

Apoptosis is particularly observed during embryogenesis in complex organs where a subpopulation of cells are selectively killed. For example many neurons die within the developing brain and self reactive T-cells are eliminated within the thymus. In the adult, apoptosis can be detected in tissues undergoing reversible expansion, for example in hormone dependent cells of the breast and prostate after hormone removal (Sandford *et al.*, 1984) or following cytokine dependent expansion of bone marrow progenitor cells. Apoptosis is a mechanism by which the immune system can kill infected cells. Moreover, regressive organ changes as e.g. toxic lead accumulation in the liver and kidney may well be accompanied by apoptosis (Columbano *et al.*, 1985). Apoptosis is observed in preneoplastic rat liver following withdrawal of tumor promoters. A similar situation occurs in keratinocytes after irradiation (Young, 1987) and in neural cells after glutamate treatment (Kure *et al.*, 1991).

Most probably the apoptotic cell death is a more common phenomenon than hitherto believed. Moreover, it appears that expanding cell populations may be dependent on autocrine or paracrine survival factors, such as peptide hormones, in order to avoid programmed cell death (Raff, 1992). Among the first identified survival factors was platelet derived growth factor (PDGF) which prevents glial cell death in the developing rat optic nerve (Barres *et al.*, 1992). More mature oligodendrocytes depend upon the ciliary neurotrophic factor (CNTF) which is supplied by the neurons which they myelinate and then undergo apoptosis in its absence (Barres *et al.*, 1994).

In conclusion, it seems reasonable to assume that cells are actively kept alive by a complex web of survival factors acting in a paracrine fashion. This web is an important toward controlling and balancing a tissue population of cells. The role of apoptosis in development remains to be clarified. We have taken advantage of a human embryonal carcinoma cell line, Tera 2, which can be induced to differentiate *in vitro*. As a xenograft it gives rise to tumors containing components from different germ layers. Tera 2 is to some extent therefore considered to mirror some aspects of embryonic stem cells (Schofield and Engstrom, 1992). Tera-2 cells have recently been shown to undergo morphologically confirmed apoptosis after serum withdrawal (Granerus *et al.*, 1994). The purpose of this study was to examine the effects of two growth factors, Insulin like growth factor I (IGF I) and Transforming Growth Factor Beta 1 (TGF Beta-1) that have been widely discussed in the context of programmed cell death (Collins *et al.*, 1994; Moulton, 1994), on proliferation and apoptosis in Tera 2 cells.

Results

Figure 1 shows the effect of growth factor addition on Tera 2 cells in a serum free medium over a 24 h assay period. Whereas lower concentrations of IGF I only exerted minor effects, the addition of 1-100 ng IGF I/ml resulted in cell numbers twice as high as in serum free control cultures. In contrast, no concentration of TGFß1 seemed to exert any stimulatory or inhibitory effect on Tera 2 cell numbers over a 24 h period.

Table 1 shows the proportion of Tera-2 cells traversing S-phase. Cell cultures were exposed to serum free medium with or without supplementation of various concentrations of IGF I or TGFß1. The proportion of cells undergoing DNA-synthesis at one given moment was assayed by pulse labeling with tritiated thymidine 24 or 48 h after medium change and subsequent

TABLE 1

Medium	% labeled cells labeling interval 23-24 h	47-48 h
10% serum	35.1	35.7
Serum free medium (SFM)	36.6	31.5
SFM+10 ng IGF I/ml	34.2	30.3
SFM+10 ng TGFß1/ml	33.8	34.8

Tera 2 cells growing on glass coverslips were exposed to media as specified above for 24 or 48 h. During the last hour prior to fixation, the cultures were exposed to 10 µCi tritiated thymidine per ml medium. After fixation the slides were washed in cold trichloroacetic acid and subjected to autoradiography. After development, the slides were counted in a light microscope. The figures represent a duplicate experiment where at least 500 cells per experimental situation were counted

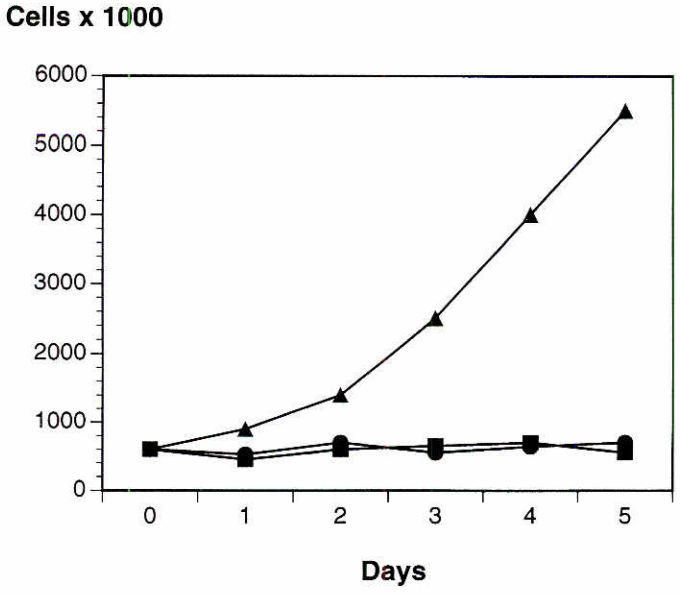

Fig. 2. Effect of TGFß1 on the proliferation of Tera 2 cells. *Cells were seeded onto 35 mm Petri dishes in 10% serum. After 24 h (day 0) the serum containing medium was removed and the cells exposed to serum free medium containing transferrin only (■), serum free medium supplemented with 10 ng TGFß1/ml (●) or 10% serum (▲). Dishes were trypsinized and counted in triplicate daily throughout a 5 day assay period. The figure represents the means of 3 different experiments.*

autoradiography. The Table clearly shows that the percentage of thymidine labeled cells was virtually unchanged when Tera-2 cells were deprived of serum. The addition of IGF I or TGFß1 did not significantly alter the proportion of cells in S-phase.

Figure 2 shows the growth of Tera 2 cells over a 5 day period under different conditions. Whereas cells growing in 10% serum undergo exponential growth, cell cultures in serum free medium are maintained on a steady state level. When 10 ng TGFß1/ml was added to the serum free cultures, no effect on cell numbers could be observed. No other concentration of TGFß1 (0,01-100 ng/ml) was found to enhance or repress Tera 2 cell proliferation in serum free medium (data not shown).

Figure 3 shows that addition of 10 ng IGF I/ml to serum free medium supports the multiplication of Tera 2 cells over a 5 day period. Similar results were obtained with higher concentrations of IGF I (50-200 ng/ml, data not shown). This multiplication stimulating effect, however, never reached that of 10% serum.

Figure 4 shows the morphological appearance of Tera 2 cells after 24 h in serum free medium. The cells were stained with acridine orange and photographed under a fluorescence microscope at 435 nm. The Figure shows some apoptotic cells with fragmented nuclei.

Figure 5 shows the effect of growth factor supplementation to a serum free medium on survival in Tera 2 cells. This was measured by staining Tera 2 cells with acridine orange and judging the proportion of intact versus fragmented nuclei with a fluorescence microscope. It was found that serum withdrawal reduced the number of intact nuclei from 90 to 35% over the first 24 h. Between the 2nd and 5th day the proportion intact nuclei stabilized at around 40%. It was found that addition of 10 ng IGF I/ml resulted in a substantially higher proportion of intact nuclei over the entire 5 day period. In contrast, supplementation with 10 ng TGFß1/ ml did not in any way alter the numbers of intact nuclei in serum free cultures. Nor did we observe any effects of any other TGFß1 concentration (data not shown). When TGFß1 was added together with 10 ng IGF I/ml there was no enhancing or suppressing effect on cell numbers or the proportion cells with intact nuclei. Nor did TGFß1 alter the growth characteristics of cell growing in 10% serum (data not shown).

Figure 6 shows nuclear DNA from serum starved Tera 2 cells visualized on an ethidium bromide gel. It shows that Tera 2 cells exposed to serum free medium for 24 h display the pattern of DNA degradation characteristic of extensive intranucleosomal cleavage, typical of, but not obligatory in, apoptosis. These short fragments disappear when IGF I or 10% serum was added to the cultures.

Finally, we examined how different culture conditions affect the expression of c-myc. Northern blot analysis of polyadenylated RNA form Tera 2 cells showed that the amount of myc-transcript was virtually unchanged after 24 h of serum depletion. The transcriptional activity was neither enhanced nor decreased by addition of IGF I or TGFß 1 (data not shown).

Discussion

In this study we have confirmed that the human embryonal carcinoma derived cell line (Tera 2) responds to serum withdrawal by morphological changes typical of apoptosis. Tera 2 cells also display a nuclear DNA fragmentation which has hith-

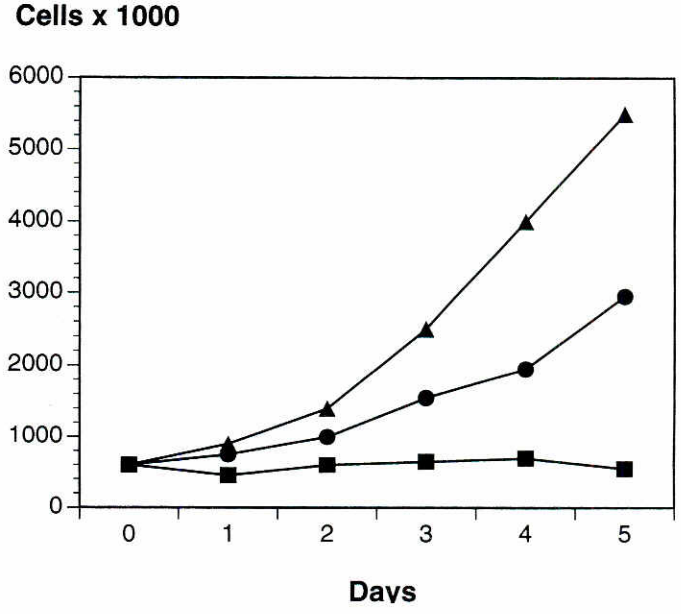

Fig. 3. Effect of IGF I on the proliferation of Tera 2 cells. *Cells were seeded onto 35 mm Petri dishes in 10% serum. After 24 h (day 0) the serum containing medium was removed and the cells exposed to serum free medium containing transferrin only (■), serum free medium supplemented with 10 ng IGF I/ml (●) or 10% serum (▲). Dishes were trypsinized and counted in triplicate daily throughout a 5 day assay period. The figure represents the means of 3 different experiments.*

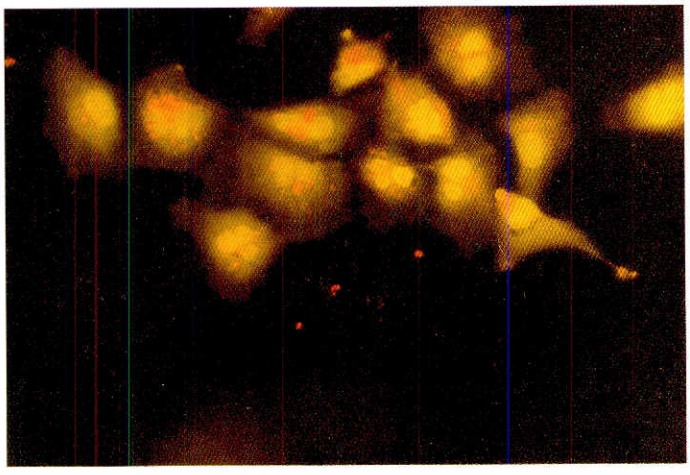

Fig. 4. Morphological appearance of Tera 2 cells 8 h after serum withdrawal. *Tera 2 cells were seeded onto glass coverslips in 10% serum. After 24 h the serum containing medium was discarded and serum-free medium supplemented with transferrin was added. After 8 h the coverslip was rapidly stained in acridine orange, rinsed and photographed in a Leitz fluorescence microscope at 435 nm.*

erto been believed to be a hallmark of apoptosis. However this concept has recently been challenged, and convincing evidence against nucleosome ladders as proof of an apoptotic process is now available (Schulze-Osthoff *et al.*, 1994). Like a variety of other cells undergoing apoptosis, serum starved Tera 2 cells shrink in volume and lose contact with their neighbors. This effect could be partly overcome by addition of insulin like growth factor I to the serum free medium. In contrast, TGFß1 had no effect – neither enhancing nor suppressing – on programmed cell death in serum starved Tera 2 cells.

The finding that IGF I promotes cell survival rather than increasing the proportion of Tera 2 cells traversing the cell cycle was first shown by Biddle *et al.* (1988). This finding is corroborated by other studies. IGF I promotes survival but not growth in glial cells in culture (Barres *et al.*, 1992). This growth factor also promotes the survival of Interleukin 3 (IL-3) dependent bone marrow-derived primary cultures and cell lines (Collins *et al.*, 1994). However, IGF I exerted a substantially lesser effect on bone marrow cell growth than IL-3.

Conversely, deprivation of single growth factors has been found to trigger apoptosis in a variety of cultured cells. These include vascular endothelial cells deprived of basic fibroblast growth factor (bFGF, Araki *et al.*, 1990), murine embryonic cells after removal of epidermal growth factor (EGF, Rawson *et al.*, 1991), rat phaeochromocytoma PC12 cells deprived of nerve growth factor (NGF, Batistatou and Greene, 1991) and glial cells deprived of platelet derived growth factor (PDGF). But perhaps the best defined examples of growth factor regulated cell death in mammals come from the hematopoietic and immune systems. Interleukin 2 (IL-2) dependent T lymphocytes enter a program of endonuclease activation and apoptosis upon IL-2 withdrawal. Likewise, removal of the critical growth factor leads to apoptosis in several IL-3 dependent progenitor cell lines (Williams *et al.*, 1991) and in activated erythroid progenitor cells after erythropoetin removal (Rodriguez-Tarduchy *et al.*, 1990). There are also striking examples of the opposite situation, i.e. where growth factor addition induces apoptosis in cells. TGFß1 was found to induce apoptosis in endometrial stromal cells (Moulton, 1994), but exerted no effect either on proliferation or cell death in Tera 2 cells.

There are several ways by which the presence or absence of growth factors may influence the cells' decision to proliferate or commit suicide. Interestingly, analysis of receptor isoforms suggested that different regions of the cytoplasmic domain of the erythropoetin receptor are coupled to proliferation and survival pathways (reviewed by Collins *et al.*, 1994). This presupposes that a growth factor receptor can switch between a "survival state" and a "proliferative state". Such an active switch is believed to depend upon the metabolic state and the expression of proliferation- or apoptosis specific gene expression. In a similar way growth factor deprivation leads to reduced receptor signalling with a decreased metabolic rate, down-regulation of survival genes and possibly the up-regulation of critical suicidal genes. However, recent attention has been focused on the interplay between two genes – the protooncogene c-myc and the tumor suppressor gene p53 in the control of apoptosis (Hermeking and Eick, 1994). A transcribed c-myc seems to be required for apoptosis in fibroblasts. Moreover, it was recently shown that apoptosis as a result of deregulated c-myc can be efficiently counteracted by IGF I. However, IGF I inhibition of apoptosis occurs in the absence of protein synthesis so it was concluded that its activity does not require immediate early gene

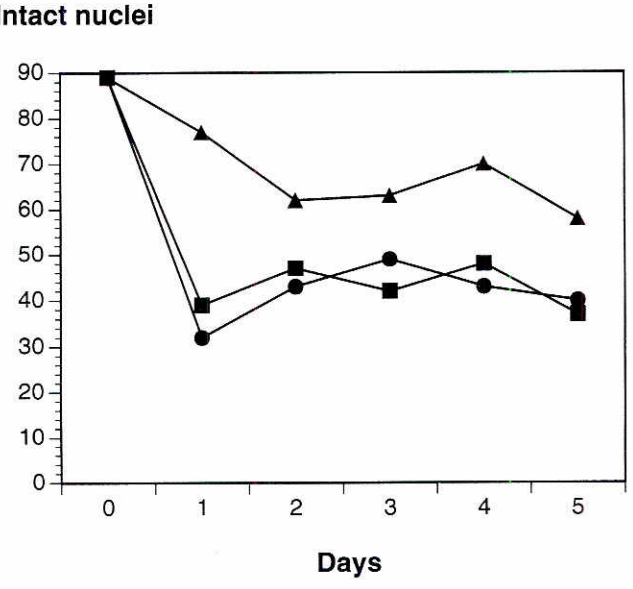

Fig. 5. Effect of growth factors on apoptosis in Tera 2 cells in serum free medium. *Cells were seeded onto gelatin treated glass slides in 35 mm Petri dishes in 10% serum. After 24 h (day 0) the serum containing medium was removed and the cells exposed to serum free medium containing transferrin only (■), serum free medium supplemented with 10 ng TGFß1/ml (●) serum free medium supplemented with 10 ng IGF I/ml (▲). Glass slides were taken daily and stained with acridine orange and examined by fluorescence microscopy (435 nm). The percentage intact nuclei was recorded by counting at least 200 cells per glass slide. The figure represents the means of 3 different experiments.*

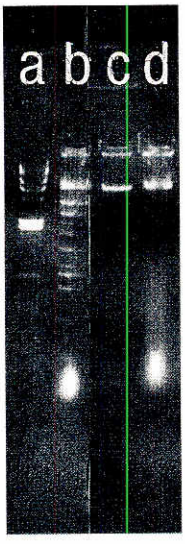

Fig. 6. Nuclear DNA from Tera 2 cells grown in serum free medium with 10 ng IGF I/ml (d), 10% serum (c) or in plain medium (b) for 24 h. *DNA was prepared according to Moulton (1994) and run on a 2% agarose gel containing 10 ng ethidium bromide/ml. The gel was transilluminated by ultraviolet light and photographed. HindIII digested lambda DNA was used as size marker* **(a)**.

expression (Harrington *et al.*, 1994). Our demonstration that c-myc is transcribed in Tera 2 cells irrespective of culture conditions does not contradict the suggested key role for this gene in controlling cell proliferation and cell survival.

Taken together present evidence suggests that growth factors not only actively promote traverse of the cell cycle, but also the probability of a cell entering successive cycles. Attention to date has focused on assessment of proliferation or differentiation on the control of embryonic stem cell population. It is now clear that a major contributing factor is the incidence of cell death within such population. It is hoped that the use of defined cell lines such as Tera 2 may rapidly augment our understanding of the nature of mechanism of these processes.

Materials and Methods

Cell culture

The human teratocarcinoma derived cell line Tera 2 was maintained as described in Thompson *et al.* (1984). Serum free culture experiments were conducted as described in Engström *et al.* (1985, 1986) and Biddle *et al.* (1988).

Growth factors and tissue culture materials

Recombinant human IGF I was purchased from Kabi (Sweden). Recombinant TGFß1 was obtained from British Biotechnology, Oxford (UK). Transferrin was acquired from Boehringer Mannheim (Sweden) and preloaded with iron according to the manufacturer's instructions. Alpha MEM trypsin and tissue culture plastic were all obtained from KEBO (Sweden).

Assessment of cell proliferation and apoptosis

The proliferation of Tera 2 cells over a 24 h or a 5 day period was monitored by counting cell numbers and pulse labeling with tritiated thymidine followed by autoradiography as described in Biddle *et al.* (1988). The assessment of intact vs. apoptotic cells was performed by acridine orange staining and fluorescence microscopy as described by Granerus *et al.* (1994). Nucleosome DNA ladders were produced and visualized as described in Moulton (1994)

Analysis of transcriptional activity

The analysis of c-myc expression was done by Northern blotting as described in Schofield *et al.* (1987)

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References

ARAKI, S., SHIMADA, Y., KAJI, K and HAYASHI, H. (1990). Apoptosis of vascular endothelial cells by fibroblast growth factor deprivation. *Biochem. Biophys. Res. Commun. 168:* 1194-1200.

BARRES, B.A., HART, I.K., COLES, S.R., BURNE, J.F., VOYVODIC, J.T., RICHARDSON, W.D. and RAFF, M.C. (1992). Cell death and control of cell survival in the oligodendrocyte lineage. *Cell 70:* 31-46.

BARRES, B.A., LAZEN, M.A. and RAFF, M.C. (1994). A novel role for thyroid hormone, glucocorticoids and retinoic acid in timing oligodendrocyte development. *Development 120:* 1097-1108.

BATES, R.C., BURET, A., van HELDEN, D.F., HORTON, M.A. and BURNS, G.F. (1994). Apoptosis induced by inhibition of intercellular contact. *J. Cell Biol. 125:* 403-415

BATISTATOU, A. and GREENE, L.A. (1991). Aurintricarbocxylic acid rescues PC12 cells and sympathic neurones from cell death caused by nerve growth factor deprivation. Correlation with suppression of endonuclease activity. *J. Cell Biol. 115:* 461-471.

BIDDLE, C., LI, C.H., SCHOFIELD, P.N., TATE, V.E., HOPKINS, B., ENGSTRÖM, W., HUSKISSON, N. and GRAHAM, C.F. (1988). Insulin like growth factors and the multiplication of Tera 2, a human teratoma derived cell line. *J. Cell Sci. 90:* 475-484.

CAELLES, C., HELMBERG, A. and KARIN, M. (1994). p53 dependent apoptosis in the absence of transcriptional activation of p53 target genes. *Nature 270:* 220-223.

COLUMBANO, A., LEDDA-COLUMBANO, G.M., CONI, P.P., FEE, G., LIGUORI, C., SANTA CRUZ, G. and PANI, P. (1985). Occurrence of cell death (apoptosis) during involution of liver hyperplasia. *Lab. Invest. 52:* 670-675.

COLLINS, M., PERKINS, G.R., RODRIGUEZ-TARCHARDU, G., NIETO, M.A. and LOPEZ-RIVAS, A. (1994). Growth factors as survival factors. Regulation of apoptosis. *BioEssays 16:* 133-137.

ENGSTRÖM, W. (1986). Differential effects of Epidermal Growth Factor on cell locomotion and cell proliferation in a cloned human embryonal carcinoma cell line *in vitro. J. Cell Sci. 86:* 55-63.

ENGSTRÖM, W., REES, A.R. and HEATH, J.K. (1985). Proliferation of a human embryonal carcinoma derived cell line in serum free medium. Interrelationship between growth factor requirement and membrane receptor expression. *J. Cell Sci. 73:* 361-373

FISCHER, D.E. (1994). Apoptosis in cancer therapy. Crossing the threshold. *Cell 78:* 539-542,

GRANERUS, M., BIERKE, P. and ENGSTRÖM, W. (1994). The effects of Leukemia Inhibitory Factor on cell proliferation migration and apoptosis in a human teratocarcinoma cell line *in vitro. Int. J. Oncol. 5:* 1419-1423.

HARRINGTON, E.A., BENNETT, M.R., FANIDI, A. and EVAN, G.I. (1994). c-myc induced apoptosis in fibroblasts is inhibited by specific cytokines. *EMBO J. 13:* 3286-3295.

HENGARTNER, M.O. and HORVITZ, H.R. (1994). Programmed cell death in *Caenorhabditis elegans. Curr. Opin. Genet. Dev. 4:* 581-586

HERMEKING, H. and EICK, D. (1994). Mediation of c-myc induced apoptosis by p53. *Science 265:* 2091-2093.

KULKARNI, G.V. and McCULLOCH, C.A.G. (1994). Serum deprivation induces apoptotic cell death in a subset of Balb/c3T3 fibroblasts. *J. Cell Sci. 107:* 1169-1179.

KURE, S., TOMINAGA, T., YOSHIMOTO, T., TADA, K. and NARISAWA, K. (1991). Glutamate triggers internucleosomal DNA cleavage in neuronal cells. *Biochem. Biophys. Res. Commun. 179:* 39-45

LAM, M., DUBYAK, G., CHEN, L., NUNEZ, G., MIESFIELD, R.L. and DISTELHORST, C.W. (1994). Evidence that Bcl-2 represses apoptosis by regulating endoplasmic reticulum associated Ca2+ fluxes. *Proc. Natl. Acad. Sci. USA 91:* 6569-6573.

McCONKEY, D.J. and ORRENIUS, S. (1994). Signal transduction pathways to apoptosis. *Trends Cell Biol. 4:* 370-375.

MOULTON, B. (1994). Transforming growth factor beta stimulates endometrial stromal apoptosis *in vitro. Endocrinology 134:* 1055-1060

NUNEZ, G and CLARKE, M.F. (1994). The BCl-2 family of proteins. Regulators of cell death and survival. *Trends Cell Biol. 4:* 399-403

OLTVAI, Z.N. and KORSMEYER, S.J. (1994). Checkpoints of duelling dimers foil death wishes. *Cell 79:* 189-192.

OSBORNE, B.A. and SCHWARTZ, L.M. (1994). Essential genes that regulate apoptosis. *Trends Cell Biol. 4:* 394-398.

PRIVES, C. and MANFREDI, J.J. (1993). The p53 suppressor protein. *Genes Dev. 7:* 529-534

RAFF, M.C. (1992). Social control on cell survival and cell death. *Nature 356:* 397-400.

RAWSON, C.L., LOO, D.T., DUIMSTRA, J.R., HEDSTROM, O.R., SCHMIDT, E.E. and BARNES, D.W. (1991). Death of serum free mouse embryo cells caused by epidermal growth factor deprivation. *J. Cell Biol. 113:* 671-680.

RODRIGUEZ-TARCHUDY, G., COLLINS, M. and LOPEZ-RIVAS, A. (1990). Regulation of apoptosis in IL-3 dependent hemopoetic cells by IL-3 and calcium ionophores. *EMBO J. 9:* 2997-3002.

RUBIN, L.L., GATCHALIAN, C.L., RIMON, G and BROOKS, S.F. (1994). The molecular mechanisms of neuronal apoptosis. *Curr. Opin. Neurobiol. 4:* 696-702.

SANDFORD, N.L., SEARLE, J.W. and KERR, J.F. (1984). Successive waves of apoptosis in the rat prostate after repeated withdrawal of testosterone stimulation. *Pathology 16:* 406-410.

SCHOFIELD, P.N. and ENGSTRÖM, W. (1992). Insulin like growth factors and human cancer. In *Insulin like Growth Factors* (Ed. P.N. Schofield). Oxford University Press, pp. 140-157.

SCHOFIELD, P.N., ENGSTRÖM, W., LEE, A., BIDDLE, C. and GRAHAM, C.F. (1987). Expression of c-myc during differentiation of a human teratocarcinoma cell line – Tera 2. *J. Cell Sci. 88:* 57-64.

SCHULZE-OSTHOFF, K., WALCZAK, H., DROGE, W. and KRAMMER, P.H. (1994). Cell nucleus and DNA fragmentation are not required for apoptosis *J. Cell Biol. 127:* 15-20.

THOMPSON, S., STERN, P.L., WEBB, M., WALSH, F.S., ENGSTRÖM, W., EVANS, E.P., SHI, W.K. and GRAHAM, C.F. (1984). Cloned human teratoma cells differentiate into neuron like cells and other cell types. *J. Cell Sci. 72:* 37-64.

WANG, L., MIURA, M., BERGERON, L., ZHU, H. and YUAN, J. (1994). Ich-1, an Ice/ced-3-relared gene, encodes both positive and negative regulators of programmed cell death. *Cell 78:* 739-750.

WHITE, E. (1993). Death defying acts. *Genes Dev. 7:* 2277-2284.

WILLIAMS, G.T., SMITH, C.A., SPOONER, E., DEXTER, T.M. and TAYLOR, D.R. (1990). Haemopoetic colony stimulating factors promote cell survival by suppressing apoptosis. *Nature 343:* 76-79.

YOUNG, A.R. (1987). The sunburn cell. *Photodermatology 4:* 127-134.