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Programmed Cell Death by Default in Embryonic Cells, Fibroblasts, and Cancer Cells

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We recently proposed that most mammalian cells constitutively express all of the proteins required to undergo programmed cell death (PCD) and undergo PCD unless continuously signaled by other cells not to. Although some cells have been shown to work this way, the vast majority of cell types remain to be tested. Here we tested purified fibroblasts isolated from developing or adult rat sciatic nerve, a mixture of cell types isolated from normal or p53-null mouse embryos, an immortalized rat fibroblast cell line, and a number of cancer cell lines. We found the following: 1) All of these cells undergo PCD when cultured at low cell density in the absence of serum and exogenous signaling molecules but can be rescued by serum or specific growth factors, suggesting that they need extracellular signals to avoid PCD. (2) The mixed cell types dissociated from normal mouse embryos can only support one another's survival in culture if they are in aggregates, suggesting that cell survival in embryos may depend on short-range signals. (3) Some cancer cells secrete factors that support their own survival. (4) The survival requirements of a human leukemia cell line change when the cells differentiate. (5) All of the cells studied can undergo PCD in the presence of cycloheximide, suggesting that they constitutively express all of the protein components required to execute the death program.

INTRODUCTION

The activities of animal cells are regulated by complex cell-cell interactions, which coordinate these activities for the good of the animal as a whole. Mammalian cells, for example, normally divide only when they are signaled to do so by other cells, ensuring that they divide only when more cells are needed (Baserga, 1985). Similarly, some types of mammalian cells, in vitro at least, survive only when they are signaled to do so by extracellular signals produced by other cells: without such survival signals the cells activate an intrinsic death program and kill themselves–a process called programmed cell death (PCD) (reviewed in Raff, 1992). In vivo, such a death by default mechanism may help ensure that cells survive only when and where they are needed (Raff, 1992; Raff *et al.*, 1993). In many types of mammalian cells, all of the proteins required to execute the death program seem to be constitutively expressed (reviewed in Martin, 1993; and Raff *et al.*, 1993).

Much less attention has been paid to the control of cell survival than to the control of cell division. Nonetheless, some general principles of cell survival control are beginning to be discovered. Extracellular signals that promote survival, for example, can be soluble (reviewed in Raff, 1992), bound to the surface of a cell (Dolci *et al.*, 1991; Flanagan *et al.*, 1991), or part of the extracellular matrix (Meredith *et al.*, 1993; Frisch and Francis, 1994; Ruoslahti and Reed, 1994). They can act in an endocrine, paracrine, or autocrine mode: adrenal cortical cells in vivo, for example, require the hormone ACTH for their survival (Wyllie *et al.*, 1973, 1980), oligodendrocytes and their precursor cells require paracrine signals from neighboring cells of a different

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type (Barres *et al.*, 1992, 1993), while lens epithelial cells (Ishizaki *et al.*, 1993) and chondrocytes (Bruckner *et al.*, 1989; Ishizaki *et al.*, 1994) can survive in culture when the only extracellular signals are provided by cells of the same type (which may be related to the in vivo situation where their only close neighbors are cells of the same type).

Despite the recent advances in understanding cell survival control, however, the extracellular signals required for the survival of the great majority of cells remain to be defined. We know the least about cells in adult tissues, largely because of the difficulty in dissociating and purifying such cells: it is unclear, for example, whether most cells in adult tissues constitutively express all the proteins required to run the death program, or to what extent the death by default mechanism applies to them. Moreover, although it is clear that cancer cells are generally less obedient than normal cells to the "social controls" that regulate cell behavior, for most cancers it is uncertain whether the transformed cells depend on extracellular signals for their survival. Nor is it clear whether cancer cells ever inactivate (by mutation) genes required to execute the cell death program and in this way gain a survival advantage over their neighbors.

In the present study we have examined the in vitro survival requirements of a variety of normal cells isolated from developing or adult tissues, as well as the survival requirements of a number of immortalized and cancer cell lines. We find that all of these cells are capable of undergoing PCD, constitutively express all of the protein components required to execute the death program, and require extracellular signals from other cells to avoid PCD in culture. We show that some cancer cells secrete factors that support their own survival, and we provide evidence for the importance of short-range signaling for cell survival in mammalian embryos.

MATERIALS AND METHODS

Animals and Materials

Sprague-Dawley rats were obtained from the breeding colony of the University College London (UCL) animal facility. BALB/c mice were purchased from Sankyo Lab Service Corporation (Tokyo, Japan). Recombinant human basic fibroblast growth factor (bFGF) was purchased from Preprotech (Rocky Hill, NJ); recombinant human platelet-derived growth factor BB (PDGF) was purchased from R&D Systems (Oxford, UK). Insulin, 3-(4,5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), Hoechst 33342, cysteine, cycloheximide, trypsin, α-chymotrypsin, collagenase, DNase-I, bovine serum albumin (BSA; highly purified, crystalline grade), poly-D-lysine, and staurosporine were purchased from Sigma Chemical (St. Louis, MO). Terminal deoxynucleotidyl transferase from calf thymus and biotinylated dUTP were purchased from Boehringer Mannheim GmbH (Germany). Fetal calf serum (FCS), Earle's balanced salt solution (EBSS), high melting point (Tm) agarose, and low Tm agarose were purchased from Life Technologies (Gaithersburg, MD). EDTA was purchased from BDH Laboratory Supplies, Merck (Poole, UK). L-15 medium, DMEM, and Ham's F-12 medium (F-12) were purchased from Flow Laboratories (Irvine, UK). OX42 monoclonal antibody (purified Ig fraction) that recognizes the leucocyte common antigen was purchased from Sera-lab (Crawley Down, UK). OX7 monoclonal anti-Thy 1.1 antibody (hybridoma culture supernatant) was a gift from A. Williams (Oxford). Rabbit anti-mouse-Ig antibodies (purified Ig fraction) were purchased from Dako (Glostrap, Denmark). Millex-GV filters (0.22 mm) were purchased from Millipore (Bedford, MA).

Cell Lines

Rat-1 cells were kindly provided by Dr. Abdallah Fanidi (Imperial Cancer Research Fund, London, UK). HeLa cells were kindly provided by Dr. Mark Marsh (Medical Research Council, Laboratory for Molecular Cell Biology, University College, London, UK). P3V1 mouse myeloma cells, C6 rat glioma cells, HL-60 human promyelocytic leukemia cells, and the PK-15 pig kidney epithelial cell line were obtained from Riken Cell Bank (Tsukuba, Japan). All of the cell lines, except the P3V1 and HL-60 cells, were maintained in 100-mm tissue culture dishes in a 1:1 mixture of DMEM and F-12 (DMEM/F-12) containing 10% FCS, in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The medium was replaced once every third or fourth day. When confluent, the cells were trypsinized (0.05% trypsin and 0.025% EDTA) and passaged. P3V1 and HL-60 cells were maintained in Falcon tissue culture flasks in DMEM/F12 with 10% FCS and RPMI 1640 medium with 10% FCS, respectively, and were subcultured every week.

Rat Sciatic Nerve Fibroblasts

Sciatic nerves were dissected from 10–20 postnatal day 7 (P7) rats or 2–4 adult rats and were finely chopped with a scalpel. They were incubated at 37°C for 45 min in EBBS containing 0.025% trypsin, 0.1% collagenase, and 0.004% DNase for P7 rats, and 0.05% trypsin, 0.2% collagenase, 0.1% chymotrypsin, and 0.004% DNase for adult rats. Digestion was stopped by addition of DMEM containing 10% FCS. The cells were resuspended in L-15 medium containing 0.1% BSA, further dissociated by trituration with a 1-ml Pipetteman (Anachem, Luton, UK), and filtered through nylon mesh (20-μm pore size, Plastok Associates, Birkenhead, UK) to remove debris and cell clumps. The fibroblasts were purified from the cell suspension by sequential immunopanning (Barres *et al.*, 1992; Cheng *et al.*, 1995). Panning dishes were prepared by incubating 100-mm petri dishes (Falcon) overnight at 4°C with 10 ml of 0.2 M carbonate-bicarbonate buffer (pH 9.5) containing 50 μg of rabbit anti-mouse Ig antibodies (Dako). The dishes were washed three times with phosphate-buffered saline (PBS) and then incubated with cell type–specific monoclonal antibodies (see below) in PBS with 0.2% BSA for at least 1 h at room temperature. The plates were washed three times with PBS before the cell suspension was added.

The cell suspension was applied first to a panning dish coated with the OX42 monoclonal antibody (1 μg/ml) to remove macrophages and other white blood cells. The nonadherent cells were then incubated in a second panning dish coated with the OX7 monoclonal antibody (hybridoma supernatant, diluted 1:20) to select the Thy-1^+ fibroblasts. The cell suspension was incubated in each dish for 30 min at room temperature and agitated vigorously at 15 and 30 min to prevent nonspecific binding. The fibroblasts that were adherent to the OX7 dish were washed at least six times until all the loosely attached cells were removed. The remaining attached cells were then incubated in a CO_2 incubator at 37°C in DMEM/F-12 containing penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% FCS. After 1 day for P7 fibroblasts and 4–5 days for adult fibroblasts, the cells were incubated in 0.125% trypsin and 0.025% EDTA in EBSS for 10 min at 37°C. DMEM/F-12 containing 10% FCS was added and the cells were squirted off the dish with a 1-ml Pipetteman. After centrifugation the cells were washed once in DMEM/F-12 containing 0.5% BSA and were then resuspended and tested in DMEM/F-12 without BSA.

Mouse Embryo "Fibroblasts" (MEFs)

MEFs were prepared from embryonic day 13 (E13) wild-type (129/0la or BALB/c) mice or E13 p53-null 129/0la mice [which have both *p53* genes inactivated by homologous recombination (Clarke *et al.*, 1993) and were kindly supplied by Abdallah Fanidi, Imperial Cancer Research Fund]. The head and liver were removed from the embryos, and the rest of the embryo was minced with a pair of scalpels. Minced embryos were incubated in EBSS containing 0.125% trypsin and 0.01% EDTA for 20 min at 37°C. After addition of DMEM/F-12 containing 10% FCS to stop the digestion, the embryos were transferred to a plastic tube with a minimum of medium. The tissue fragments were disrupted mechanically by repeated trituration with a Pasteur pipette, and the released cells were passed through a nylon mesh (100-μm pore size), washed once in DMEM/F-12 containing 10% FCS, and then resuspended and plated in the same medium in a tissue culture dish. After 1 or 2 days, the cells that adhered to the dish were washed with EBSS and incubated with a mixture of trypsin (0.05%) and EDTA (0.025%) in EBSS for 3 min at 37°C. The incubation was stopped by the addition of DMEM/F-12 containing 10% FCS, and the cells were washed once with DMEM/F-12 containing 0.5% BSA and resuspended in the same medium at the appropriate cell density for culture. MEFs from normal mice were passaged one to six times before they were used in survival assays, while MEFs from p53-null mice were passaged 11–15 times.

Induction of HL-60 Cell Differentiation

HL-60 cells were induced to differentiate into granulocyte-like cells by treating the cells in RPMI 1640 and 10% FCS with either 1 μM retinoic acid or 1.25% dimethyl sulfoxide for 5 days (Collins *et al.*, 1978; Breitman *et al.*, 1979). HL-60 cells were induced to differentiate into monocyte-like cells by treatment with 50 nM calcitriol for 5 days (Bar-shavit *et al.*, 1983; Mangelsdorf *et al.*, 1984). In both cases, differentiation was confirmed by the characteristic changes in cell morphology.

Cell Culture

For survival assays, the cells were mainly cultured in agarose gels, as described by Benya and Schaffer (1982), with some modifications as described in Ishizaki *et al.* (1994). Cell suspensions were prepared in low Tm agarose (2% in water), which was autoclaved for 20 min and then cooled to 37°C before being mixed with an equal volume of 2× concentrated DMEM at 37°C to give a final concentration of 1% agarose in DMEM. Cells suspended in F-12 with or without 0.5% BSA were mixed with an equal volume of 1% agarose in DMEM at 37°C to give the appropriate cell densities in 0.5% agarose in DMEM/F-12. Ten microliters of the cell suspension was added to each well of a Terasaki microwell plate (Nunc, Roskilde, Denmark). The culture plate was held at 37°C in a CO_2 incubator for 15 min before the agarose was allowed to gel at 4°C for 15 min. After gelation, an equal volume (10 μl) of DMEM/F-12 was added and the culture plate was returned to a CO_2 incubator.

For experiments comparing the survival of dissociated MEFs with reaggregated MEFs, cells dissociated from E13 BALB/c mouse embryos were passaged once and suspended at 2×10^6 cells/ml in F-12. The cell suspension was mixed with either the same volume of 1% low Tm agarose in DMEM (for dissociated cell cultures) or with the same volume of DMEM (for reaggregated cell cultures),and then plated into 96-well Falcon culture plates that had been coated with high Tm agarose. For dissociated cell cultures the plates were left at 4°C for 5 min to allow the agarose to gel and were then placed in a CO_2 incubator after addition of the same volume of DMEM/F-12. For reaggregate cultures the plates were kept at 37°C for 30 min to allow the cells to reaggregate, after which the medium was gently aspirated and 0.5% low Tm agarose in DMEM/F-12 was added to each well; the plates were then left at 4°C for 5 min to allow the agarose to gel, DMEM/F-12 was then added, and the plates were returned to a CO_2 incubator.

In some experiments cells were cultured on poly-D-lysine (PDL)-coated plastic, either in Terasaki microwell plates or in 96-well Falcon tissue culture plates, rather than in agarose gels. For photography, cells were grown on PDL-coated glass coverslips in 24-well Falcon tissue culture plates.

Single Cell Agarose-Gel Culture

For single cell culture experiments, a cell suspension in F-12 containing 0.5% BSA was serially diluted to the concentration of 2×10^2 cells/ml, mixed with the same volume of 1% low Tm agarose in DMEM, and 10 μl was placed in each well of a Terasaki microculture plate. After gelation, 10 μl of DMEM/F-12 was added; the wells that contained a single cell were selected (using an inverted microscope) and marked, and the culture plate was returned to a CO_2 incubator. Approximately 30 of 60 wells of each plate contained a single cell and were used for the survival assay. The experiments were repeated at least three times with similar results.

Schwann Cell-conditioned Medium

The Schwann cells remaining after immunopanning on OX42 and OX7 antibody-coated plates were cultured at 1.5×10^5 cells/ml in PDL and laminin-coated wells of a 24-well Falcon culture plate in DMEM containing insulin, transferrin, progesterone, putrescine, selenium, thyroxine, tri-iodothyonine, and BSA, as previously described (Cheng *et al.*, 1995). The medium was collected and replaced every 2 days. The conditioned medium was used immediately and was undiluted.

MTT Cell Survival Assay

Cell survival was assessed mainly by the MTT assay, which measures intracellular dehydrogenase activity (Mosmann, 1983). MTT was dissolved in DMEM/F-12 at 5 mg/ml and sterilized by passage through a 0.22-μm Millipore filter. This stock solution was added (one part to 10 parts of medium) to each microwell of the Terasaki plate or to each well of the 96-well culture plate, and the plate was incubated at 37°C for 1 h. Viable cells cleave the tetrazolium ring into a visible dark blue formazan reaction product, whereas dead cells remain uncolored. In all high-density ($\geq 10^5$ cells/ml) cultures and in low-density ($\leq 10^4$ cells/ml) cultures in 96-well plates, at least 100 cells were counted in an inverted Zeiss or Olympus microscope and the fraction of live cells was determined. In low-density ($\leq 10^4$ cells/ml) cultures in agarose gel in Terasaki plates, the total numbers of live and dead cells were counted. Although cells did not generally divide in serum-free cultures, they did in the presence of 10% FCS, where colonies of cells in agarose gel were frequently observed; in these cases each colony was counted as a single cell.

Propidium Iodide (PI) Staining

In some cases cell survival was assessed by staining cell nuclei with Hoechst 33342 (40 μg/ml in DMEM/F-12) or with PI (4 μg/ml in PBS containing 100 μg/ml DNase-free RNase A) for 30 min at 37°C after fixation in 70% ethanol for 10 min at −20°C (Barres *et al.*, 1992). The cells were assessed in an Olympus inverted fluorescence microscope.

Terminal Deoxynucleotidyl Transferase-mediated dUTP-Biotin Nick-end Labeling (TUNEL)

In some cases in situ DNA cleavage was assessed by the TUNEL technique, described by Gavrieli *et al.* (1992), with some modifications, as described by Ishizaki *et al.* (1994). Cells cultured on PDL-coated glass coverslips in 24-well Falcon tissue culture plates were

fixed in 4% paraformaldehyde in 10 mM Tris-HCl, pH 8.0, for 30 min at 4°C, washed extensively in the same buffer, and then permeabilized in 0.1% Triton X-100 in the same buffer for 5 min at room temperature. After extensive washing in the same buffer, the cells were preincubated for 10 min at room temperature in the reaction buffer for terminal deoxynucleotidyl transferase supplied by Boehringer Mannheim GmbH (200 mM potassium cacodylate, 0.25 mg/ml BSA, 25 mM Tris-HCl, pH 6.6). The reaction was started by removing the preincubation buffer and adding the reaction mixture containing 500 U/ml terminal deoxynucleotidyl transferase, 2.5 mM $CoCl_2$, and 40 μM biotinylated dUTP. After 60 min at 37°C, the reaction was terminated by the addition of 300 mM NaCl and 30 mM sodium citrate. After 25 min at room temperature, cells were washed with PBS and incubated with streptavidin fluorescein (diluted 1:100, Amersham, Buckinghamshire, UK) for 60 min at room temperature in the dark. After extensive washing in PBS, the cells were examined in an Olympus inverted fluorescence microscope.

RESULTS

Assessment of Cell Survival

In most cases cell survival was assessed in agarose gel microcultures, where dissociated cells can be cultured at various cell densities under conditions in which they do not contact their neighbors and cannot migrate; even at the highest cell densities tested (10^6 cells/ml), the cells were not in contact (see Figure 5A). In such cultures dead cells are not phagocytosed, so that both live and dead cells can be readily counted. Moreover, cell divisions produce colonies that can be counted as single units, so that cell proliferation does not confound the assay, although in the absence of serum or added signaling molecules, only transformed cells divided in agarose gel (our unpublished observations). For all of the nontransformed cells studied, cell survival was also assessed on PDL-coated plastic, rather than in agarose gels. In all cases cell survival was generally assessed by the MTT assay, as described in MATERIALS AND METHODS, where individual live cells were distinguished by their dehydrogenase activity (Figure 1, A and B).

For all cell types and cultures studied, when the cells died, they did so with the morphological characteristics of apoptosis: when visualized by phase-contrast or differential-interference-contrast microscopy, or by fluorescence microscopy after staining with the DNA-binding dyes Hoechst 33342 or PI, the cells were shrunken and the nucleus was condensed and sometimes fragmented (Figure 1, B–H). Moreover, when tested by the in situ end-labeling TUNEL technique, the nucleus of the dead cells was labeled (Figure 1, G–H), indicating that DNA fragmentation had occurred.

Rat Sciatic Nerve Fibroblasts

To study the survival of fibroblasts in the absence of other cell types, we purified fibroblasts from P7 rat sciatic nerves by sequential immunopanning, first removing the macrophages and other leucocytes on one

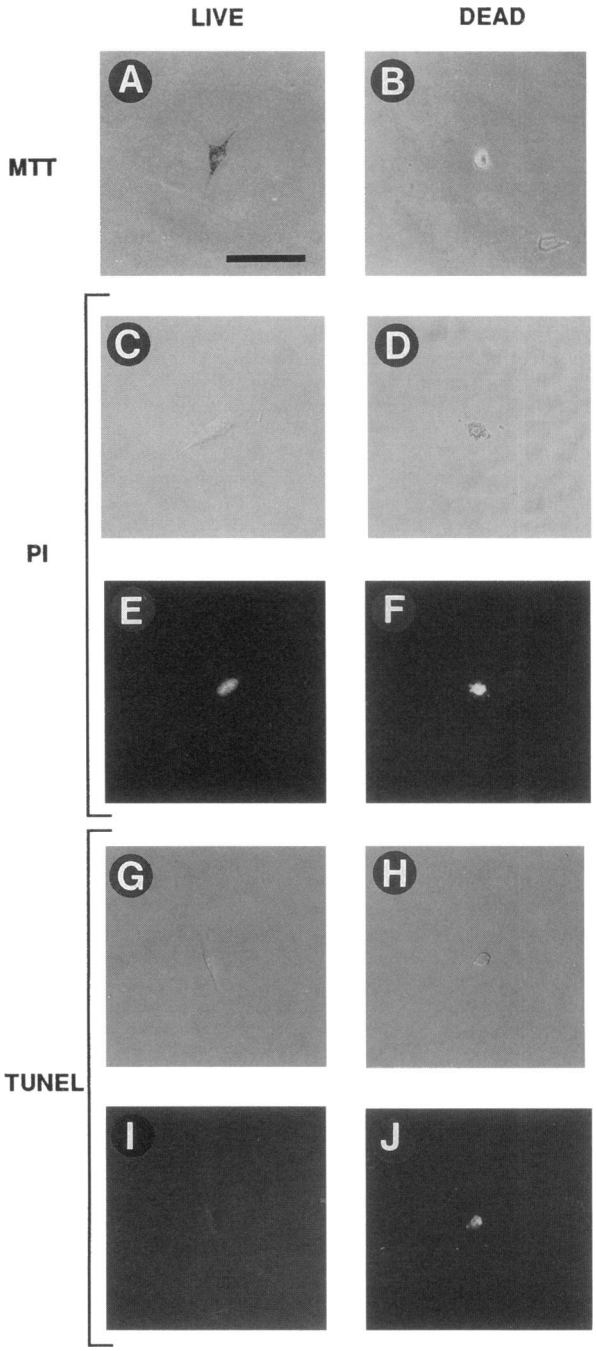

Figure 1. PCD of MEFs assayed in different ways. MEFs from E13 BALB/c mouse embryos were cultured on PDL-coated glass coverslips in 24-well plates in DMEM/F-12 at 10^4 cells/ml in the presence (A, C, E, G, and I) or absence (B, D, F, H, and J) of growth factors (5 μg/ml insulin, 10 ng/ml PDGF-BB, and 10 ng/ml bFGF). Their viability was assessed after 3 days by MTT assay (A and B), propidium iodide (PI) staining (C–F), or TUNEL (G–J). The cells in panels C and D and in panels G and H, visualized by differential interference contrast, are the same as in panels E and F and in panels I and J, respectively. Bar, 100 μm.

antibody-coated panning dish and then selecting the Thy-1$^+$ fibroblasts on an anti-Thy-1 antibody–coated dish. The resulting cells, which were > 99% pure as assessed by anti-Thy-1 staining, were cultured in DMEM/F-12 medium, either on PDL-coated plastic or in agarose gel microcultures, in the absence of both serum and exogenous signaling molecules. Cells were assessed for survival by the MTT assay after various times. Most of the cells died by 3 days (Figure 2A) with the morphological features of apoptosis. Although cell survival was greater at higher cell densities, even at the highest density tested (10^6 cells/ml), most cells died within 3 days (Figure 2A). Although cysteine, presumably acting as an antioxidant, has been shown to promote the survival of lens epithelial cells (Ishizaki *et al.*, 1993) and chondrocytes (Tschan *et al.*, 1990; Ishizaki *et al.*, 1994), the addition of 1 mM cysteine to these cultures had no effect (our unpublished observations). On the other hand, in the presence of either FCS or PDGF-BB, 90% of the purified fibroblasts survived for 3 days, and 80% survived for 7 days, while bFGF or a high concentration of insulin had much less survival-promoting effect (Figure 2B). Conditioned medium from cultures of purified sciatic nerve Schwann cells, the normal neighbors of the fibroblasts in the nerve, did not promote cell survival (Figure 2B); the fibroblasts also died when they were cultured with Schwann cells in unpurified sciatic nerve cell cultures (our unpublished observations). The results were similar whether the fibroblasts were cultured on PDL-coated plastic (Figure 2) or in agarose gels (our unpublished observations). Importantly, similar results were obtained with fibroblasts purified from adult sciatic nerves: they died in the absence of serum and signaling molecules (Figure 3A) but survived in FCS or PDGF-BB (Figure 3B).

Mouse Embryo Cells (MEFs)

We next tested a commonly studied population of cells–commonly referred to as mouse embryo fibroblasts (MEFs)–prepared by dissociating cells from whole E13 mouse embryos from which only the head and liver were removed. After one to six passages in 10% FCS, the cells were removed from the culture dish with trypsin and tested at various cell densities in agarose gel microwell cultures in the absence of serum and exogenous signaling molecules. Even at the highest cell density (10^6 cells/ml), more than 95% of the cells died within 3 days with the characteristics of apoptosis; at lower cell densities, all of the cells died within 1 day (Figure 4A). The addition of 1 mM cysteine (our unpublished observations), PDGF-BB, or

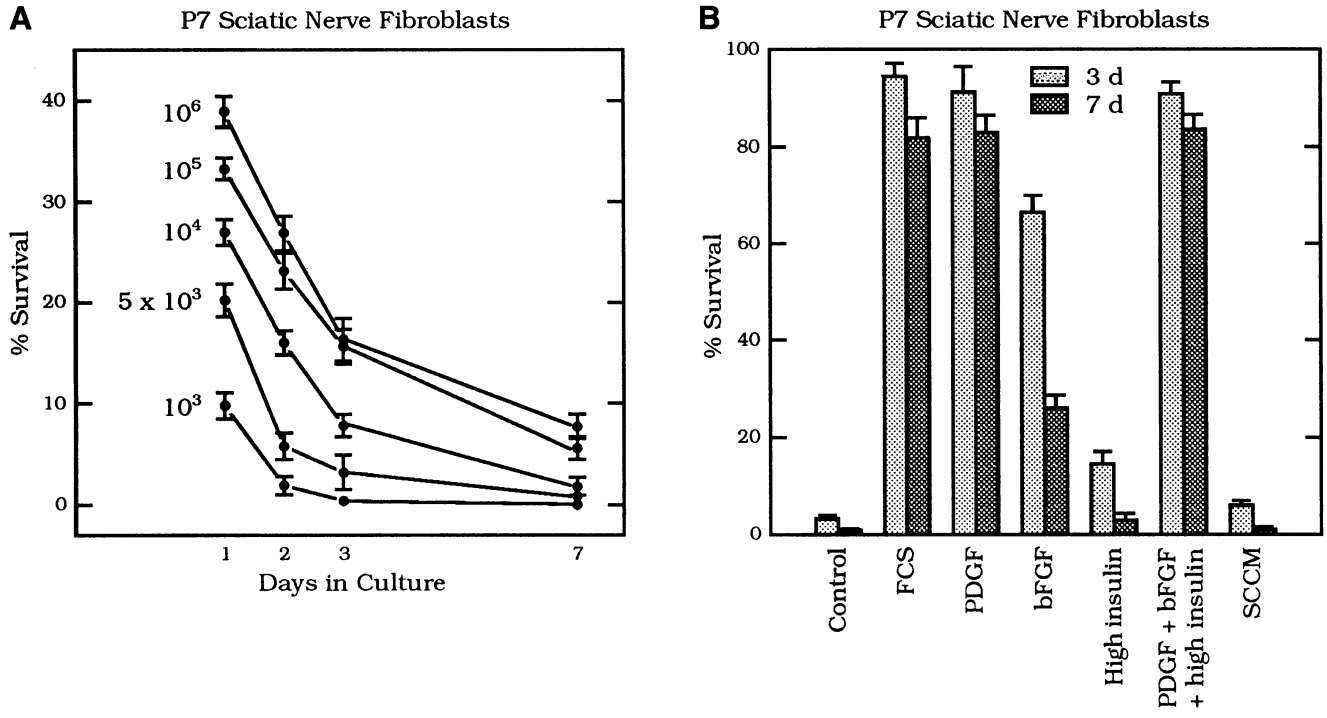

Figure 2. Survival of purified P7 rat sciatic nerve fibroblasts. The cells were cultured on PDL-coated plastic in 96-well culture plates in DMEM/F-12 at various cell densities (A) or at 5×10^3 cells/ml in various growth factors or neat Schwann-cell-conditioned medium (SCCM) (B). Cell viability was assessed by MTT assay after various times. The initial cell viability was > 95% in all cases. In this and the following figures (except Figure 7), the results are expressed as means ± SE of four to six experiments.

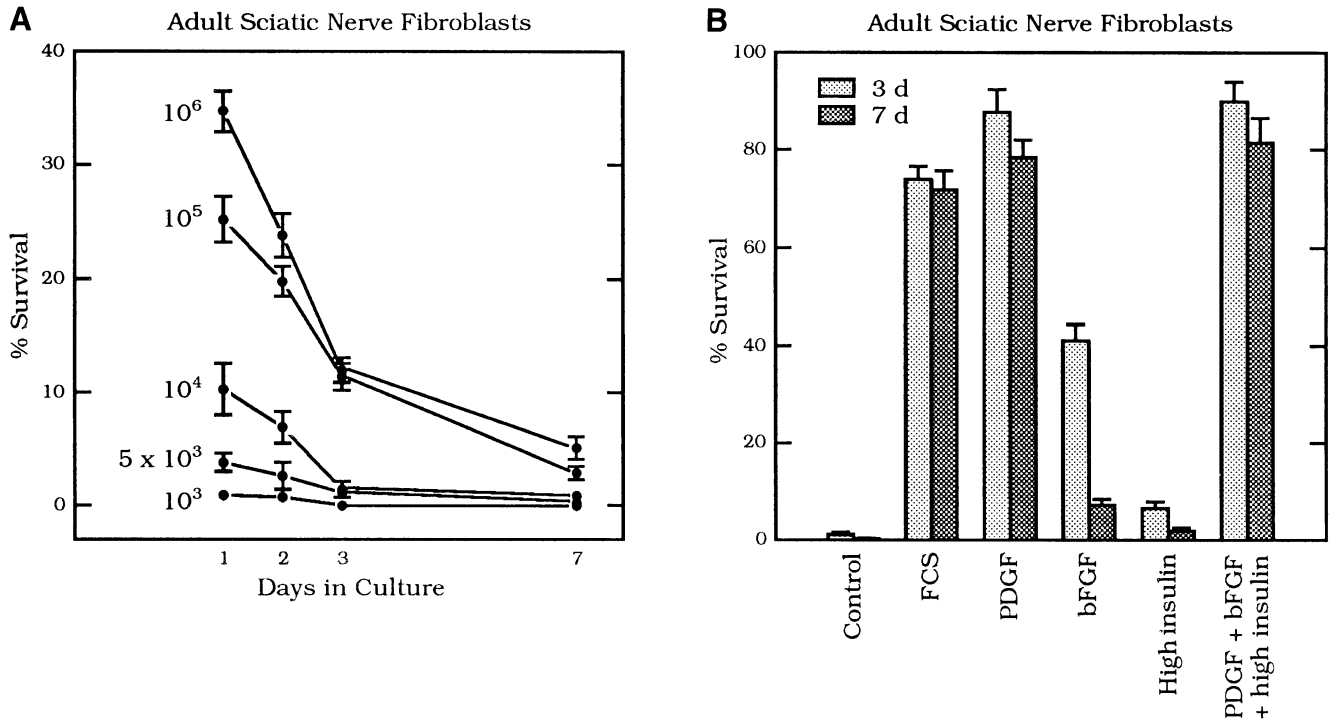

Figure 3. Survival of purified adult rat sciatic nerve fibroblasts. The cells were cultured and assayed as in Figure 2. The initial cell viability was > 95%.

high insulin did not promote survival; bFGF, and to a lesser extent FCS, did promote survival (Figure 4B).

When the cells were cultured at high density (10^6 cells/ml) on PDL-coated plastic, however, many cells died in the absence of serum or exogenous signaling molecules, although others formed a confluent monolayer and survived for weeks (our unpublished observations). This finding suggested that embryonic cells in a mixed population cannot support one another's survival in culture in the absence of serum and exogenous signals unless they are in contact, or at least very close to one another. To test this suggestion directly, MEFs were plated onto high-Tm agarose-coated plastic for 30 min at 37°C, during which time many of the cells formed aggregates. The cells were then overlaid with low-Tm agarose in DMEM/F-12 and cultured for 3 days before their viability was assessed by the MTT assay. As illustrated in Figure 5, cells in aggregates survived, whereas isolated cells did not.

Cells prepared in the same way from E13 mouse embryos in which both copies of the p53 gene were inactivated by homologous recombination (p53-null mice) behaved similarly in agarose gel cultures: although they survived a little better than wild-type cells, most died within 3 days in the absence of serum and signaling molecules (Figure 4C) but survived in FCS or bFGF (our unpublished observations). Thus the PCD that occurs under these conditions does not seem to depend on p53.

Rat-1 Fibroblasts

To determine whether immortalization confers autonomy for survival on fibroblasts, we studied the immortal rat fibroblast cell line Rat-1. These cells behaved similarly in agarose gel cultures to sciatic nerve fibroblasts and MEFs in that in the absence of serum and exogenous signaling molecules, even at high density and in the presence or absence of 1 mM cysteine, they died rapidly (Figure 6A) with the characteristic morphology of apoptosis. FCS promoted their survival, as did the combination of high insulin, bFGF, and PDGF-BB, although the individual growth factors had little effect on their own (Figure 6B). When cultured at high density (10^6 cells/ml) on PDL-coated plastic, however, many of the cells died in the absence of serum and signaling molecules, although some formed confluent clusters and survived for several weeks (our unpublished observations). Thus immortalization does not confer autonomy for fibroblast survival, although Rat-1 cells seem to be able to support one another's survival as long as they are in contact or in very close proximity.

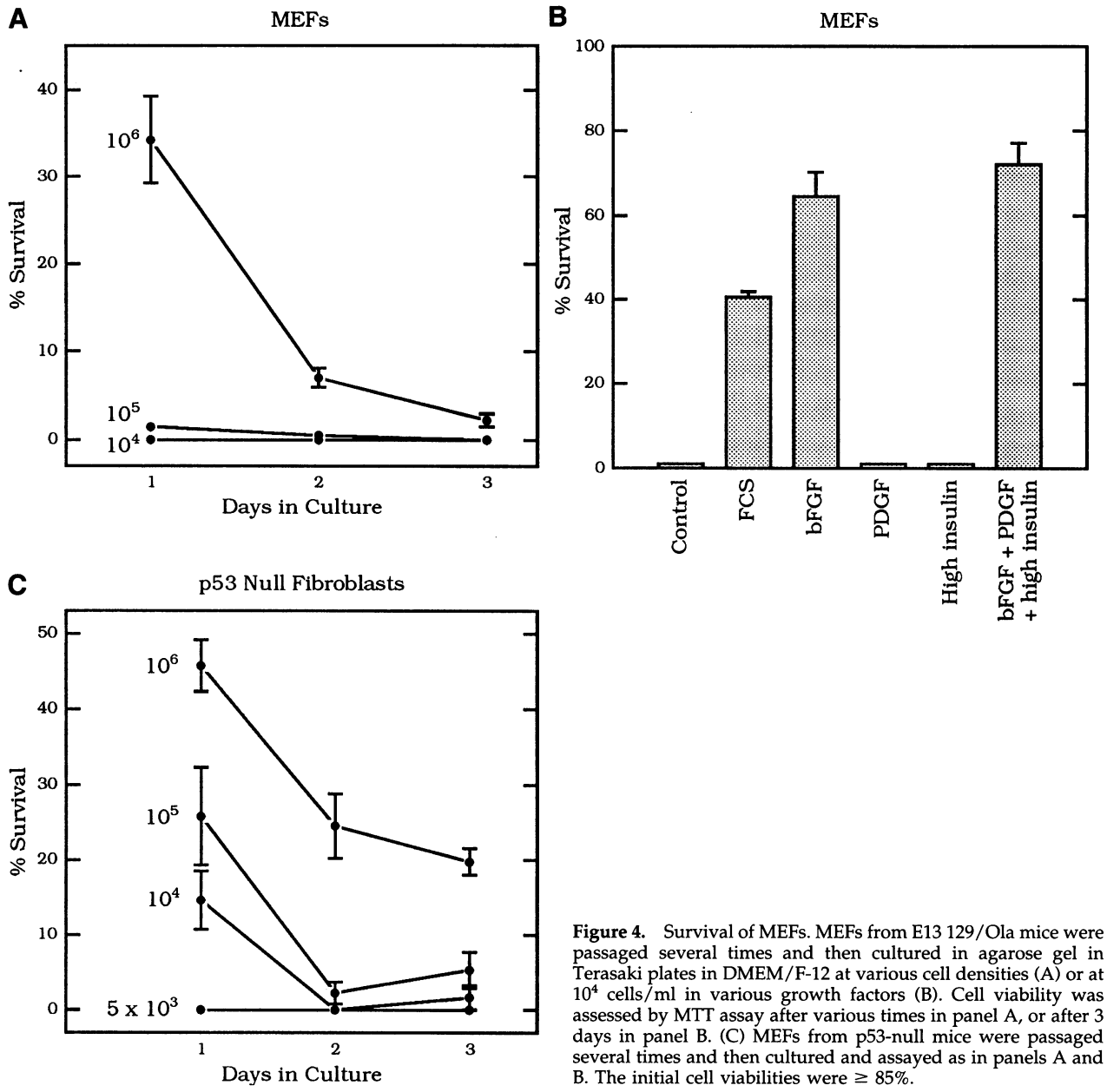

Figure 4. Survival of MEFs. MEFs from E13 129/Ola mice were passaged several times and then cultured in agarose gel in Terasaki plates in DMEM/F-12 at various cell densities (A) or at 10^4 cells/ml in various growth factors (B). Cell viability was assessed by MTT assay after various times in panel A, or after 3 days in panel B. (C) MEFs from p53-null mice were passaged several times and then cultured and assayed as in panels A and B. The initial cell viabilities were ≥ 85%.

Transformed Cells

To determine whether transformation into a cancer cell confers autonomy for survival, we studied a number of cancer cell lines, including the mouse myeloma cell line P3V1, the rat glioma cell line C6, the human cervical carcinoma cell line HeLa, and the human promyelocytic leukemia cell line HL-60. In addition, we studied a transformed porcine renal epithelial cell line, PK-15. All of these cells were tested in agarose gel microcultures. The myeloma cells behaved just like normal fibroblasts and Rat-1 cells: even at high density and in the presence of 1 mM cysteine, they died by 3 days in the absence of serum and signaling molecules but were saved by FCS (our unpublished observations). C6, PK-15, and HeLa cells, however, survived far better than normal cells in the absence of serum and exogenous signals: at high cell densities most survived for 7 days (Figure 7) and 30–40% survived for 1 mo (our unpublished observations). HL-60 cells behaved similarly (see below). When C6, PK-15, or HeLa cells were cultured on their own as isolated single cells, however, they survived for 7 days in 10% FCS and 1 mM cysteine, but almost all died by 7 days in the absence of serum, even if cysteine was present;

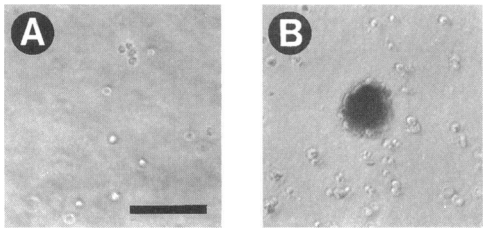

Figure 5. Survival of dissociated (A) or reaggregated (B) MEFs. Cells dissociated from E13 BALB/c mouse embryos were passaged once and cultured at 10^6 cells/ml in agarose gel in 96-well culture plates in protein-free medium either as dissociated cells (A) or after the cells were allowed to aggregate (B). Cell viability was assessed after 3 days by MTT assay. Note that the viable cells in the aggregate metabolized the MTT into an insoluble dark blue reaction product. Bar, 100 μm.

about 40% of C6 and 70% of HeLa survived as single cells for 4 days in the absence of serum and signaling molecules if cysteine was present (Figure 8). Results with 0.4 mM *N*-acetyl cysteine were similar to those with 1 mM cysteine (our unpublished observations). Thus even cancer cells, it seems, require extracellular signals to avoid PCD.

The ability of some transformed cells to survive in agarose gel at high density but not low density in the absence of serum and exogenous signaling molecules suggested that these cells secrete autocrine survival signals. To test this possibility we cultured the cells in agarose at very low density (1000 cells/ml) in the presence or absence of conditioned medium from high-density cultures. As shown in Figure 9, conditioned medium from either C6, PK-15, or HeLa cells promoted the survival of both C6 and PK-15 cells in low-density cultures for at least 7 days, but all were much less effective at promoting the survival of HeLa cells under the same conditions. Thus some transformed cells secrete signals that can support their own survival, as well as the survival of some other types of transformed cells.

Differentiated HL-60 Cells

HL-60 cells differentiate into granulocyte-like cells when treated with retinoic acid (RA) (Breitman *et al.*, 1979) or dimethyl sulfoxide (DMSO) (Collins *et al.*, 1978) and into monocyte-like cells when treated with the active form of vitamin D3 (calcitriol) (Bar-shavit *et al.*, 1983; Mangelsdorf *et al.*, 1984). When HL-60 cells were treated with either RA (Figure 10B) or DMSO (our unpublished observations) for 5 days and then tested at high cell density in agarose gel, none of the cells survived for 4 days in the absence of serum and exogenous signaling molecules, whereas 50% of untreated (undifferentiated) cells survived for 4 days under these conditions (Figure 10A). By contrast, when HL-60 cells were treated with calcitriol for 5 days and then tested in the same way, 80% of the cells

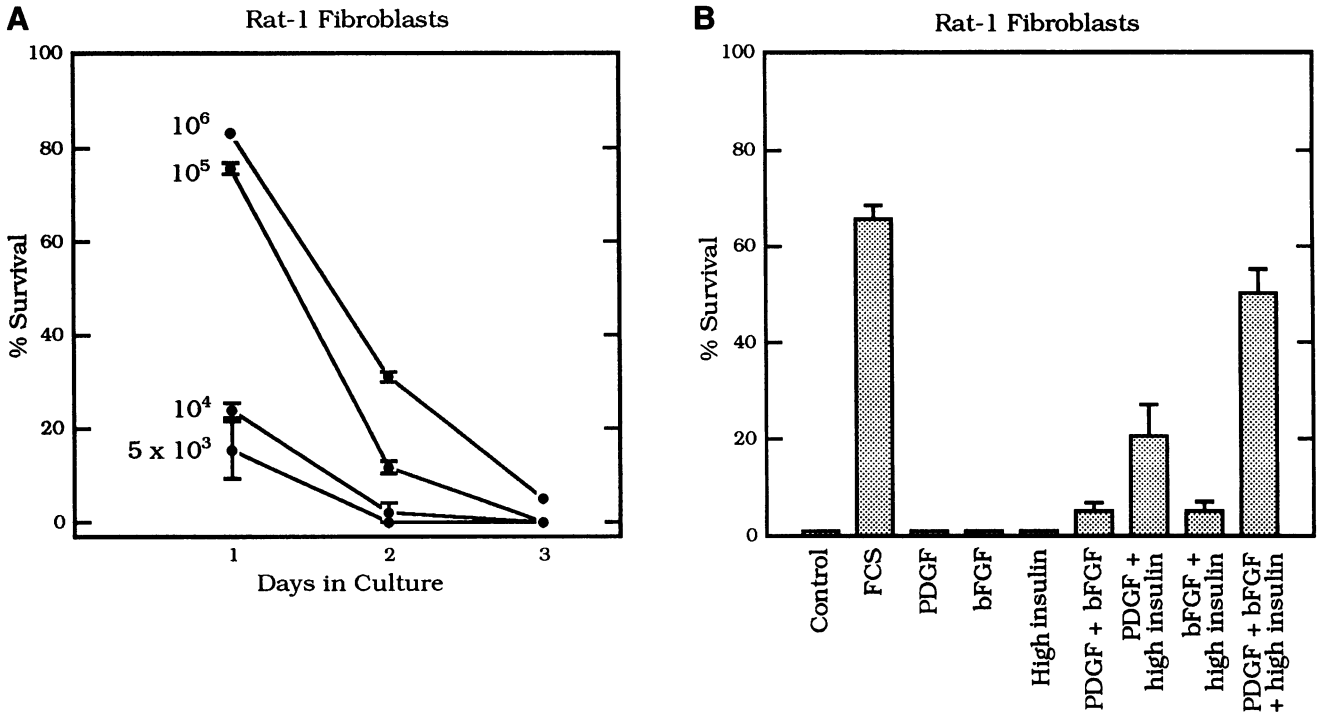

Figure 6. Survival of Rat-1 fibroblasts. The cells were cultured and assayed as in Figure 4. The initial cell viability was ≥ 85%.

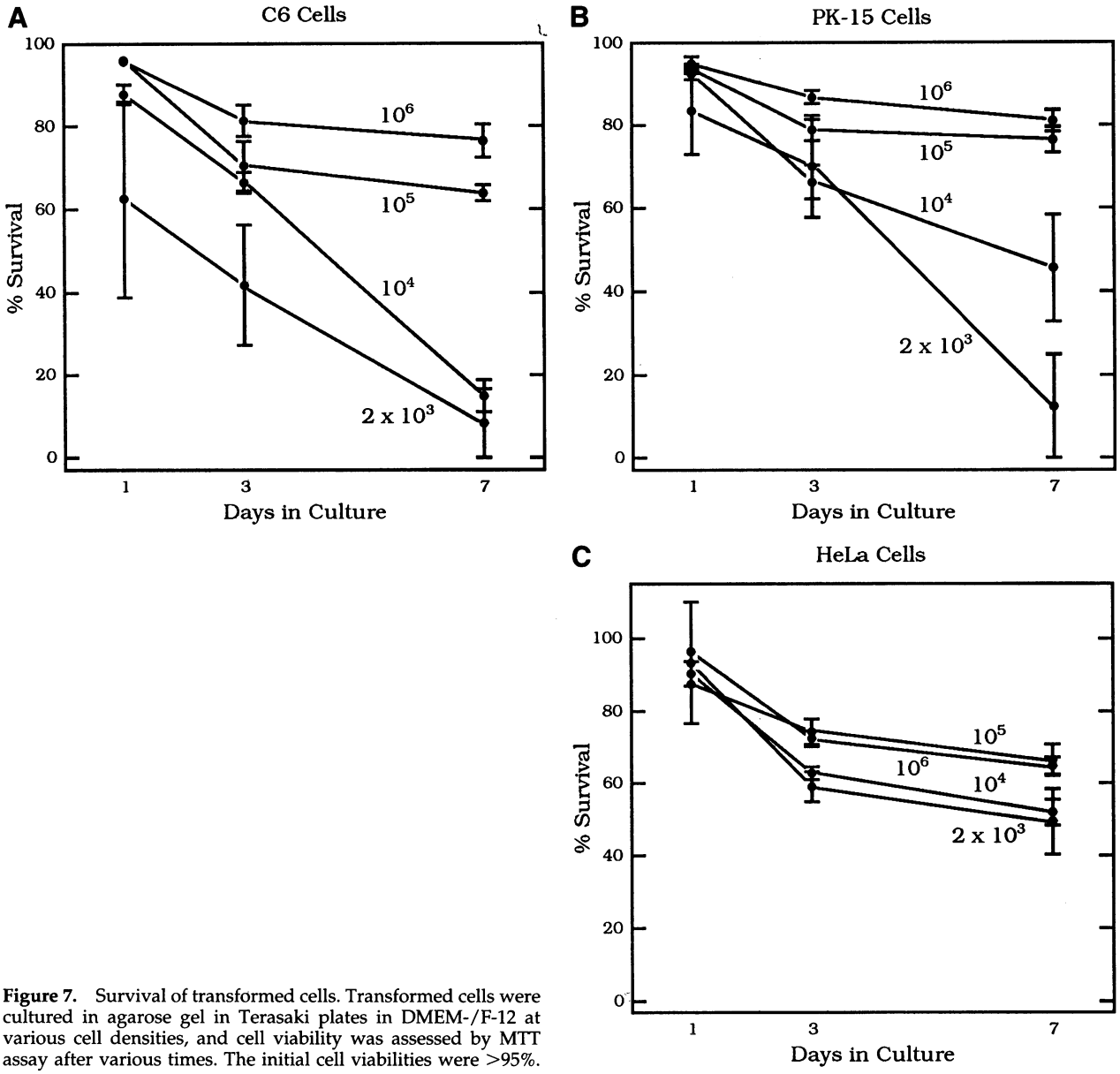

Figure 7. Survival of transformed cells. Transformed cells were cultured in agarose gel in Terasaki plates in DMEM-/F-12 at various cell densities, and cell viability was assessed by MTT assay after various times. The initial cell viabilities were >95%.

survived for 4 days (Figure 10C). Thus the survival requirements of HL-60 cells change when they differentiate, and they change in different ways depending on the differentiation pathway they follow.

Staurosporine-induced PCD

We showed previously that, even in the presence of FCS, a variety of cell types are induced to undergo PCD when treated with μM concentrations of the broad-spectrum protein kinase inhibitor staurosporine for 12–24 h and that pretreatment of the cells with the protein synthesis inhibitor cycloheximide increased their sensitivity to staurosporine-induced PCD (Ishizaki *et al.*, 1993, 1994; Jacobson *et al.*, 1993, 1994). These findings suggest that many cell types constitutively express all of the protein components required to run the death program. We tested all of the normal and cancer cells discussed in this paper with staurosporine, in the presence or absence of cycloheximide, for 24 h. As expected, most purified sciatic nerve fibroblasts, Rat-1 cells, and wild-type MEFs died when treated with staurosporine in the presence of FCS, and even more did so when treated with both staurosporine and cycloheximide (Figure 11A); the cells died with the morphological characteristics of apoptosis (our unpublished observations). MEFs from p53-null

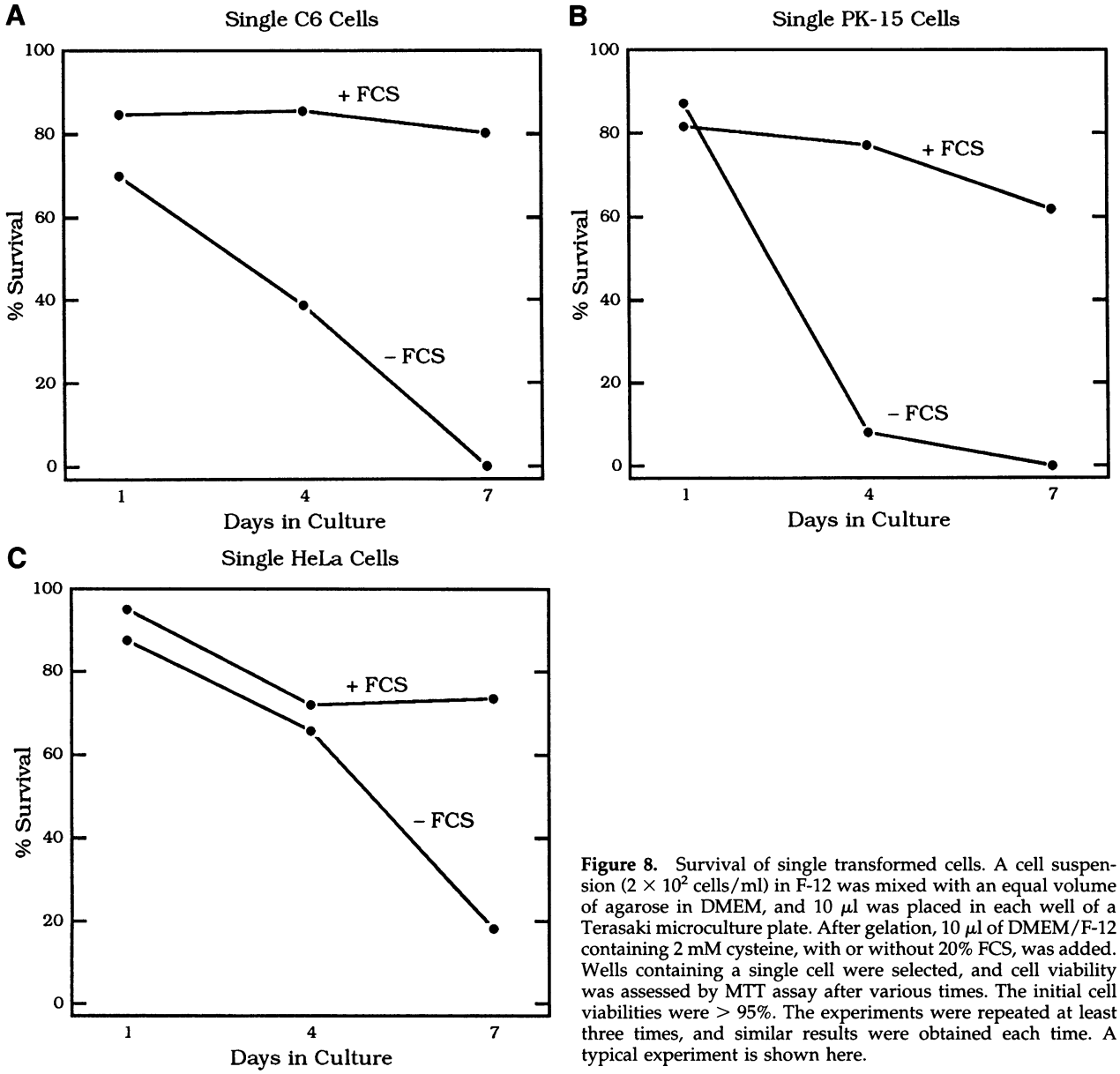

Figure 8. Survival of single transformed cells. A cell suspension (2×10^2 cells/ml) in F-12 was mixed with an equal volume of agarose in DMEM, and 10 μl was placed in each well of a Terasaki microculture plate. After gelation, 10 μl of DMEM/F-12 containing 2 mM cysteine, with or without 20% FCS, was added. Wells containing a single cell were selected, and cell viability was assessed by MTT assay after various times. The initial cell viabilities were > 95%. The experiments were repeated at least three times, and similar results were obtained each time. A typical experiment is shown here.

mice were also sensitive (Figure 11A), showing for the first time that staurosporine-induced PCD is p53 independent, at least in MEFs. We also tested unpassaged cells freshly isolated from E13 wild-type mice: in the presence of 1 μM staurosporine and 10 μg/ml cycloheximide, all of the cells underwent PCD within 1 day (our unpublished observations).

All mouse myeloma cells and HL-60 cells (both undifferentiated and differentiated) (our unpublished observations), as well as HeLa cells (Figure 11B), died when treated with either staurosporine alone or staurosporine plus cycloheximide for 1 day. Most C6 and PK-15 cells, by contrast, were resistant to such treatment, but all died in the presence of cycloheximide when treated with a 10 times higher concentration of staurosporine (10 μM) for 2 days in the absence of FCS (Figure 11B). In all cases the cells died with the characteristic features of apoptosis (our unpublished observations). Thus all five transformed cell lines can undergo PCD and constitutively express all the proteins required to run the death program.

DISCUSSION

It has been proposed that all nucleated mammalian cells constitutively express all the proteins required to undergo PCD (Raff *et al.*, 1993) and, except for blastomeres (Biggers *et al.*, 1971), may need continuous

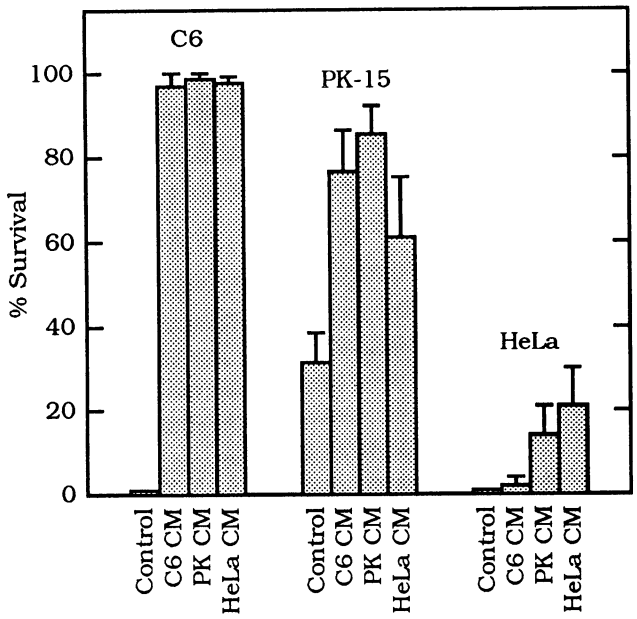

Figure 9. Effects of conditioned medium (CM) on transformed cells. The survival-promoting effects of neat CM from high-density (10^6 cells/ml) transformed cell cultures was tested on low-density (10^3 cells/ml) transformed cell cultures. The medium was removed and replaced by fresh conditioned medium every day. MTT assays were performed after 7 days. Initial cell viabilities were > 95%.

signaling from other cells to keep the death program suppressed (Raff, 1992). This death by default strategy may have evolved, in part at least, to help control cell numbers and to ensure that cells survive only when and where they are needed (Raff, 1992; Raff *et al.*, 1993).

Most of the evidence suggesting a death by default mechanism has come from in vitro experiments where cells deprived of signals from their normal neighbors and from endocrine cells die with the characteristic morphology of apoptosis (reviewed in Raff, 1992; Raff *et al.*, 1993). Some evidence, however, has also come from in vivo experiments, where it has been shown that treatment with specific survival signals can prevent some normal cell deaths that occur either during development (Levi-Montalcini and Booker, 1960; Hamburger *et al.*, 1981; Hofer and Barde, 1988; Oppenheim *et al.*, 1988; Barres *et al.*, 1992; Coles *et al.*, 1993) or in tissue homeostasis (Wyllie *et al.*, 1973, 1980) and that depletion of these signals can increase these cell deaths (Cohen, 1960; Levi-Montalcini and Booker, 1960; Klingman and Klingman, 1967; Wyllie *et al.*, 1973, 1980; Barbacid, 1995). The findings that high concentrations of staurosporine, which would be expected to inhibit many of the intracellular signaling pathways activated by extracellular survival signals, induce PCD in a number of cell lines and developing cell types in culture (Ishizaki *et al.*, 1993, 1994; Jacobson *et al.*, 1993; Bertrand *et al.*, 1994) are also consistent with a death by default mechanism. The findings that staurosporine induces PCD even in cells in which protein synthesis has been inhibited by cycloheximide treatment suggest that the proteins required to execute the death program are constitutively expressed (Raff *et al.*, 1993). The present findings provide further support for the generality of both the death by default mechanism and the constitutive expression of the cell death machinery and suggest that they apply to most immortalized and transformed cells, as well as to normal cells in developing and adult tissues.

We show that fibroblasts purified from newborn as well as adult sciatic nerve die by PCD when cultured at either low or high cell density in the absence of serum and exogenous signaling molecules but survive, even at low density, in the presence of FCS or PDGF. They also undergo PCD if cultured in FCS in the presence of 1 μM staurosporine, even when protein synthesis is inhibited by treatment with cycloheximide. It seems likely, therefore, that fibroblasts in both developing and mature peripheral nerves constitutively express all of the protein components required to undergo PCD and require continuous signaling from other types of cells to keep their intrinsic death program suppressed. Interestingly, their main neighbor in nerve–the Schwann cell–seems unable to promote their survival in vitro: even in mixed cultures of fibroblasts and Schwann cells, the fibroblasts rapidly die in the absence of serum or exogenous signals. It is possible, therefore, that the fibroblasts in peripheral nerves depend on survival signals from axons or endocrine cells, or on survival signals that Schwann cells and/or the fibroblasts themselves produce only when signaled to do so by axons or endocrine cells.

We also show that when E13 MEFs are cultured as dissociated cells in agarose gels (where they cannot contact their neighbors) in the absence of serum and exogenous signaling molecules, even at high cell density, they undergo PCD. In this case, bFGF or FCS, but not PDGF, promotes their survival. When the cells are cultured on PDL-coated plastic at high density in the absence of serum or exogenous signals, however, many cells undergo PCD, although some form a confluent monolayer and survive for weeks. Moreover, if the cells are cultured in agarose gel after some of them have been allowed to reaggregate into clusters, the cells in clusters survive in the absence of serum and exogenous signals, but the cells in the same culture that are not in clusters undergo PCD. As the cultures are prepared from whole E13 embryos from which only the head and liver are removed, it seems likely that they contain a large variety of cell types. These findings therefore suggest that most nucleated cells in the E13 embryo require signals from other cells to

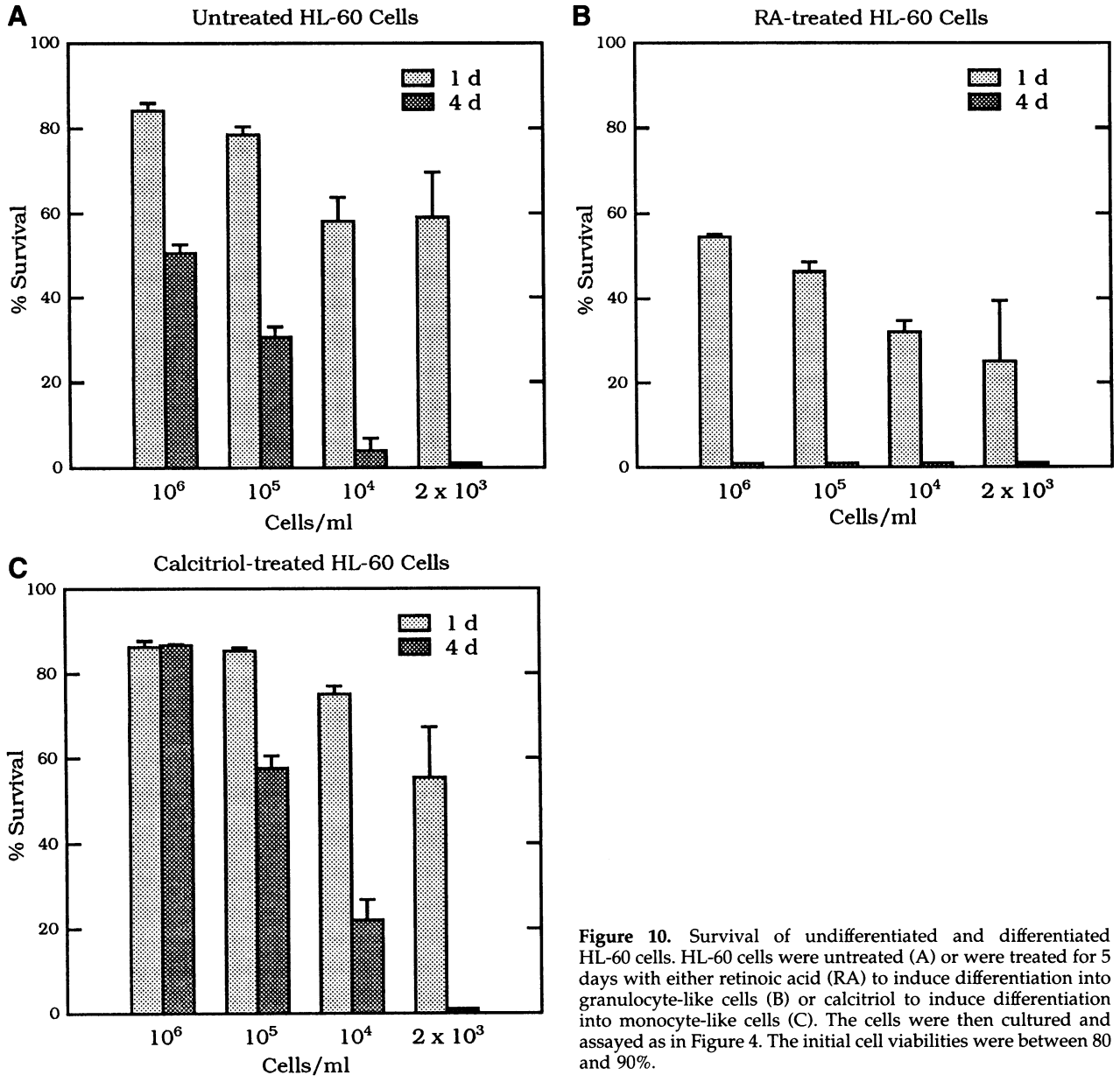

Figure 10. Survival of undifferentiated and differentiated HL-60 cells. HL-60 cells were untreated (A) or were treated for 5 days with either retinoic acid (RA) to induce differentiation into granulocyte-like cells (B) or calcitriol to induce differentiation into monocyte-like cells (C). The cells were then cultured and assayed as in Figure 4. The initial cell viabilities were between 80 and 90%.

avoid PCD and at least some of these signals can act only over a very short distance. As all of the nucleated cells dissociated from these embryos also rapidly undergo PCD when treated with cycloheximide and staurosporine in the presence of FCS, it seems likely that they constitutively express all the proteins required to run the death program.

Rat-1 cells, an immortal fibroblast cell line, behave similarly in agarose gel cultures to nerve fibroblasts and mouse embryo cells in that, even at high cell density, they undergo PCD when deprived of serum and exogenous signaling molecules. Thus immortalization does not in itself confer autonomy for cell survival. Interestingly, a combination of high insulin [presumably acting through the insulin-like growth factor 1 receptor (Sara and Hall, 1990; Harrington *et al.*, 1994)], PDGF, and bFGF promotes the survival of Rat-1 cells, whereas the individual growth factors on their own do not. Why do single growth factors promote the survival of nerve fibroblasts and embryo cells but not Rat-1 cells? One possibility is that, because nerve fibroblast and embryo cultures are more heterogeneous than Rat-1 cell cultures, endogenous paracrine signals, which are insufficient on their own to allow survival, may cooperate with the single growth factor to allow the survival of the normal cells

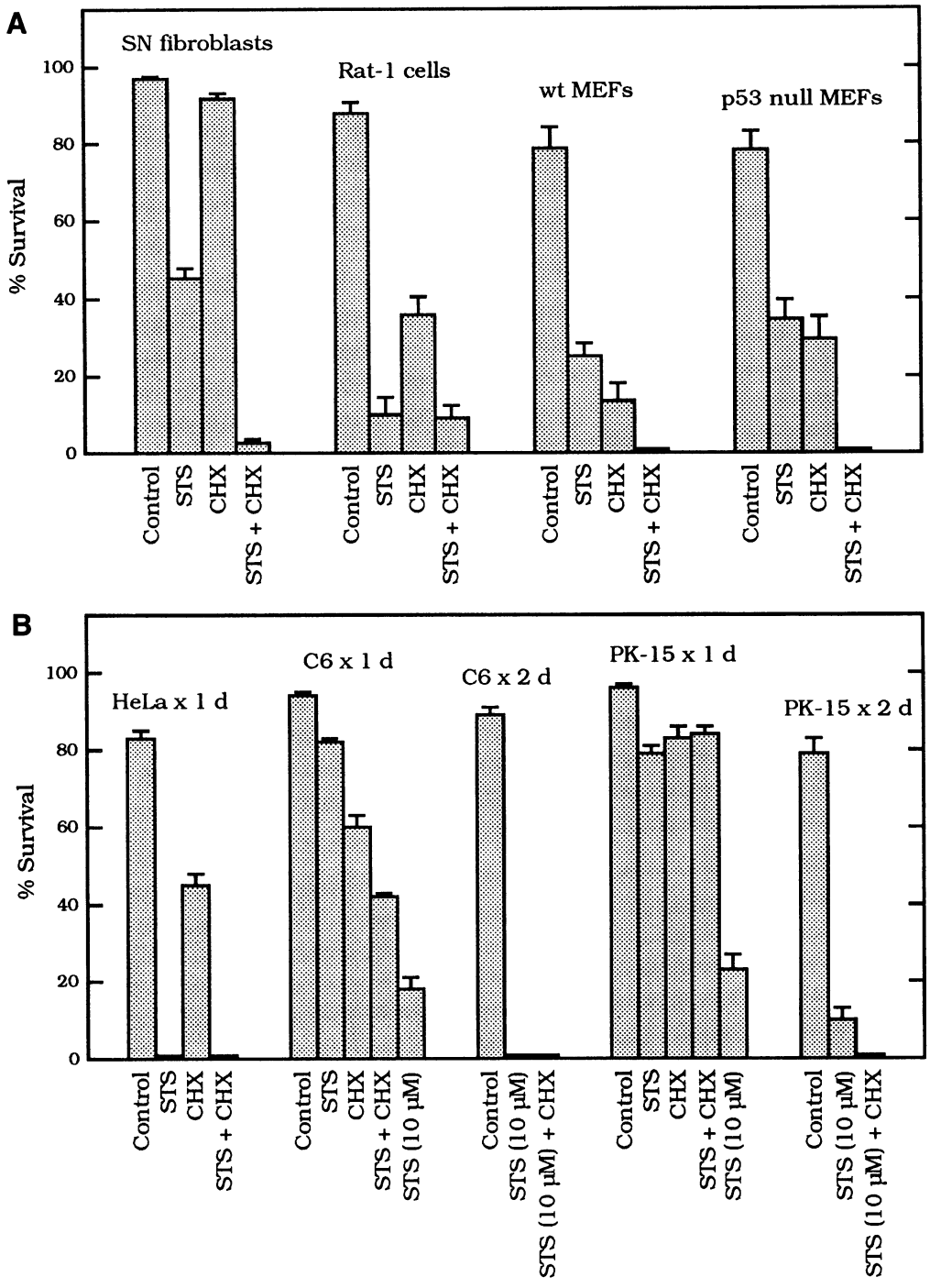

Figure 11. Effects of staurosporine (STS) plus cycloheximide (CHX) on cell survival. CHX was used at 10 μg/ml, and unless indicated otherwise, STS was used at 1 μM. Cells in panel A were cultured at 5000 cells/ml in 10% FCS, while the cells in panel B were cultured at 10,000 cells/ml without FCS. CHX was added for 1 h before the addition of STS. The initial cell viabilities were > 95%.

in culture. Some Rat-1 cells can survive for several weeks as small confluent clusters when cultured at very high cell density (10^6 cells/ml) on PDL-coated plastic in the absence of serum or exogenous signals, suggesting that they can support one another's survival as long as they are in contact, or at least very close together.

Cancer is thought to result from accumulated mutations that alter the properties of cancer cells so that the cells' survival, proliferation, and migration are less dependent on the extracellular controls that regulate the behaviors of normal cells (Varmus and Weinberg, 1993). Just as mutations that enhance the signaling pathways involved in cell proliferation

(anywhere from growth factors and their receptors to the nucleus and the cell-cycle apparatus) can promote cancer by enabling the mutant cells to proliferate independently of normal extracellular controls (Bishop, 1991), so one might expect mutations that enhance the signaling pathways involved in cell survival (anywhere from extracellular survival signals and their receptors to the intracellular death program) to promote cancer by enabling the mutant cells to survive independently of normal extracellular controls. As each individual cancer has its own unique set of mutations, one would expect different cancer cell lines to vary in their survival requirements. It is not surprising, therefore, that some transformed cells, like the mouse myeloma cells we tested, cannot survive in agarose gel culture, even at high cell density, in the absence of exogenous signals and serum, whereas others, such as C6 rat glioma cells, HeLa human cervical carcinoma cells, HL-60 human promyelocytic leukemia cells, and transformed PK-15 porcine kidney epithelial cells, can survive for weeks on their own at high cell density in these conditions. Similarly, it is not unexpected that some transformed cells, such as C6 and PK-15 cells, secrete signals that promote their own survival, just as some transformed cells have been shown to secrete growth factors that stimulate their own proliferation. It is more surprising, however, that none of the cancer cells we have studied seem able to survive for very long at low cell density in the absence of serum and exogenous signaling molecules. Apparently, most cancer cells do not have constitutively activated intracellular signaling pathways that enable the cells to survive in the absence of extracellular signals. This finding provides hope that treatments that deprive cancer cells of their particular survival signals could prove to be useful in many types of cancer, rather than just in those few, such as breast and prostate cancers, that are hormone dependent.

All of the transformed cells we tested are induced to undergo PCD when treated with staurosporine and cycloheximide, although a higher concentration of staurosporine and a longer period of treatment than usual is required to induce PCD in C6 and PK-15 cells. Thus even cancer cells seem to express constitutively all of the proteins required to undergo PCD. Why do cancer cells not acquire mutations that inactivate their death program? Although inactivation of this ultimate checkpoint would give them a selective survival advantage, to our knowledge, no one has reported a cancer cell that is unable to undergo PCD. Perhaps too few cancers have been examined to pick up the rare ones that have inactivated their death program. Or perhaps there is sufficient redundancy in the protein components of the death machinery that total inactivation of the death program is highly unlikely. Or perhaps the components are also used for some other essential function in cells, such as cell cycle progression (Ucker, 1991; Rubin *et al.*, 1993; Shi *et al.*, 1994).

Although the death program is present in many cancer cells, its intracellular regulation is often abnormal. Fifty percent of human cancers, for example, have inactivating mutations in both copies of the p53 gene (Hollstein *et al.*, 1991; Vogelstein and Kinzler, 1992) and are therefore less sensitive to DNA damage–induced PCD (Lowe *et al.*, 1993; Zambetti and Levine, 1993; Lane *et al.*, 1994). Moreover, some cancer cells overexpress the *bcl-2* gene (Cleary and Sklar, 1985; Tsujimoto *et al.*, 1985) (and some may overexpress one or more *bcl-2*-related genes) and are therefore less susceptible to various PCD-inducing stimuli (Reed, 1994). This could be one reason why C6 and PK-15 cells are less sensitive than most normal cells to staurosporine-induced PCD.

We found that the survival requirements of HL-60 cells change when the cells are induced to differentiate. They survive less well without exogenous signals when they are induced to differentiate into granulocyte-like cells by treatment with retinoic acid or DMSO but survive better in these conditions when induced to differentiate into monocyte-like cells by treatment with calcitriol. This finding is consistent with previous reports on normal cells that indicate that cells change their survival requirements as they develop (Barres *et al.*, 1992; Davies, 1994). Solary *et al.* (1993) found that HL-60 cells induced to differentiate into monocyte-like cells by phorbol esters are less sensitive to PCD induced by topoisomerase inhibitors.

The present study adds significantly to the growing list of mammalian cells that have been shown to constitutively produce all the protein components required to execute the death program and to undergo PCD in culture when deprived of extracellular signals from other cells. It remains to be demonstrated that there are any nucleated mammalian cells that do not constitutively express these protein components and, besides blastomeres (Biggers *et al.*, 1971), do not depend on extracellular signals for their survival.

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