Serum deprivation induces apoptotic cell death in a subset of Balb/c 3T3 fibroblasts

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INTRODUCTION

At least two distinct modes of cell death have been recognized in vivo: apoptosis and necrosis. Apoptosis, a mode of physiologic cell death in which single cells are deleted in the midst of otherwise healthy tissue (Wyllie, 1992), has a complementary but opposite role to mitosis in the regulation of animal cell populations (Kerr et al., 1972, 1987). As apoptosis is a rare, asynchronous event (Williams et al., 1992), study of its initiation and regulatory mechanisms is difficult in vivo. Withdrawal of stimulatory growth factors induces apoptotic cell death in several cell types in vitro (Araki et al., 1990; Galli and Fratelli, 1993) and is especially effective in those cells in which growth factors are an obligate requirement for survival and proliferation. Indeed many growth factors play dual roles as mitogens and survival factors (Holley and Kiernan, 1971; Macpherson and Montagnier, 1964). In quiescent cultures, growth factors maintain cell viability even at very low concentrations (Pardee, 1989). After complete withdrawal of growth factors, some cell lines exhibit features that are char-
acteric of physiologic cell death in vivo. For example, withdrawal of acidic and basic fibroblast growth factors induces cell death in Balb/c 3T3 fibroblasts (Tamm et al., 1991). Based on the failure to generate oligonucleosomal length fragmentation of nuclear DNA they and others (Collins et al., 1992; Cohen et al., 1992) have concluded that cell death is not necessarily accompanied by DNA fragmentation and was not apoptotic in nature. However, as no ultrastructural evidence was provided, the exact mode of cell death in the fibroblast model system remains unclear. Several workers have emphasized the importance of morphology in the definition of apoptosis in other cell systems (Collins et al., 1992; Cohen et al., 1992) but definitive morphological evidence in the Balb/c 3T3 model is lacking.

We have characterized ultrastructural, cytological and several metabolic changes in quiescent cultures of Balb/c 3T3 fibroblasts after serum withdrawal. These data indicate that only a subset of cells indeed undergo apoptotic cell death and without internucleosomal degradation of DNA.

MATERIALS AND METHODS

Balb/c 3T3 fibroblasts (ATCC CCL 163 Balb/c 3T3 clone A31) were seeded at ~400 cells/cm² in 60 mm² tissue culture dishes (Becton Dickinson Labware, Lincoln Park, NJ) and grown in Dulbecco’s modified essential medium (DMEM; Gibco Laboratories, Grand Island, NY) supplemented with 15% fetal calf serum and antibiotics in a 95% air and 5% CO2 atmosphere at 37°C in a humidified incubator. Cells were allowed to grow to density-inhibited monolayer, cultures were videotaped for up to 48 hours with one second time intervals between frames, using a Nikon Diaphot-TMD inverted phase-contrast microscope (Nikon, Tokyo, Japan) immediately after serum withdrawal. Serum- and CO₂-free DMEM (Gibco Laboratories, Grand Island, NY) was used for videomicroscopy. Temperature was maintained at 37°C throughout the video recording.

Video microscopy

To study morphological changes, detachment and kinetics of detachment, cultures were videotaped for up to 48 hours with one second time intervals between frames, using a x40 objective on a Nikon Diaphot-TMD inverted phase-contrast microscope (Nikon, Tokyo, Japan) immediately after serum withdrawal. Serum- and CO₂-free DMEM (Gibco Laboratories, Grand Island, NY) was used for videomicroscopy. Temperature was maintained at 37°C throughout the video recording.

Electron microscopy

Transmission electron microscopy (TEM) was performed to provide ultrastructural evidence about the nature of cell death. Control cells collected by trypsinization and non-adherent cells from 3 hour serum-deprived cultures were fixed with 2.5% formaldehyde and 1% glutaraldehyde, post-fixed with osmium tetroxide, dehydrated in an ethanol series and embedded in epoxy resin. Thin sections (7 nm) were stained with uranyl acetate and lead citrate and examined under a Hitachi H-7000 electron microscope.

Scanning electron microscopy (SEM) provided comparisons of overall shape and surface characteristics of control and serum-deprived fibroblasts. Control and serum-deprived cells were fixed in 3.7% formaldehyde in PBS overnight at 4°C, washed 3x in PBS, dehydrated in graded alcohols, critical-point dried, and examined by SEM.

Confocal microscopy

To obtain information on nuclear structure and filamentous actin content, control and experimental cells grown on coverslip chamber slides (Nunc, Roskilde, Denmark) were fixed with 3.7% formaldehyde in PBS plus 0.01% Triton X-100 for 10 minutes, stained with propidium iodide and FITC-phalloidin, and examined using a ×63, 1.3 NA oil immersion objective under epifluorescent optics and confocal imaging (Leitz, Heidelberg, Germany).

DNA extraction and agarose gel electrophoresis

Gel electrophoresis of DNA from control and serum-deprived cells was performed to detect nuclear cleavage in dying cells. Approximately 1x10⁵ cells were collected by serum-supplemented cultures by trypsinization with 0.01% trypsin. A similar number of non-adherent cells from 3 and 24 hour serum-deprived cultures were collected by centrifugation at 300 g for 10 minutes, and then lysed in 1.5 ml of 1 mM Tris, 1 mM EDTA buffer containing 10% SDS, on ice. The lysates were then digested with 1 mg/ml protease K overnight at 37°C. DNA was extracted by a standard phenol-chloroform-isomyl alcohol extraction procedure (Ausubel et al., 1987). Electrophoresis of RNase-treated DNA was carried out on 1.7% agarose gels in 1 mM Tris-EDTA buffer (pH 8.0) and stained with ethidium bromide. As controls, an equivalent amount of DNA was digested for 5 minutes on ice with nuclease S7 (1 pg/μg DNA) (Boehringer Mannheim, Montreal, Quebec, Canada) and DNA fragments from HaeIII-digested φX174-RF DNA were used as molecular mass standards (Pharmacia, Montreal, Quebec, Canada). As positive controls for oligonucleosomal DNA degradation, DNA was extracted from HL-60 lymphocyte hybridoma cells (ATCC) that had been treated previously with 10 μM of the topoisomerase II inhibitor etoposide (Barry et al., 1993).

Flow cytometry

To assess membrane damage in populations of viable and non-viable cells over varying times of serum deprivation, single cell suspensions of adherent (viable) and non-adherent (apoptotic/non-viable) cells were stained with fluorescein diacetate (FDA) and propidium iodide (PI) and analyzed on a FACSTAR Plus flow cytometer (Becton Dickinson Immunocytocchemistry Systems, Mountainview, CA; Jones and Senft, 1985). A 570 nm beam splitter was used for two-color fluorescence analysis. A 625/35 nm filter was used to collect red fluorescence (PI) and a 530/30 nm filter was used for green fluorescence (fluorescein). Relative fluorescence intensities were compared over 0, 1, 2 and 3 hour periods.

Cell volume changes

As reduction in cell size is recognized as one of the classical features of apoptotic cell death (Wyllie, 1992), mean cellular volumes of apoptotic (non-adherent) and normal (adherent) cell suspensions were measured electronically over 30 minutes, 1, 2, 3 and 4 hours using a Coulter Channelizer (Coulter Electronics, Hialeah, FL). Adherent cells collected by trypsinization and non-adherent cells collected at the indicated time periods were analyzed immediately after suspension in an isotonic buffer (Isoton II; Coulter Electronics, Burlington, ON, Canada).

Cell survival assay

To determine whether cells that lost attachment upon serum withdrawal also lost their viability, cells were transferred to serum-supplemented medium after 0.5, 1, 2, 3 and 4 hours of serum deprivation. The ratio of non-adherent to adherent cells of serum-
supplemented cultures provided an estimate of the proportion of cells that lost viability and the time frame over which these changes occurred.

**Calcium measurements**
The availability of intracellular calcium ions has been implicated as an important determinant of DNA digestion mediated by endogenous endonucleases in apoptosis (Cohen and Duke, 1984). To study the role of intracellular Ca\(^{2+}\) in early cellular responses to serum withdrawal, intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(i\)) in single cells at baseline and immediately following serum withdrawal was estimated in cells loaded with 2 \(\mu\)M fura 2/AM (Molecular Probes, Eugene, OR). Cells were measured for 15 minutes in DMEM using a \(\times40\), 1.3 NA oil immersion objective on a Nikon Diaphot epifluorescent microscope. The cells were excited at 345/380 nm wavelengths alternating at 100 Hz. Fluorescence emission at 510 nm was analyzed with a PTI microspectrofluorimetry system (PTI, Photon Technology International, London, ON, Canada). Ratiometric calcium measurements were converted to molar concentrations using previously described methods (Grynkiewicz et al., 1985). The same cell was followed for 15 minutes after replacement of the serum-supplemented growth medium by serum-free growth medium.

**Protein synthesis inhibition**
The requirement for de novo protein synthesis was assessed by serum withdrawal from cultures that were treated with cycloheximide (final concentration = 10 \(\mu\)g/ml). Experiments were conducted in which cycloheximide was added prior to serum withdrawal or prior to and after serum withdrawal.

**RESULTS**

**Phase-contrast microscopy**
Adherent fibroblasts with a typical stellate morphology (Fig. 1A) manifested dramatic shrinkage in size within minutes of serum withdrawal (Fig. 1B). The cells rounded up, lost attachment to the substrate and floated to the top of the liquid medium. While some of these latter cells reattached momentarily once or twice over 24 hours, increasing numbers remained floating in the medium (Fig. 1C). The momentary reattachment, rounding and reattachment of cells in serum-free medium bore striking resemblance to premitotic cells in serum-supplemented conditions except that cells in serum-free conditions never completed mitosis, remained rounded and ultimately floated in the medium. In serum-free medium several cells were seen to form strictures in the middle and exhibited the appearance of cells undergoing cytokinesis, however actual division was not seen and these bilobed structures remained floating for several hours. These changes (which we liken to aborted mitoses) were detected between 5 and 7 hours after serum withdrawal. In serum-supplemented cultures, the first detectable cell divisions occurred between 4 and 6 hours.

In non-confluent cultures of similar age obtained by low initial plating densities, there was no morphological evidence for the type of cell death observed in contact-inhibited cultures after serum-withdrawal (Fig. 2). The cells exhibited a more distinct cytoplasmic structure than cells in serum-supplemented cultures.

**Electron microscopy**
In TEM, serum-deprived fibroblasts exhibited nuclear morphology classically described for apoptotic cells (Fig. 3). The nuclear chromatin in serum-deprived cells was compacted along the nuclear periphery in contrast to controls in which the chromatin was dispersed throughout the nucleus. The nuclear
membrane in apoptotic cells appeared intact with patent nuclear pores especially in regions where the compacted chromatin was absent. In a number of cells the nuclei exhibited dilatations (Fig. 3) or complete fragmentation with compacted chromatin within the fragments. Organelles such as mitochondria appeared morphologically normal with intact cristae. There was a conspicuous absence of large vacuoles or swollen organelles in both the control and serum-deprived cells. While the majority of the cells (approx. 60%) in 3 hour serum-deprived samples demonstrated these features, typical of apoptosis, the remaining cells exhibited a spectrum of features, which ranged from near normal morphology to frankly necrotic or lysed cells. These data indicate that only a subset of the cells in these cultures underwent apoptosis.

Scanning electron micrographs of control cells in serum-containing medium exhibited typical stellate morphologies with wide lamellopodiae (Fig. 4A). In contrast, the 3 hour serum-deprived cells were rounded, and demonstrated marked surface blebbing and possible apoptotic body formation (Fig. 4B). Consistent with TEM observations, some of the cells retained normal stellate shapes.

Prevention of ab initio substrate attachment by plating of cells on microbiological plastic failed to induce apoptotic ultrastructural changes as those seen in serum withdrawn adherent cultures (Fig. 5). Cells that were plated on non-adherent plastic exhibited numerous large vacuoles and loss of cytoplasmic structure, features suggestive of necrosis.

Fluorescence microscopy
Apoptotic nuclei were stained by propidium iodide or DAPI more intensely than normal cells and the nuclei were shrunk (Fig. 6C and A, respectively). In contrast with normal cells (Fig. 6B), the filamentous actin of apoptotic cells was either poorly defined, or was collapsed around the nucleus, or formed blebs around the nucleus (Fig. 6D). In optical sections obtained by confocal microscopy, there were approximately two-fold reductions in the cross-sectional nuclear area and the overall cell area in serum-deprived cells compared to control cells.

Fig. 2. Five hour serum-deprived 5-day old non-confluent cultures showing absence of rounding and loss of attachment. Note the distinct cytoplasmic structure in these cells (arrowheads). ×300.

Fig. 3. (A) Transmission electron photomicrograph of control fibroblasts showing normal nuclear morphology and organelle ultrastructure. ×3705. (B) Electron photomicrograph of fibroblasts after 3 hours in serum-free DMEM showing compacted, marginated nuclear chromatin and normal membrane and organelle ultrastructure. ×4235. (C) Higher magnification (×5294) of nucleus in B showing intact nuclear membrane and cell organelles.
DNA agarose gel electrophoresis

There was no evidence of internucleosomal DNA degradation in 3 hour serum-deprived cultures (Fig. 7) or at 24 hours (data not shown). Serum-deprived cells exhibited intact high molecular mass DNA that was similar to DNA from control cells. Brief exposures of DNA to micrococcal nuclease S7 readily degraded the DNA to oligonucleosomal length fragments (Fig. 7, lanes 2 and 3). DNA from control and etoposide-treated HL-60 cells (Fig. 7, lanes 5 and 6, respectively) showed, respectively, intact DNA or the typical oligonucleosomal ladder associated with apoptosis. Further, application of the in situ terminal transferase assay for detection of endonuclease activity in single cells (Gavrieli et al., 1992) failed to demonstrate staining in cytospin preparations of apoptotic cells (data not shown). These data indicate that the absence of detectable oligonucleosomal length fragmentation in fibroblasts was not due to insensitivity of the assays.

Flow cytometry

Cells from serum-deprived cultures formed two distinct populations (R1 and R2; Fig. 8) based on forward and orthogonal light scatter. Cells gated in the R1 region were relatively large, viable cells with high fluorescence intensities due to fluorescein diacetate (FDA) and low fluorescence due to propidium iodide (PI). Cells in the R2 region were relatively small in size, exhibited high PI and low FDA fluorescence intensities and represented the putative dead cell population. Over the first 3 hours of serum deprivation, an increasing proportion of the serum-deprived cells in the R2 region showed elevated PI and reduced FDA uptake, findings that were suggestive of loss of viability. Cells gated in the R1 region, showed only minimal changes over the same period, indicating that the majority of cells in this population maintained cell membrane integrity. To test the hypothesis that cells gated in the R2 region were apoptotic, we used flow cytometry to sort FDA and PI-stained populations of serum-deprived cells and imaged them on a confocal microscope. The mean cross-sectional areas of cells sorted into the R1 and R2 regions were 1072.7 \( \mu m^2 \) and 191.0 \( \mu m^2 \), respectively, indicating that cell size and intensity of PI staining of the R2 population were consistent with those of apoptotic cells.

Cell volume changes

Over the first four hours of serum deprivation there was a reduction in the mean and modal diameter of cells in suspen-
sion (Fig. 9; at 0 hours, mean diameter = 17.95 μm; at 4 hours, mean diameter = 15.46). There was also a concurrent increase in the number of particles less than 4 μm in diameter, possibly representing an increase in the formation of apoptotic bodies.

**Cell survival assay**

There was a linear increase of approximately 1×10³ cells/hours in the number of cells that lost attachment after serum withdrawal (Fig. 10). To determine whether cells that detached from the culture dish after serum deprivation were really non-viable, the numbers of cells that reattached after plating in serum-supplemented medium were converted into a ratio of the number of non-viable to viable cells. The ratio of non-viable to viable cells increased linearly over time indicating that increasing durations of serum-deprivation were associated with increasing loss in cell viability (Fig. 10). Notably, floating cells in serum-containing, density-inhibited, 5/6 day old cultures were virtually all dead (time 0, Fig. 10B).

**Calcium fluxes**

Intracellular calcium ion concentration was initially ~110 nM but within 5 minutes of serum deprivation there was a reduction in [Ca²⁺]; from 110 nM to 75 nM in those cells that exhibited the typical morphological changes of rounding (Fig. 11). These calcium changes were not observed in cells that failed to demonstrate the morphological changes typical of apoptosis.

**Protein synthesis inhibition**

Cycloheximide treatment either before or during serum withdrawal failed to inhibit the morphological (Fig. 12) or cytoskeletal changes observed in serum-deprived cultures without cycloheximide treatment.

**c-myc expression**

As high levels of c-myc expression have been associated with entry into the apoptotic pathway in fibroblasts, we stained cells with monoclonal antisera to mouse c-myc (Santa Cruz Biotechnologies, Santa Cruz, CA) and found that both cells exposed to fresh serum and serum-deprived cells expressed high levels of the protein (Fig. 13).

**DISCUSSION**

Historically (Kerr et al., 1972; Kerr and Harmon, 1991) and up to the present (Alles et al., 1991; Vaux, 1993), the concept of apoptosis has relied on morphological distinctions between necrosis and apoptosis. The serum-deprived cells in this study demonstrated several characteristic morphological features of apoptotic cell death (Bowen and Bowen, 1990) including rounding, loss of cell-to-cell contact, cellular condensation, compaction of nuclear chromatin along the nuclear periphery, nuclear fragmentation, preservation of membrane integrity and organelle structure. Consistent with the findings of earlier work with the same cell line (Tamm et al., 1991) and in other fibroblast cell lines (Ucker et al., 1992), we did not detect DNA fragmentation. The inclusion of positive controls and the use of 2 different types of assays suggest that the failure of detection was not simply due to inadequate technique.

Although DNA fragmentation has been regarded as a hallmark of apoptosis, there is some consensus on the dispensability of this characteristic in certain cell types under defined conditions (Cohen et al., 1992; Collins et al., 1992). Conceivably, the regulation and kinetics of DNA degradation in the fibroblast model of apoptosis described here may be different from that of thymocyte (Cohen and Duke, 1984; Yonish-Rouach et al., 1991) or lymphocyte (Colotta et al., 1992; Wyllie, 1987) models. Alternatively, the observed reduction of intracellular calcium ion concentration after serum deprivation could explain the failure of activation of endogenous, calcium-dependent endonucleases (Cohen and Duke, 1984; Gaido and Cidlowski, 1991; Compton, 1991). Several findings are consistent with an active extrusion of calcium including first, the ultrastructural integrity of cellular membranes; second, the survival of cells during the early period following serum-withdrawal; and third, the reduction of intracellular calcium measured by ratio fluorimetry. What
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purpose calcium depletion serves or what mediates blockade of calcium flux from the extracellular environment is unknown but it does suggest a mechanism by which DNA degradation could be regulated in apoptosis.

Several different assays have been used to quantify cell death including trypan blue exclusion (McConkey et al., 1989; Roy et al., 1992; Kruman et al., 1992), neutral red uptake (Dijmans and Billiau, 1991), propidium iodide uptake (Barbieri et al., 1992), release of radiolabeled protein or DNA (Ojcius et al., 1991; Ucker et al., 1992; Zychlinsky et al., 1992) and release of lactate dehydrogenase (Geier et al., 1992).

Assays using non-vital dye uptake detect loss of cell membrane integrity, which is characteristic of necrotic or late apoptotic cells and thus may fail to detect early apoptotic cells or to differentiate between apoptosis and necrosis. When used alone,
survival assays can be criticized since the outcome of viability or non-viability relies on the measurement of a single parameter. In this study we have used a battery of tests to assess apoptosis. The concordance of these data lends strong support to the notion that not all apparently dead cells are in fact dead. Indeed, the survival assay we have used is considered the most rigorous assay (Cook and Mitchel, 1989) and minimizes under

Fig. 8. Plots of forward scatter (y-axis) against propidium iodide (x-axis) fluorescence intensity in control cultures and 1, 2 and 3 hours after serum withdrawal. Note the shift of cells into the electronically gated R2 region over 3 hours, indicative of cell death.

Fig. 9. Cellular diameter distributions of suspended cells in control (0 hours) and 1, 2, 3 and 4 hours after serum deprivation. Note the reduction of mean cell channel number (diameter) from 0 to 4 hours (arrows).

Fig. 10. (A) Number of cells that, over time, lost attachment after serum withdrawal. (B) Ratio of number of non-viable to viable cells. Cells that lost attachment (A) after indicated periods in serum-free conditions were returned to serum-supplemented conditions. The cells that reattached (A) after indicated periods in serum-free conditions were considered viable. Those that remained non-adherent at the end of 24 hours were counted as non-viable. Note the linear increase in the ratio of non-viable to viable cells over time. Time zero represents floating cells from a 5-day-old density-inhibited culture containing serum. Note that nearly all of the floating cells in these cultures are non-viable.

Fig. 11. Time plot of intracellular calcium ion concentration in a single cell after serum withdrawal. Note the approximately 35 nM reduction in [Ca^{2+}], in the first 300 seconds after serum withdrawal. A steady baseline was established before serum withdrawal (arrow).
or overestimation of cell death. The survival assay data were consistent with TEM findings and clearly showed that serum deprivation in quiescent Balb/c 3T3 fibroblasts yielded a mixed population of apoptotic and potentially apoptotic but still viable cells. Thus apoptosis was induced in only a subset of the cells at a given time but we are unable presently to determine which characteristics might mark specific cells for deletion by apoptosis.

Apoptosis likely requires active participation of the cell (Williams et al., 1992). As a corollary to this contention, de novo protein synthesis is necessary for cell death to occur in some model systems (Wyllie, 1980). On the basis of the cycloheximide experiments, fibroblasts can apparently undergo apoptosis without protein synthesis and this blockade may actually enhance cell death. Indeed, a previous study of fibroblast apoptosis used cycloheximide to induce entry into the death pathway (Evan et al., 1992). It has been suggested that some cells are ‘primed’ to undergo apoptosis and that the effect of cycloheximide in depleting the cell of ‘blocking factors’ may trigger apoptosis (Ghibelli et al., 1992). In this context, deregulated c-myc expression and failure of growth arrest as a possible explanation for apoptosis in serum-deprived fibroblasts (Evan et al., 1992) is of considerable interest. The expression of c-myc in serum-deprived cells indicates that c-myc is expressed at least at the same level as serum-supplemented cells, indicating that this protooncogene is expressed in the absence of serum and points to other, non-protein synthesis-dependent mechanisms for induction of apoptosis.
Substrate attachment is a prerequisite for most of the metabolic processes in fibroblasts. Cells that were prevented from gaining substrate attachment at the outset did not exhibit morphological features suggestive of apoptosis, suggesting that at least some intact metabolic pathways are required for apoptosis in fibroblasts. Consistent with this concept, cells on the apoptotic pathway must remain viable for a finite period of time before losing the ability to remain attached to the substrate and proliferate. Indeed substrate attachment is an absolute requirement for mitosis in diploid fibroblasts (Macpherson and Montagnier, 1964). Conceivably, there might be sharing of a short, common pathway by pre-mitotic and ‘pre-apoptotic’ cells during which they continue to exhibit many signs of viability. Subsequently, pre-mitotic and pre-apoptotic cells may diverge on two distinct courses with diametrically opposite results, possibly due to an aborted entry into the cell cycle by the pre-apoptotic cells (Rubin et al., 1993). Notably, we observed attempted mitoses by cells in serum-deprived cultures prior to detachment and presumably, cell death.

Similar to the results of Tamm et al. (1991, 1992), the gross morphological changes upon serum withdrawal occurred rapidly, starting within minutes and continuing over several hours. Earlier studies using phase-contrast microscopy of cells in culture have shown that the condensation and budding to form apoptotic bodies were completed within several minutes (Russell et al., 1972; Sanderson, 1976; Mather, 1979) to a few hours. One of the consistent findings in our flow cytometric and cell size measurement data was the presence of two easily distinguishable populations. We speculate that the smaller cells might contain numerous apoptotic bodies. To test this hypothesis directly, we sorted FDA and PI stained populations of serum-deprived cells by flow cytometry and then examined these cells on a confocal microscope. The small size of the putative apoptotic population and the finding of dense, brightly staining nuclei support the validity of the flow cytometric method for detection of apoptotic cells and point to the formation of apoptotic bodies in this model system, a contention also supported by the finding of small particles with the Coulter Channelizer.

Another important characteristic of apoptotic cells in tissues is the loss of contact with neighboring cells (Kerr et al., 1972), possibly the result of loss of specialized surface elements such as microvilli and desmosomal cell-cell junctions. Consistent with these previous observations, our SEM data showed profound alterations in cell surface structure including blebbing. Although not directly comparable to the in vivo situation, withdrawal of serum from fibroblast cultures also caused the cells to lose cell-to-cell attachment although it is not known if this is an essential step prior to rounding and loss of attachment to the substrate.

The question of why serum deprivation produces apoptosis in confluent cultures remains to be answered. Clearly, the degree of confluence exerts a profound effect on the induction of apoptosis as low density cultures did not exhibit apoptotic cells. Conceivably, competition for autocrine, survival factors in high density cultures could be a determinant. This is of particular interest as fibroblasts produce autocrine factors (Paulsson et al., 1987), which can promote cell survival (Gospodarowicz et al., 1987). The fact that serum deprivation induces apoptosis and that exogenous serum is required for survival of Balb/c 3T3 fibroblasts is consistent with the untransformed nature of these cells (Wharton and Smyth, 1989) and suggests that production of autocrine factors may be at a low level in these cells. Further, and as suggested by recent data (Gustinich and Schneider, 1993), serum deprivation induces a serum deprivation gene in NIH3T3 cells that may provide a second block to passage through the cell cycle in addition to growth factor depletion. Conceivably, the existence of two blocks to cell cycle progression (e.g. growth factor deprivation plus protein synthesis inhibition or induction of specific genes) is an obligate requirement for induction of apoptosis in fibroblasts.

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REFERENCES


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