Death of Serum-free Mouse Embryo Cells Caused by Epidermal Growth Factor Deprivation

Cathleen L. Rawson, Deryk T. Loo, Julie R. Duimstra, Olaf R. Hedstrom, Edward E. Schmidt, and David W. Barnes

Department of Biochemistry and Biophysics, Environmental Health Sciences Center, Oregon State University, Corvallis, Oregon 97331-6503

Abstract. Serum-free mouse embryo (SFME) cells, derived in medium in which serum is replaced with growth factors and other supplements, are proastroblasts that are acutely dependent on epidermal growth factor (EGF) for survival. Ultrastructurally, an early change found in SFME cells deprived of EGF was a loss of polysomes which sedimentation analysis confirmed to be a shift from polysomes to monosomes. The ribosomal shift was not accompanied by decreased steady-state level of cytoplasmic actin mRNA examined as an indicator of cellular mRNA level. With time the cells became small and severely degenerate and exhibited nuclear morphology characteristic of apoptosis. Genomic DNA isolated from cultures undergoing EGF deprivation–dependent cell death exhibited a pattern of fragmentation resulting from endonuclease activation characteristic of cells undergoing apoptosis or programmed cell death. Flow cytometric analysis indicated that cultures in the absence of EGF contained almost exclusively G1-phase cells. Some of the phenomena associated with EGF deprivation of SFME cells are similar to those observed upon NGF deprivation of nerve cells in culture, suggesting that these neuroectodermal-derived cell types share common mechanisms of proliferative control involving peptide growth factor–dependent survival.

W E have described the long-term culture of mouse embryo cells derived and passaged under conditions in which the serum supplement to the basal nutrient medium is replaced by growth factors and other supplements: insulin, transferrin, EGF, high-density lipoprotein, and fibronectin (Loo et al., 1987, 1989a, b). These serum-free mouse embryo (SFME)[1] cells exhibit a number of unusual properties. Unlike mouse embryo cells derived in conventional, serum-supplemented media which undergo growth crisis and immortalization, SFME cells do not lose proliferative potential or develop gross chromosomal aberration when cultured for more than 10 times the number of population doublings that can be achieved with mouse embryo cells in conventional, serum-containing medium (Todaro and Green, 1963; Loo et al., 1987, 1989b; Ernst et al., 1990).

Proliferation of SFME cells is reversibly inhibited by serum (Loo et al., 1987, 1990; Rawson et al., 1991), and treatment of SFME cells with serum or transforming growth factor β leads to the appearance of glial fibrillary acidic protein (GFAP), an intermediate filament protein that is a specific marker for astrocytes (Sakai et al., 1990). Cells with properties like those of SFME cells also can be isolated directly from mouse brain (Sakai et al., 1990), and a modification of the SFME medium formulation allows the multipassage serum-free culture of human brain cells that express GFAP (Loo et al., 1991). These results suggest that SFME cells are proastroblasts.

SFME cells are acutely dependent on EGF for survival, and cycloheximide or actinomycin D prevents death caused by EGF deprivation (Rawson et al., 1990), suggesting that EGF-dependent survival may depend on suppression of synthesis of proteins that cause cell death, a phenomenon similar to that reported for neuronal "programmed cell death" in the absence of nerve growth factor (NGF) (Martin et al., 1988). Thus, multiple cell types of neuroectodermal origin may share common mechanisms of proliferative control involving peptide growth factor–dependent survival. SFME cell death in the absence of EGF also is delayed by orthovanadate, an inhibitor of phosphotyrosine phosphatases, and 12-O-tetradecanoylphorbol 13-acetate, an activator of protein kinase C (Rawson et al., 1990).

Greater than 90% of SFME cells are dead within 48 h after removal of EGF from the culture medium, and decreased cell survival is observed after EGF deprivation for as little as 8 h (Loo et al., 1987, 1989a). Individual omission of any of the other medium supplements leads to reduced SFME

---

1. *Abbreviations used in this paper:* FALS, forward angle light scatter; GFAP, glial fibrillary acidic protein; HDL, high density lipoproteins; MVB, multivesicular bodies; NGF, nerve growth factor; SFME, serum-free mouse embryo.

cell growth, but does not induce cell death (Loo et al., 1989a). NGF, platelet-derived growth factor, and transforming growth factor β will not substitute for EGF in this system, while fibroblast growth factor will allow survival and slow growth of SFME cells in the absence of EGF (Loo et al., 1989a). We have suggested that at least two alternative mechanisms for the control of cell proliferation may exist: one related to intrinsic limitation of proliferative potential observed in conventional cell cultures, and the other governed by growth factor–dependent survival observed in SFME cell cultures (Sakai et al., 1990).

We examined ultrastructural, biochemical, and physiological responses of SFME cells to EGF deprivation as a step toward defining the primary mechanisms leading to death of these cells. Early changes included a shift of ribosomes from a polysomal organization to monosomes. The ribosomal shift was not accompanied by decreased steady-state level of actin messenger RNA in the cytoplasm. With time the cells became small and degenerate, and pyknotic nuclei appeared. Flow cytometric analysis indicated that cultures in the absence of EGF contained almost exclusively a diploid amount of DNA (G1-phase). Genomic DNA isolated from cultures undergoing EGF deprivation–dependent cell death exhibited a pattern of fragmentation associated with programmed cell death in other systems (Arends et al., 1990).

Materials and Methods

Cell Culture

Detailed procedures for the initiation and continuous culture of BALB/c SFME cells have been published (Loo et al., 1989a, b). The basal nutrient medium was a one to one mixture of DME containing 4.5 g/l glucose and Ham's F12 (Mather and Sato, 1979; Ham and McKeehan, 1979) supplemented with 15 mM Hepes, pH 7.4, 1.2 g/l sodium bicarbonate, sodium selenite (10 nM), penicillin (200 U/ml), streptomycin (200 μg/ml) and ampicillin (25 μg/ml) (F12:DME). Stock cultures of SFME cells were cultured in F12:DME supplemented with insulin (10 μg/ml), transferrin (10 μg/ml), human high density lipoprotein (HDL) (10 μg/ml) and EGF (50 ng/ml) in dishes or flasks precoated with bovine fibronectin (10 μg/ml). Methods for preparation, storage, and use of medium supplements and fibronectin precoating have been published (Loo et al., 1989a).

Plating was accomplished by adding cells in prewarmed medium to plates or flasks previously precoated with fibronectin and preincubated in the incubator (5% CO_2–95% air atmosphere at 37°C) for ~15 min with the remaining portion of medium. Insulin, transferrin, EGF, and HDL were added directly to the individual culture vessels as small aliquots from concentrated stocks immediately after plating the cells. Bovine insulin, human transferrin, trypsin, and soybean trypsin inhibitor were obtained from Sigma Chemical Co. (St. Louis, MO). Mouse EGF was obtained from Upstate Biotechnologies (Lake Placid, NY). HDL (density = 1.068–1.21 g/cc) was prepared by KBr ultracentrifugation as described (Gospodarowicz, 1984; Loo et al., 1989a) and filter sterilized after dialysis. Preparation of fibronectin has been described (Loo et al., 1989a).

EM

SFME cells were plated in 10-cm-diam plates (2 × 10^7 cells/plate) in serum-free medium with 5 ng/ml EGF. The following day some plates were changed to serum-free medium without EGF (10 ml/plate). Control plates were changed to serum-free medium with 50 ng/ml EGF. At the indicated times 8 ml of the culture medium were carefully removed from a plate, 3 ml of fixative (1.75% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.40) were added, and plates were then incubated at room temperature for 30 min. After fixation, the cells were removed from the dishes with a rubber policeman and were resuspended in fixative, pelleted, and the pellet was minced into pieces 1 mm^3. The samples were washed through two changes of 0.2 M sodium cacodylate buffer, pH 7.4, and were postfixed for 1 h in 1.0% OsO$_4$ in 0.15 M sodium cacodylate buffer.

Samples were washed through two changes of 0.2 M sodium cacodylate buffer and dehydrated through a graded acetone series (X3), infiltrated through several mixtures of acetone/resin, and embedded in freshly prepared Medcast-Araldite 502 resin (Ted Pella, Inc., Irvine, CA) and polymerized at 60°C for 24 h (Hayat, 1986). Ultrathin sections were cut on an ultramicrotome (model MT-500; Sorvall Instruments Div., DuPont Co., Newton, CT) with a sapphire knife. Sections 60-70 nm in thickness were collected on 300 hexagonal mesh copper grids and stained with saturated ethanolic uranyl acetate followed by bismuth subnitrate. The sections were examined with a transmission electron microscope (EM 10/A; Zeiss, Oberkochen, Germany) at an accelerating voltage of 60 kV.

DNA Fragmentation Analysis

SFME cells (2 × 10^7/plate) were seeded in 10-cm-diam plates in serum-free medium with 5 mg/ml EGF. The following day some plates were changed to serum-free medium without EGF (10 ml/plate). Control plates were changed to serum-free medium with 50 ng/ml EGF. At the indicated times duplicate cells were harvested by scraping into cold phosphate-buffered saline without calcium or magnesium (PBS) followed by centrifugation at 4°C in 1,000 g for 5 min. The supernatant was aspirated and the cell pellets were immediately stored at −86°C.

Cell pellets were thawed on ice and incubated at 37°C for 4 h in 4 ml of lysis buffer (200 mM Tris, pH 8.5, 100 mM EDTA, 50 μg/ml proteinase K, 1% SDS). The DNA solution was extracted twice with phenol. The aqueous phase was dialyzed overnight against 10 mM Tris HCl, pH 7.5, 1 mM EDTA. After dialysis the DNA solution was incubated at 37°C for 5 h with 50 μg/ml of DNAse-free RNAse A. Proteinase K (50 ng/ml) was added and the DNA solution was further incubated at 37°C for 5 h. The DNA solution was extracted once with phenol and once with phenol:chloroform:isoamyl alcohol (25/24/1). DNA was precipitated in ethanol and resuspended in distilled water. DNA concentration was determined from the absorbance at 260 nm.

For analysis of fragmentation, DNA (20 μg/lane) was fractionated by electrophoresis for 16 h at 40 V on a 1.0% agarose gel with 90 mM Tris HCl, 90 mM boric acid, 2 mM EDTA, pH 8.0 as running buffer. DNA was visualized by shortwave UV illumination after ethidium bromide staining (Maniatis et al., 1982). A 123 bp DNA ladder (Bethesda Research Laboratories, Gaithersburg, MD) was used as a standard.

Enzyme Assay

Culture medium (0.5 ml) from BALB SFME or BALB 3T3 cultures maintained for the indicated times under serum-free conditions in the presence or absence of EGF was added to an equal volume of lactate dehydrogenase assay reagent (100 mM lactate, 14 mM nicotinamide adenine dinucleotide, pH 8.9; #228-10; Sigma Diagnostics, St. Louis, MO). Enzyme units were quantified by determining the rate of absorbance change at 340 nm over a 60-s time interval.

Flow Cytometry

SFME cells were seeded at 2 × 10^7 cells per 10-cm-diam dish and cultured overnight in serum-free medium with 5 ng/ml EGF. The following day some plates were changed to serum-free medium without EGF (10 ml/plate). Control plates were changed to serum-free medium with 50 ng/ml EGF. At the indicated times cells were analyzed for cell size by measuring forward angle light scatter (FALS) or cell cycle by measurement of DNA staining with chromomycin A3 (IGFL).

For size analysis cells were removed from plates with 0.25% crude trypsin/1 mM EDTA in PBS and diluted into an equal volume of F12:DME containing 1 mg/ml soybean trypsin inhibitor. Cells were washed with ice-cold PBS by centrifugation and resuspension in 5 ml PBS. Formalin (550 μl, 37%) was added while vortexing and fixed cells were stored at 4°C.

For chromomycin staining cells were removed from plates, centrifuged, resuspended in 5.0 ml ice-cold PBS as described above, and then pelleted by centrifugation. The supernatant was aspirated, leaving 0.5 ml in the bottom of the tube, and 5.0 ml of ice-cold 70% EtOH was added dropwise while vortexing the cell pellet. Cells were stored in ethanol at 4°C until all samples could be processed together. Fixed cells were centrifuged, resuspended in chromomycin A3 (20 μg/ml) with 15 mM MgCl$_2$, and incubated for 0.5 hour at room temperature in the dark. Cells were centrifuged from solution, resuspended (5 × 10^5 to 5 × 10^6 cells/ml) in ice-cold PBS, and filtered through a 40 μm nylon filter.

Basic procedures for flow cytometry were as described by Gray and Coffino (1979). Flow cytometry was carried out with an Epics flow
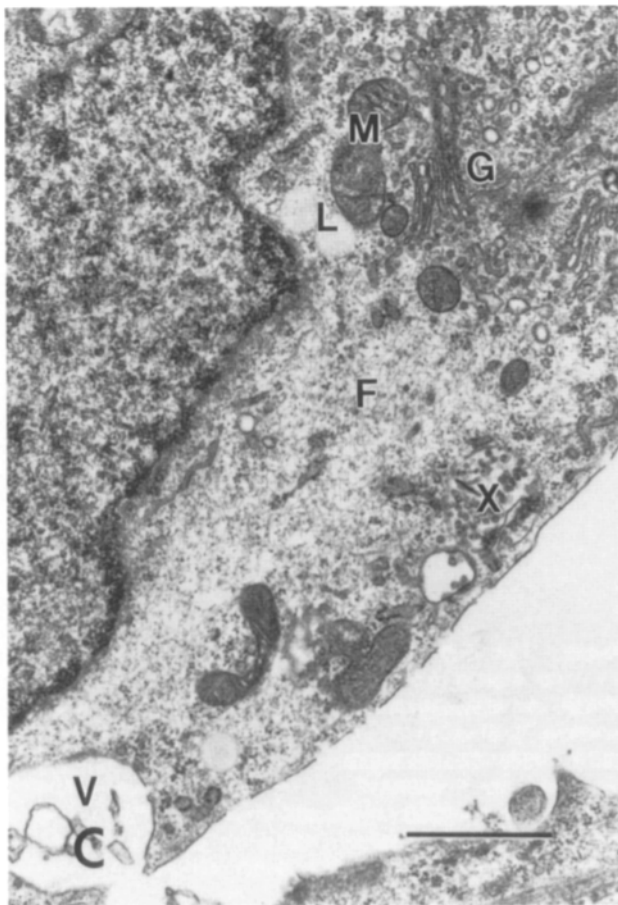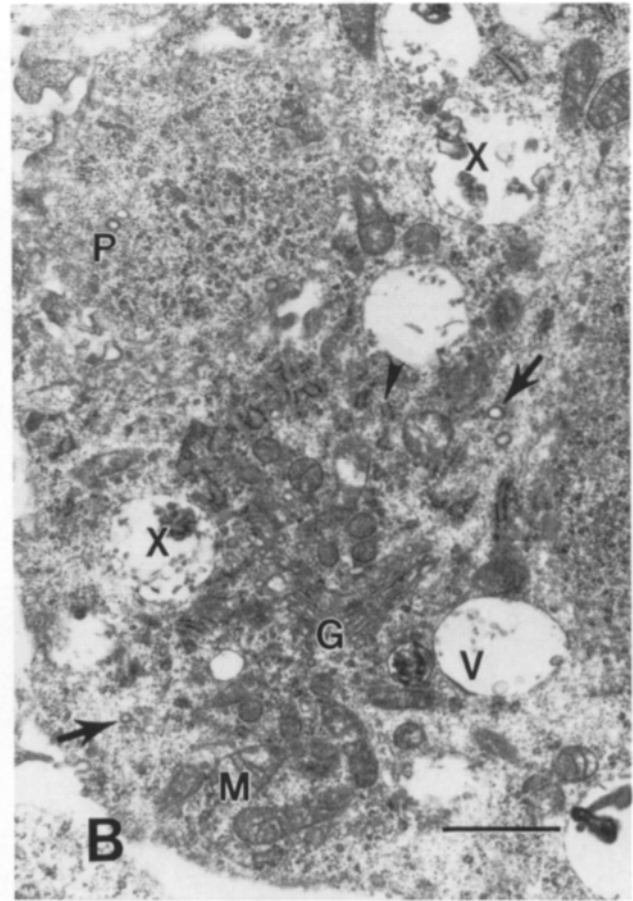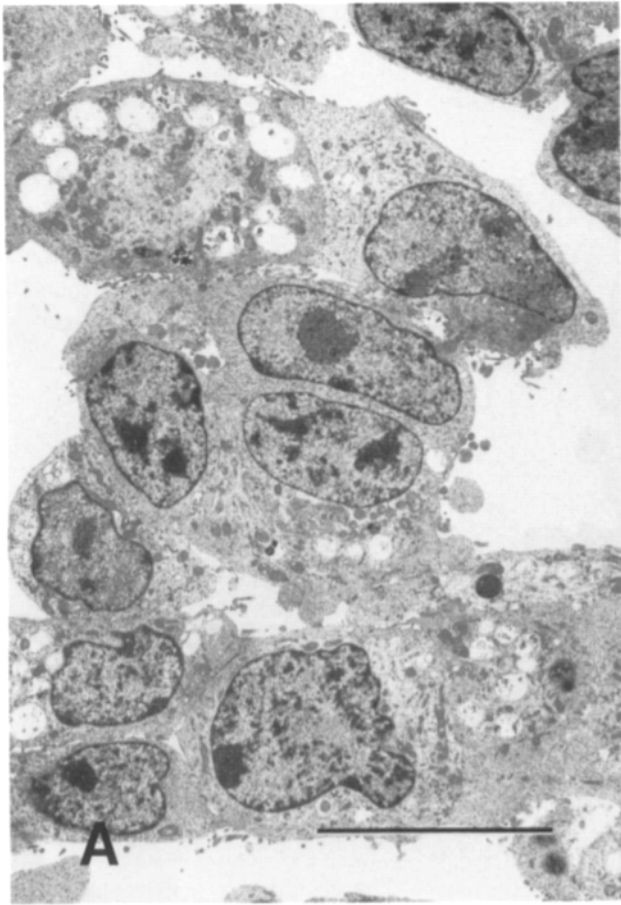
Figure 1. SFME cells cultured with EGF or without EGF for 4 h. (*A*) With EGF, low power. Polygonal or rounded cells, found in sheets, contain large, round to oblong, slightly indented nuclei. Nuclei usually have a thin peripheral rim of heterochromatin, abundant finely dispersed chromatin, and prominent nucleoli. (*B*) With EGF, at higher magnification, the cytoplasm is rich in organelles: abundant polyribosomes (*P*), prominent clear vacuoles (*V*) containing delicate membranous-like material, multivesicular bodies (*X*), well-developed Golgi complex (*G*), coated vesicles (*arrows*), short, slightly dilated profiles of RER (*arrowhead*), and mitochondria (*M*). (*C*) Without EGF for 4 h. The first significant alteration detected was decreased polyribosomic aggregates giving the cytoplasm an increased electron lucency. A few cytoplasmic lipid droplets (*L*) were also found. A well-developed Golgi complex (*G*), clear vacuoles (*V*), multivesicular bodies (*X*), intermediate filaments (*F*), and mitochondria (*M*) were ultrastructurally similar to corresponding control organelles. Bars: (*A*) 10 μm; (*B*) 1 μm; (*C*) 1 μm.
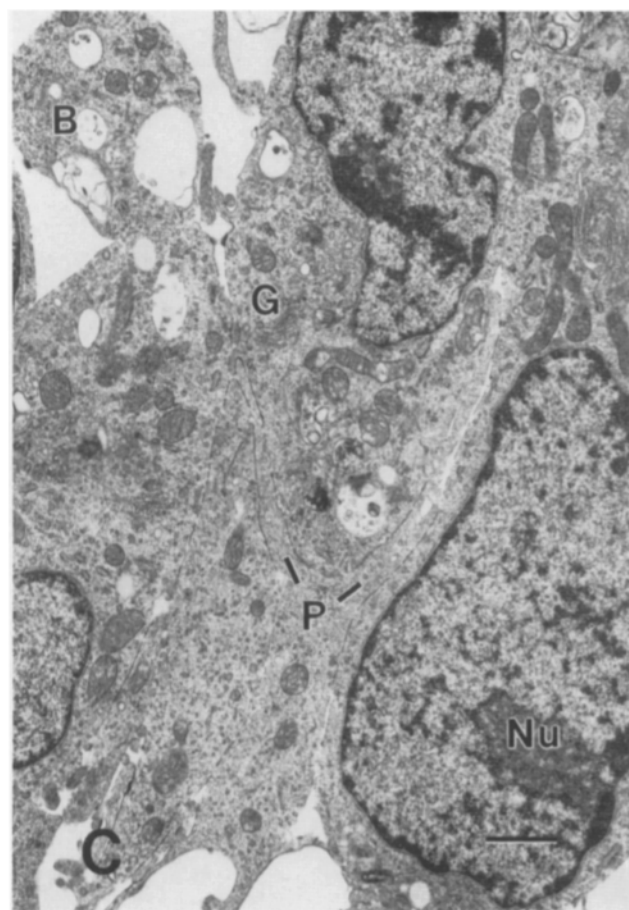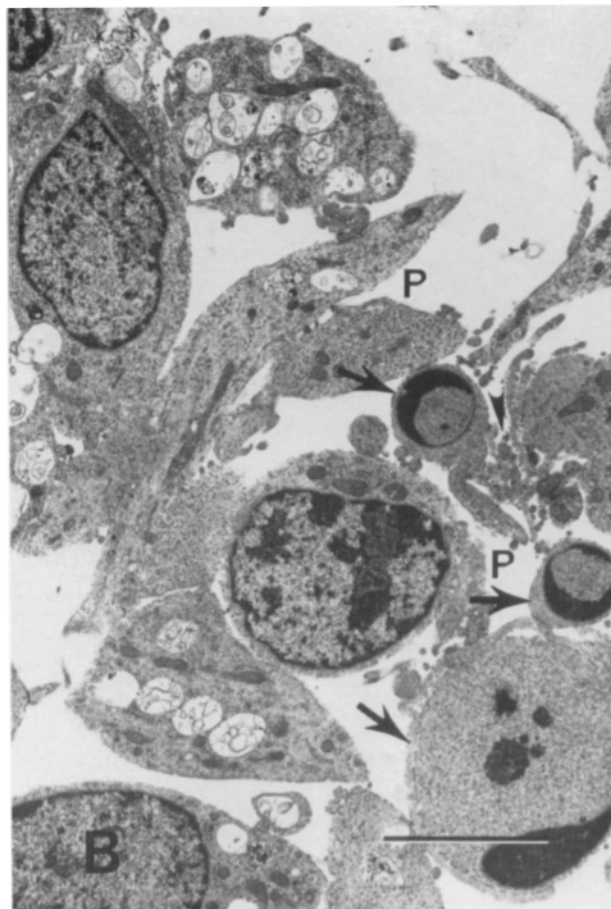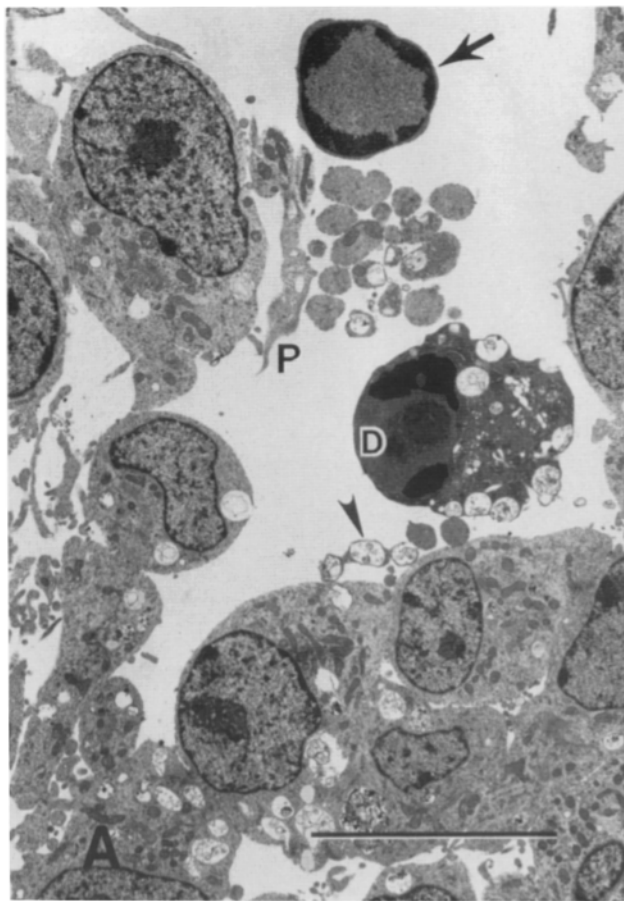
Figure 2. Cell death and degeneration in SFME cells cultured for 8 h in the absence of EGF. (*A* and *B*) Low-power magnification of SFME cells depicting pleomorphic cells, severely degenerate cells (*arrows*), dead cells (*D*), cellular debris (*arrowhead*), and elongated cytoplasmic projections with the remaining portion of the cell out of the plane of section (*P*). (*C*) Higher magnification of SFME cells showed elongated cytoplasmic projections (*P*), cytoplasmic blebs (*B*), atrophied Golgi (*G*), and lack of aggregates of polyribosomes. Note the prominent light granular nucleolus (*Nu*) peripherally located.

674

cytometer-cell sorter (Coulter Electronics Inc., Hialeah, FL). Chromomycin A3-stained cells were excited with an output of 100 mW of 457-nm light emitted from an argon laser. The resulting cell-associated fluorescence was captured through a 495-nm-long pass absorbance filter (Coulter Electronics Inc.) and blocking interference filter (Corion Corp., Holliston, MA). Cell sizing was analyzed with a ND-1 filter for FALS.

Polysome Profiles

SFME cells were plated at 2×10^7 cells/10-cm-diam dish and cultured for 24 h in serum-free medium with 5 ng/ml EGF. The medium was changed on one plate to serum-free conditions with 50 ng/ml EGF and on the other plate to serum-free conditions without EGF. After 6 h the medium was aspirated, plates were washed twice with PBS containing 10 μg/ml cycloheximide, and then stored frozen at −86°C. All subsequent manipulations were performed on ice or at 4°C. Plates were scraped in 2× lysis buffer (500 mM NaCl, 50 mM MgCl$_2$, 100 mM Tris, pH 7.5, 1% Triton X-100, 400 U/ml RNasin, and 40 μg/ml cycloheximide) and if necessary sterile water was added to achieve a 1× lysis buffer concentration.

Lysates were centrifuged for 10 min at 13,000 rpm in a precooled (model SS34; Sorvall Instruments Div., DuPont Co.) rotor. The supernatant was carefully transferred to the top of a freshly prepared 15–50% sucrose gradient (250 mM NaCl, 25 mM MgCl$_2$, 50 mM Tris, pH 7.5, 20 μg/ml cycloheximide, and 15–50% sucrose). Gradients were centrifuged for 130 min in a precooled rotor (model SW40; Beckman Instruments, Inc., Palo Alto, CA) at 32,000 rpm and scanned from top to bottom at 254 nm using a model 185 density gradient fractionator (ISCO, Lincoln, NE) at a flow rate of 0.375 ml/min (Gross and Merrill, 1989). Absorbance was monitored by a UA-5 absorbance/fluorescence detector and type 6 optical unit (ISCO), which was calibrated to an internal standard immediately before use.

Northern Blot Hybridization Analysis

For isolation of cytoplasmic RNA, plates containing SFME cells were washed with ice-cold PBS, cells were scraped into cold PBS and centrifuged (4°C, 1,000 g, 5 min). Cell pellets were resuspended in 10 mM Tris HCl, pH 7.6, 140 mM NaCl, 1.5 mM MgCl$_2$, 200 μg/ml heparin, lysed by added 0.5% NP-40, and vortexed for 20 s, and then centrifuged at 4,000 g for 10 min to pellet nuclei. 1% SDS and 10 mM EDTA were added to the supernatant followed by extraction twice each with phenol, phenol:chloroform: isoamyl alcohol (25/24/1), and chloroform:isoamyl alcohol (24/1). RNA was precipitated in ethanol and stored in diethylpyrocarbonate-treated water at −86°C. Poly(A)+ RNA was prepared from cytoplasmic RNA using standard methods (Maniatis et al., 1982).

Poly(A)+ RNA (1.0 μg/lane) was fractionated by electrophoresis on a 1.2% agarose gel containing 6.6% formaldehyde and transferred for 24 h to a nitrocellulose membrane (Maniatis et al., 1982). The plasmid pCD-β actin was linearized with HindIII and labeled (5×10^8 cpm/μg DNA) by oligonucleotide-primed extension (kit from Boehringer Mannheim Diagnostics, Indianapolis, IN). The membrane was prehybridized for 4 h, and then hybridized with probe (2×10^6 cpm/ml) for 20 h in 50% formamide, 50 mM sodium phosphate, pH 6.5, 0.8 M NaCl, 1 mM EDTA, 0.1% SDS, 0.05% BSA, 0.5% Ficoll, 0.05% polyvinylpyrrolidone, 250 μg/ml sonicated salmon sperm DNA, and 500 μg/ml yeast RNA at 57°C. Filters were washed twice with 1× SSC and 0.1% SDS at room temperature for 30 min each, and then twice with 0.5× SSC and 0.1% SDS at 65°C for 30 min each. Autoradiography was carried out on film (X-OMAT AR; Kodak Co., Rochester, NY) for 12–36 h at −86°C.

Results

Ultrastructural Changes Associated with EGF Deprivation

SFME cells were cultured in the absence of EGF and fixed for EM 4, 8, 16, and 24 h later. Control cells grown in the presence of EGF were fixed at the beginning and end of the experiment. Cells grown in the presence of EGF had a high nuclear to cytoplasmic ratio relative to most cultured cells and contained abundant aggregates of polyribosomes that were generally uniformly distributed (Fig. 1, a and b). An additional prominent cytoplasmic feature of control cells was frequent large vacuoles and multivesicular bodies (MVB) that tended to be regionally located. A well-developed Golgi

complex and abundant coated vesicles were present and intermediate filaments and microtubules were prominent. Cytoplasmic lipid droplets were rarely seen. Nuclear chromatin appeared as a thin peripheral rim of heterochromatin with small aggregates of heterochromatin also randomly dispersed throughout the nuclear matrix. Euchromatin was predominant and found throughout the nuclear matrix. In most nuclei, single or multiple compact nucleoli were seen either centrally or marginally located adjacent to the nuclear envelope.

SFME cells cultured in the absence of EGF for 4 h exhibited a loss of polyribosomes (Fig. 1 c) and predominance of monosomes. When SFME cells were cultured for 8 h without EGF, cellular degeneration, cell death, and other ultrastructural pathologic alterations were visible. Cellular morphology was pleomorphic; some cells were small and round, while other cells were larger and elongated with distinct ameboid-like cytoplasmic projections (Fig. 2, a and b).

Some of the small cells were severely degenerate and contained electron-dense nuclei with a thick band of marginated chromatin in a circumscribed or crescent form with nucleoli condensed or absent. Cytoplasm in these degenerate cells was condensed and cytoplasmic organelles were scant but recognizable; occasional cytoplasmic blebs or projections were found. A portion of cytoplasmic and nuclear matrix of most degenerate cells displayed a light-grey granular matrix. The few mitochondria found in degenerate cells with light grey cytoplasmic matrix were normal, whereas the few profiles of rough ER observed were slightly dilated or vesicular with a loss of polyribosomes.

Dead cells were distinguished by prominent electrondense cytoplasm containing lucent cytoplasmic vacuoles and pyknotic nuclei. The nuclei had either a wide, electrondense marginated band of chromatin with the remaining nuclear matrix of similar electron density to that of the cytoplasm, or the nucleus was round and filled with electrondense chromatin. Nucleoli were not found in these cells.

The predominant population of cells in the absence of EGF appeared smaller than control cells cultured in the presence of EGF because of a loss of cytoplasm. Cytoplasmic projections with the remaining portion of the cell out of the plane of section and cytoplasmic debris were scattered throughout the fields, suggesting that cells may have been in an early phase of disintegration. Frequently, nuclei were found that contained large nucleoli composed primarily of a light granular component (Fig. 2 c).

At 16 h, a greater proportion of SFME cells grown without EGF were ultrastructurally altered than at 8 h; however, the extent of the ultrastructural changes, except for minor differences, were similar to those changes described at 8 h. Severely degenerate cells, elongated cytoplasmic projections with the remaining portion of the cell out of the plane of section, amorphous cellular debris, dead cells, and fragmentation of dead cells with only remnants of pyknotic nuclei and cytoplasmic debris remaining were more commonly found at 16 than at 8 h.

In the remaining cells the nuclear envelope was frequently deeply indented or invaginated resulting in segmented nuclei. Nucleoli, if found, were condensed and small. Cytoplasmic vacuoles appeared expanded and occupied a larger proportion of the cytoplasm. Lipid droplets were commonly found, Golgi were atrophied, intermediate filaments, and coated vesicles were difficult to find, and aggregates of poly-
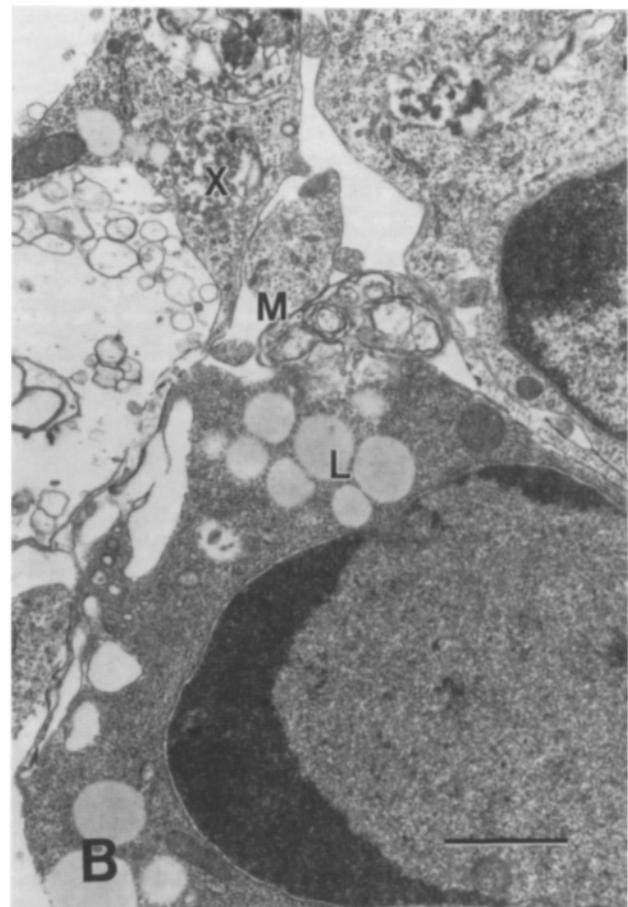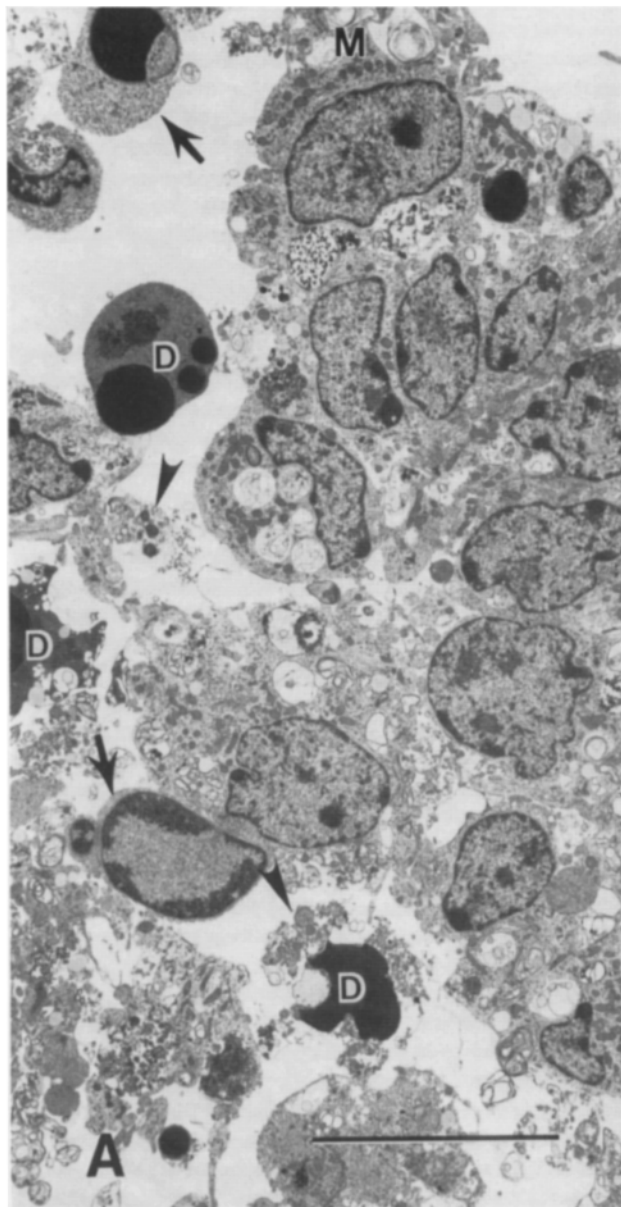
Figure 3. SFME cell death and severe degeneration after 24 h of EGF deprivation. (*A*) Dead (*D*) or severely degenerate cells (*arrows*), and dispersed cellular debris (*arrowheads*) were a major feature found at this time period. The cytoplasm of remaining cells contained abundant large vacuoles, some of which compressed adjacent structures (i.e., nucleus). Nuclei of these cells were usually indented or partially segmented. (*B*) The presence of abundant cytoplasmic lipid droplets was an additional feature observed during this time period. Note the numerous lipid droplets (*L*), peripheral collapsed vacuoles, and condensed granular cytoplasm of a severely degenerate cell. In adjacent cells, cytoplasmic vacuoles contained a predominance or a mixture of the following structures: circular membranous material, vesicles or cellular debris. Multivesicular bodies (*X*) or in some vacuoles, membranous whorls (myelin figures) (M) were also seen. Bars: (*A*) 10 μm: (*B*) 1 μm.
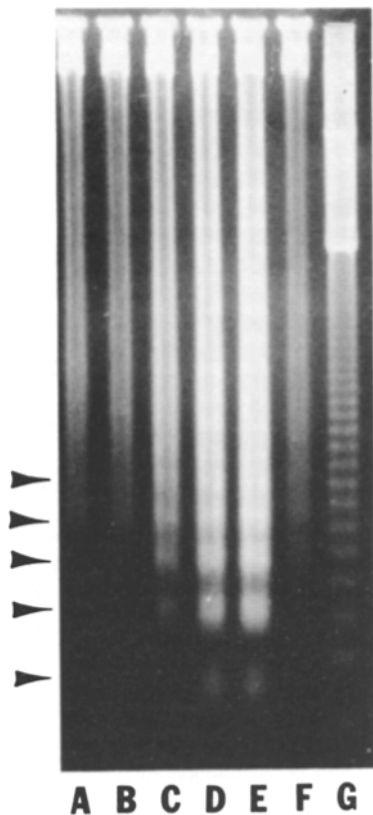
ribosomes were sparse. Nuclear karyorrhexis, and apparent segmental loss of the nuclear envelope was occasionally seen in some early degenerate cells, but cytoplasmic organelles in these cells were recognizable. This finding suggests that nuclear condensation and eventual pyknosis occurred before cytoplasmic condensation and increased electron density.

The majority of SFME cells cultured without EGF for 24 h were either dead or degenerate (Fig. 3 *a*). Dead cells, severely degenerate cells, cytoplasmic projections, and cellular debris were similar in appearance to that described above, but were a greater fraction of the total population rel-

ative to 8 and 16 h samples. The remaining cells appeared to be degenerate because the cytoplasm was filled with large vacuoles and abundant lipid droplets. Vacuoles often contained abundant membranous material in circular or concentric myelin figure profiles (Fig. 3 *b*). Vacuoles also contained variable amounts of electron-dense amorphous debris and vesicles.

Biochemical and Physiological Changes Associated with EGF Deprivation

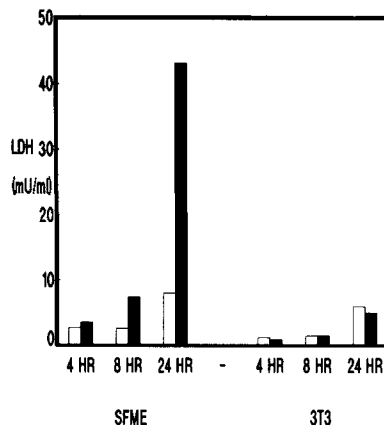Fragmentation of DNA isolated from SFME cells cultured

Figure 5. Release of cytoplasmic enzyme in culture medium by SFME cells in the absence of EGF. SFME cells or 3T3 cells were incubated the indicated times in serum-free medium with or without EGF and culture medium assayed for lactate dehydrogenase as described. 3T3 cells are mouse embryo control cells that are not acutely dependent on EGF for survival. (□) with EGF; (■), without EGF.
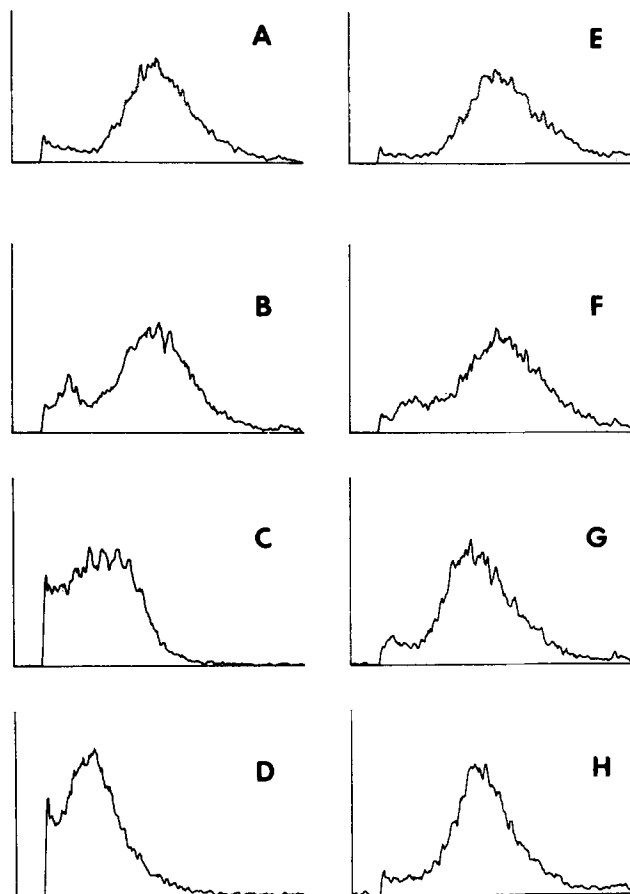
Figure 4. DNA fragmentation in EGF-deprived SFME cells. Cultures were changed to medium without EGF and incubated for up to 24 h. DNA was isolated from samples during this time course, fractionated by electrophoresis, and visualized by ethidium bromide staining. (A) 0 h without EGF; (B) 4 h without EGF; (C) 8 h without EGF; (D) 16 h without EGF; (E) 24 h without EGF; (F) 24 h with EGF; (G) 123-bp DNA ladder standard. Arrowheads mark bands comprising oligonucleosome ladders indicative of DNA fragmentation by endonuclease (Arends, 1990).

without EGF was detected 8 h after removal of the growth factor (Fig. 4). The pattern of degradation into oligonucleosome-length fragments, generating a regularly spaced "ladder," is characteristic of programmed cell death (Arends, 1990) resulting from activation of endonuclease cleaving chromatin into polynucleosomes. Lactate dehydrogenase released by cells into the culture medium was assayed as an indication of cell lysis. A large increase in enzyme activity was only observed in SFME cell medium at relatively late times after removal of EGF from the cultures (Fig. 5). No major release of lactate dehydrogenase over the same time course was detected in identical experiments with 3T3 mouse embryo cells, which are not acutely dependent on EGF for survival (Shipley and Ham, 1983).
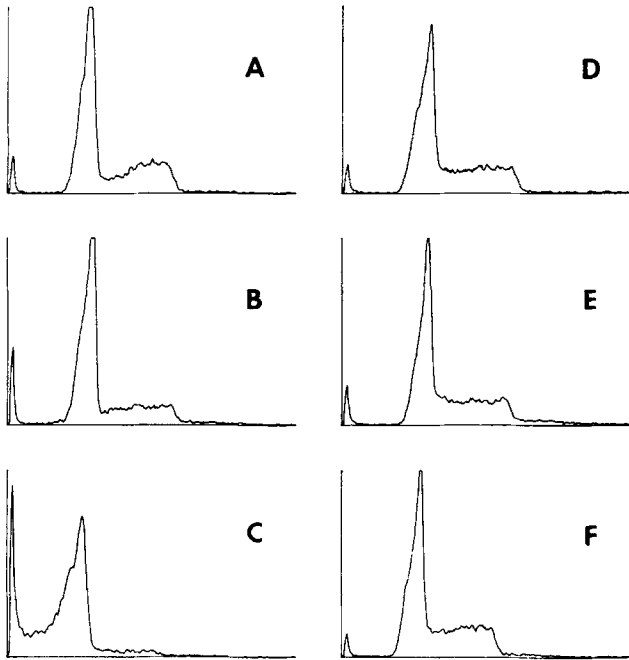
Nuclease assay of medium from SFME cells cultured in the absence of EGF did not detect increased activity before the time in which a general release of cellular enzymes was detected by assay of lactate dehydrogenase, indicating that nuclease causing the cleavage pattern of Fig. 4 probably was not released as cells underwent the progressive changes in the early phases leading to cell death resulting from EGF deprivation. Incubation of SFME cells in the presence of EGF with extracts of SFME cells cultured without EGF for 24 h did not lead to cell death.

Figure 6. Flow cytometric size analysis of SFME cells. Cultures were incubated with or without EGF and processed as described for flow cytometry size analysis by measurement of forward angle light scattering. Tracings show relative cell number (Y axis) versus relative volume per cell (X axis). (A) no EGF, 0 h; (B) no EGF, 8 h; (C) no EGF, 24 h; (D), no EGF, 48 h; (E-H), EGF present, 0, 8, 24, and 48 h.

Figure 7. Flow cytometric DNA analysis of SFME cells. Cultures were incubated with or without EGF and processed as described for analysis of DNA content per cell. Tracings show relative cell number (Y axis) versus relative amount of DNA per cell (X axis). (A) no EGF, 0 h; (B) no EGF, 8 h; (C) no EGF, 24 h; (D-F) EGF present, 0, 8, and 24 h.

Light and electron microscopic examination suggested that SFME cells became smaller after removal of EGF. This was demonstrated quantitatively by flow cytometric analysis of forward angle light scatter. The average size of the population of cells became smaller with time after removal of EGF, while SFME cells from parallel cultures incubated with EGF did not show this change (Fig. 6). Analysis of DNA content of SFME cells incubated without EGF showed that the percentage of cells in G2 and S phase of the cell cycle markedly decreased with time after removal of EGF (Fig. 7). Most of the cells 24 h after removal of EGF were in the G1 stage of the cell cycle. No time-dependent change in the relative percentages of cells in cell cycle stages was seen over the course of the experiment when EGF was included in the medium.

To confirm the electron microscopic observations that polysomal aggregation decreased in SFME cells upon removal of EGF, we generated polysome profiles by gradient centrifugation and spectrophotometrically quantified the relative polysome and monosome content of SFME cells cultured for 6 h with or without EGF (Fig. 8). Integration of the spectrographic tracings revealed that polysome-associated ribosomes were reduced ~50% in EGF-deprived cells, relative to controls, with a corresponding increase in monosomes and ribosomal subunits. It is unlikely that the shift from polysomes to monosomes was because of an overall reduction in steady state level of mRNA in the cultures incubated without EGF, because Northern blot hybridization analysis did not detect a change in actin mRNA in SFME cells after 16 h without EGF (Fig. 9).

Discussion

NGF-dependent neurons in vitro represent a well-studied

growth factor-dependent cell culture system. NGF is necessary to establish cultures of these cells and neurons die within 48 h in vitro if the growth factor is omitted from the medium (Yanker and Shooter, 1982; Thoenen and Barde, 1980; Martin et al., 1988). The behavior of these cells in vitro has been compared to programmed neuronal cell death in vivo, and the ability of NGF to promote neuronal survival in vivo has been demonstrated (Hamburger et al., 1981; Yip and Johnson, 1984). EGF-dependent SFME cells represent another growth factor-dependent culture system of neural tissue origin (Sakai et al., 1990). Growth factor-dependent systems also exist among hematopoietic stem cells: interleukin-dependent lymphocytes and erythropoietin-dependent reticulocyte progenitors (Koury and Bondurant, 1990; Rodriguez-Tarduchy and Lopez-Rivas, 1989; Nicola, 1989).

Identification of SFME cells as astrocyte precursors suggests mechanistic and developmental relationships may exist between the NGF dependence of neurons and the EGF dependence of SFME cells. In one respect, however, the SFME culture system more closely resembles the hematopoietic cells. Like lymphocyte and reticulocyte precursors, and unlike neurons, the cells are capable of proliferation in vitro, and the growth factor promotes both proliferation and survival. A common feature among all the growth factor-dependent cell types is the loss of the growth factor requirement after oncogenic transformation; in some instances malignant transformation is accompanied by autocrine production of growth factor or activation of a receptor (Shirahata et al., 1990; Isfort, 1990; Bishop, 1987).

Analysis of DNA from SFME cells cultured without EGF revealed a pattern consistent with the notion that the cells underwent degradation of chromatin in the regions unprotected by nucleosomes. This pattern is characteristic of cells undergoing apoptosis or programmed cell death, through which an endogenous endonuclease is activated (Arends, 1990). The time course of appearance of fragmented DNA in cultures incubated without EGF indicates that increased levels of fragmented DNA occurred coordinately with increased
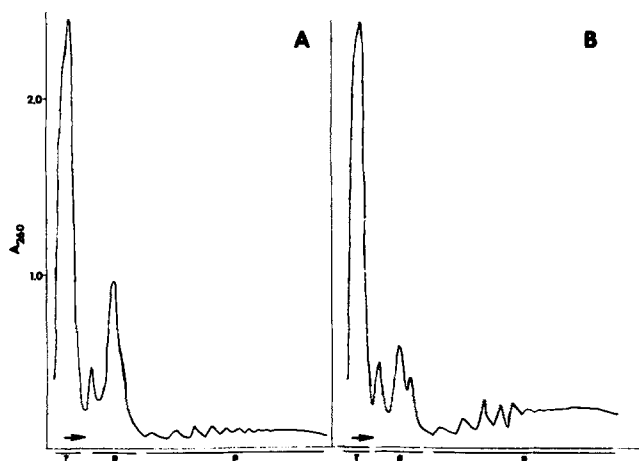
Figure 8. Polysome profiles of SFME cells. Cells were incubated with or without EGF, lysed in the presence of cycloheximide, and polysome distributions were determined by sedimentation in a sucrose gradient and spectroscopic measurement as described. (A) without EGF, 6 h; (B) control, with EGF. Arrows show direction of sedimentation. (T) Absorptive material at the top of the gradient; (R) monosomes and ribosomal subunits; (P) polysomes.

◄ 28 S

◄ 18 S

A B
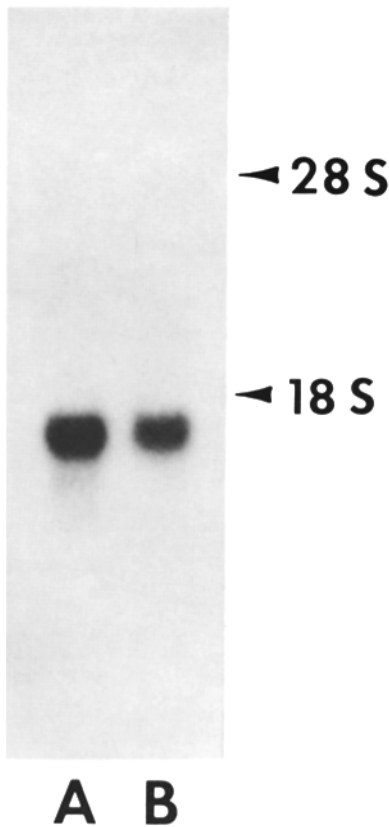
Figure 9. Northern blot hybridization analysis of actin mRNA from SFME cells. SFME cells were cultured as described in serum-free medium with (*A*) or without (*B*) EGF for 16 h followed by isolation of poly A–containing RNA and blot hybridization as described. The blot was probed with labeled plasmid containing the gene for β actin.

frequency of appearance of dead cells with pyknotic nuclei. Degenerate cells in our cultures show ultrastructural similarities to "phase 1" apoptotic cells, identified by Arends et al. (1990) as undergoing early phases of programmed cell death, and may not yet have undergone extensive DNA fragmentation associated with pyknotic nuclei. Cells identified as degenerate appeared before the large scale manifestation of dead cells with pyknotic nuclei, also suggesting that degenerate cells are precursors to apoptotic cells.

In experiments in which EGF was removed for 8 to 24 h and then added back, we found that incubation for only 8 h in the absence of EGF reduced by ~50% the number of cells that ultimately were recovered after EGF deprivation (Loo et al., 1989*a*), suggesting that the degenerate cells traverse an irreversible pathway leading to death. Incubation for 24 h in the absence of EGF reduced by 80% the number of cells that recovered after EGF deprivation (Loo et al., 1989*a*; Rawson et al., 1990). At this time most of the cells in the cultures appeared either dead or degenerate by ultrastructural analysis and significant leakage of cytoplasmic enzyme into the culture medium was detected.

Assay of cytoplasmic enzyme release into the medium did not detect a large release at earlier time points when ultrastructural and some biochemical changes were obvious, indicating that plasma membrane integrity was maintained through this early period and implying that degenerate cells were not highly permeable. Nuclease activity was not

released in a specific manner before the general breakdown of membrane integrity and extracts of dying cells did not kill SFME cells in the presence of EGF, suggesting that cell death resulting from EGF deprivation is not recruited in some cells by release of enzyme from other cells. Flow cytometry showed that the cultures in the absence of EGF for 24 h were entirely in the G1 phase of the cell cycle. This probably reflects in inability of cells to progress into S phase in the absence of EGF, and may also indicate an initial preferential loss of cells in S or G2 in the absence of EGF.

Ultrastructural analysis of SFME cells cultured in the absence of EGF identified a time-dependent reduction in polysomes, alterations in nuclear morphology, a reduction in cell size and cytoplasmic volume and the appearance of lipid or membrane-containing vacuoles and apoptotic nuclei. The appearance of lipid-containing vacuoles and multivesicular bodies and a progression to small cell size has been noted in neurons deprived of NGF (Martin et al., 1988). In both culture systems apoptotic nuclei appear with little evidence of a transition state, suggesting that this change occurs rapidly (Martin et al., 1988). Primary disturbances in ER are observed in the neuronal system, but were not prominent in SFME cells without EGF, and the shift from polysomes that we observed with SFME cells is not seen in neurons cultured without NGF (Martin et al., 1988).

The reduction in SFME cell size perceived microscopically was confirmed by flow cytometric analysis. This effect appears to be the result of fragmentation and loss of cytoplasm from the cells; the small cells are essentially nuclei surrounded by a minimal amount of cytoplasm and plasma membrane. A loss of polysomes was the earliest change in SFME cells observed in the electron micrographs after removal of EGF, and the EGF-dependent polysome shift was confirmed in sedimentation profiles. mRNA remained intact in these cells, suggesting that an EGF-dependent regulation of translation may be responsible.

EGF and other peptide-growth factors stimulate phosphorylation of ribosomal protein S6 in other cell culture systems (Oliver et al., 1988), but the functional significance of this effect has not been established. The relationship of the ultrastructural, biochemical, and physiological alterations we have observed in EGF-deprived SFME cells to the primary mechanism of cell death in this system, as well as the means by which inhibitors of protein and RNA synthesis prevent cell death, remains to be determined.

References

Arends, M. J., R. G. Morris, and A. H. Wyllie. 1990. Apoptosis: the role of the endonuclease. *Am. J. Pathol.* 136:593–608.
Bishop, J. M. 1987. The molecular genetics of cancer. *Science (Wash. DC).* 235:305–311.
Ernst, T., C. Jackson, and D. Barnes. 1990. Factors influencing karyotypic stability of serum-free mouse embryo (SFME) cells. *Cytotech.* In press.
Gospodarowicz, D. 1984. Preparation and uses of lipoproteins to culture normal diploid and tumor cells under serum-free conditions. *Cell Cult. Meth. Mol. Cell. Biol.* 1:69–88.

Gray, J. W., and P. Coffino. 1979. Flow cytometry. *Methods Enzymol.* 58: 233–238.

Gross, M. K., and G. F. Merrill. 1989. Thymidine kinase synthesis is repressed in nonreplicating muscle cells by a translational mechanism that does not affect the polysomal distribution of thymidine kinase mRNA. *Proc. Natl. Acad. Sci. USA.* 86:4987–4991.

Ham, R. G., and W. L. McKeehan. 1979. Media and growth requirements. *Methods Enzymol.* 58:44–93.

Hamburger, V., J. K. Brunso-Bechtold, and J. W. Yip. 1981. Neuronal death in the spinal ganglia of the chick embryo and its reduction by nerve growth factor. *J. Neurosci.* 1:60–71.

Hayat, M. A. 1986. Basic Techniques for Transmission Electron Microscopy. Academic Press, Inc., Orlando, FL. p. 226.

Isofort, R. J. 1990. Frequency and mechanisms of factor independence in IL-3-dependent cell lines. *Somatic Cell Mol. Genet.* 16:109–121.

Koury, M. J., and M. C. Bondurant. 1990. Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells. *Science (Wash. DC).* 248:378–381.

Loo, D. T., J. I. Fuquay, C. Rawson, and D. Barnes. 1987. Extended culture of mouse embryo cells without senescence: inhibition by serum. *Science (Wash. DC).* 236:200–202.

Loo, D., C. Rawson, A. Helmrich, and D. Barnes. 1989a. Serum-free mouse embryo (SFME) cells: growth responses in vitro. *J. Cell. Physiol.* 139: 484–491.

Loo, D. T., C. L. Rawson, T. Ernst, S. Shirahata, and D. W. Barnes. 1989b. Primary and multipassage culture of mouse embryo cells in serum-containing and serum-free media. *In* Cell Growth and Cell Division: A Practical Approach. Baserga, R., editor. IRL Press Limited, Eynsham, Oxon, England. 17–34.

Loo, D., C. Rawson, M. Schmitt, K. Lindburg, and D. Barnes. 1990. Glucocorticoid and thyroid hormones inhibit proliferation of serum-free mouse embryo (SFME) cells. *J. Cell. Physiol.* 142:210–217.

Loo, D., Y. Sakai, C. Rawson, and D. Barnes. 1991. Serial passage of embryonic human astrocytes in serum-free, hormone-supplemented medium. *J. Neurosci. Res.* 28:101–109.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Martin, D. P., R. E. Schmidt, P. S. DiStefan, O. H. Lowry, J. G. Carter, and E. M. Johnson, Jr. 1988. Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. *J. Cell Biol.* 106:829–844.

Mather, J. P., and G. H. Sato. 1979. The use of hormone-supplemented serum-free media in primary cultures. *Exp. Cell Res.* 124:215–221.

Nicola, N. A. 1989. Hemopoietic cell growth factors and their receptors. *Annu. Rev. Biochem.* 58:45–77.

Olivier, A. R., L. M. Ballou, and G. Thomas. 1988. Differential regulation of S6 phosphorylation by insulin and epidermal growth factor in Swiss mouse 3T3 cells: insulin activation of type 1 phosphatase. *Proc. Natl. Acad. Sci. USA.* 85:4720–4724.

Rawson, C., C. Casola-Smith, and D. Barnes. 1990. Death of serum-free mouse embryo cells caused by epidermal growth factor deprivation is prevented by cycloheximide, 12-o-tetradecanoylphorbol 13-acetate or vanadate. *Exp. Cell. Res.* 186:177–181.

Rawson, C., D. Loo, A. Helmrich, T. Ernst, T. Natsuno, G. Merrill, and D. Barnes. 1991. Serum inhibition of proliferation of serum-free mouse embryo (SFME) cells. *Exp. Cell Res.* 192:271–277.

Rodriguez-Tarduchy, G., and A. Lopez-Rivas. 1989. Phorbol esters inhibit apoptosis in IL-2-dependent T-lymphocytes. *Biochem. Biophys. Res. Commun.* 164:1069–1075.

Sakai, Y., C. Rawson, K. Lindburg, and D. Barnes. 1990. Serum and transforming growth factor beta regulate glial fibrillary acidic protein in serum-free mouse embryo cells. *Proc. Natl. Acad. Sci. USA.* 87:8378–8382.

Shipley, G. D., and R. G. Ham. 1983. Multiplication of Swiss 3T3 cells in a serum-free medium. *Exp. Cell Res.* 146:249–260.

Thoenen, H., and Y. -A. Barde. 1980. Physiology of nerve growth factor. *Physiol. Rev.* 60:1284–1335.

Todaro, G. J., and H. Green. 1963. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J. Cell Biol.* 17:299–313.

Yanker, B. A., and E. M. Shooter. 1982. The biology and mechanism of action of nerve growth factor. *Annu. Rev. Biochem.* 51:845–868.

Yip, H. K., and E. M. Johnson. 1984. Developing dorsal root ganglion neurons require trophic support from their central processes: evidence for a role of retrogradely transported nerve growth factor from the central nervous system to the periphery. *Proc. Natl. Acad. Sci. USA.* 81:6245–6249.