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Acidic and basic fibroblast growth factors are survival factors with distinctive activity in quiescent BALB/c 3T3 murine fibroblasts

(protein synthesis/protein kinase C/platelet-derived growth factor/membrane vesiculation/nuclear condensation and disruption)

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ABSTRACT **Platelet-derived growth factor (PDGF), epidermal growth factor, and insulin-like growth factor have previously been identified as survival factors with distinctive activities for the density-inhibited quiescent BALB/c 3T3 murine fibroblasts. Fibroblast growth factor (FGF), like PDGF, renders quiescent BALB/c 3T3 cells competent to respond to epidermal growth factor and insulin-like growth factor, which mediate cell-cycle traverse through G_1 into S phase [Stiles, C. D., Pledger, W. J., VanWyk, J. J., Antoniades, H. N. & Scher, C. D. (1979) *Proc. Natl. Acad. Sci. USA* 76, 1279–1283]. We now show that FGF possesses marked cell survival-enhancing activity distinctive from that of PDGF. Both acidic FGF (aFGF) and basic FGF (bFGF) markedly enhance short-term (3-hr) survival of quiescent cells. bFGF is the more active of the two factors and shows marked long-term (20-hr) survival-promoting activity alone, whereas aFGF requires heparin for long-term activity. Protection by bFGF or aFGF plus heparin is not associated with cell-cycle traverse into S phase. Both the short-term (3-hr) and long-term (20-hr) protective actions of aFGF and bFGF critically depend on protein synthesis, whereas those of PDGF do not. The accumulated evidence shows that several growth factors can contribute to maintenance of the integrity of quiescent murine fibroblasts and that their action can involve protein kinase A- and C-mediated processes as well as protein synthesis. Different growth factors display distinctive modes of action.**

Density-arrested quiescent murine BALB/c 3T3 fibroblasts provide a striking example of the dependence of cell survival on growth factors. Upon withdrawal of serum from the medium most of the cells die within 3 hr unless the medium is supplemented with platelet-derived growth factor (PDGF) or insulin-like growth factor (IGF-1) (1). This type of death is not dependent on macromolecular synthesis (1). In the presence of only PDGF a majority of the cells survive for at least 20–24 hr, whereas with IGF-1, the additional presence of epidermal growth factor (EGF) is required to assure long-term survival. The finding (56) that survival of quiescent cells can also be achieved in serum-free culture medium by adding second-messenger agonists that activate protein kinase A or C supports the hypothesis that activation of signal-transduction pathways plays a major role in the cell survival-enhancing activity of growth factors (1). We have shown that some survival-enhancing effects of growth factors and second-messenger agonists are mediated via processes not involving *de novo* protein synthesis, whereas other effects require protein synthesis (1, 56). These findings are consistent with the following model: growth factors in serum regulate the activity of metabolic processes essential for the survival of quiescent density-inhibited BALB/c 3T3 fibroblasts, and this regulation involves second-messenger pathways.

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Acidic FGF (aFGF) and basic FGF (bFGF) are expressed in a wide variety of cell types and are potent inducers of DNA synthesis in diverse diploid cell types from mesodermal and neuroectodermal lineages (for reviews, see refs. 2 and 3). Bovine aFGF and bFGF share 55% sequence identity (4). Binding to heparin potentiates the mitogenic activity of aFGF (5, 6) but not that of bFGF (7). bFGF does not itself appear to be actively degraded by cells (8) or to behave like a typical secretory protein (see, e.g., refs. 9 and 10). FGF, like PDGF, renders quiescent BALB/c 3T3 cells competent to respond to EGF and IGF-1, which mediate traverse through G_1 into S phase (11).

It has been reported recently that FGFs promote *in vitro* survival of neurons from different parts of the nervous system (12–16). In addition, neuronal survival-enhancing effects have also been seen after lesion *in vivo* (17, 18).

We report that aFGF and bFGF have marked short- and long-term survival-promoting activity for quiescent BALB/c 3T3 fibroblasts. The long-term activity of aFGF requires heparin. In contrast to results obtained with PDGF, both short- and long-term protective activities of aFGF and bFGF critically depend on protein synthesis. Characteristic aspects of the mode of death of serum- and growth factor-deprived quiescent cells have been defined.

MATERIALS AND METHODS

Cell Culture. Density-inhibited quiescent cultures of BALB/c 3T3 cells were prepared in 96-well microtiter plates as described (1). Cultures were washed once with serum-free Dulbecco's modified Eagle's medium (DMEM) before setting up experiments. Cell survival-enhancing activity was measured in serum-free DMEM.

Materials. Human PDGF was purchased from R & D Systems (Minneapolis, MN); recombinant human aFGF and bFGF were purchased from Upstate Biotechnology (Lake Placid, NY) and from Biosource International (Westlake Village, CA); heparin was from Organon; [^{3}H]thymidine (6.7 Ci/mmol; 1 Ci = 37 GBq) and ^{125}I-labeled deoxyuridine were from New England Nuclear/DuPont; cycloheximide (CHX) and Hoechst dye 33258 came from Sigma; staurosporine was from Kamiya Biomedical (Thousand Oaks, CA); valinomycin was from Calbiochem; and 1-kilobase (kb) DNA marker was from BRL. Actinomycin D and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) were from Merck Sharp & Dohme.

Assays for Cell Survival and DNA Synthesis. DNA synthesis was measured by [^{3}H]thymidine incorporation as described

Abbreviations: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; IGF-1, insulin-like growth factor; BS, bovine serum; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; CHX, cycloheximide; DMEM, Dulbecco's modified Eagle's medium.

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(1). Cell survival was assayed by measurement of neutral red uptake (1, 19, 56). Survival in serum-free medium with or without added growth factors was expressed as percentage of survival in 10% bovine serum (BS)-supplemented medium. Determinations of the survival-enhancing activity of growth factors by neutral red-uptake measurement and cell enumeration give equivalent results (56).

Fluorescence Staining for DNA. Cells were grown to confluence, washed once, and incubated for 1 hr in DMEM with or without 10% BS. Monolayers were fixed directly by adding an equal volume of methanol/acetic acid, 3:1 for 5 min, fixed twice more with methanol/acetic acid, and air dried. Samples were then stained for 10 min with Hoechst dye 33258 at 0.5 μg/ml of phosphate-buffered saline and examined by fluorescence microscopy (excitation wavelength of 365 nm; emission wavelength of 480 nm). Photographs were taken of the same fields by using phase-contrast and fluorescence optics.

Agarose Gel Analysis for DNA Fragmentation. Cells (4×10^6) were incubated for 1 hr in DMEM with or without IGF-1 at 40 ng/ml plus EGF at 10 ng/ml. Cells were lysed at room temperature with the addition of an equal volume of 2× TET lysis buffer (2× TET buffer is 20 mM Tris, pH 7.5/20 mM EDTA/0.4% Triton X-100) and processed for analysis as described (20). Positive controls were prepared from P815 mastocytoma cells treated with 100 μM valinomycin for 4 hr (20).

Labeling with ^{125}I-Labeled Deoxyuridine and Assay for DNA Release. Three days after seeding, cultures were labeled for 2 hr with ^{125}I-labeled deoxyuridine. The label was removed, cultures were washed approximately five times with medium, DMEM/10% BS was added, and incubation was continued for 4 days at which time cultures were quiescent. Medium was removed, and monolayers were washed once with serum-free medium before addition of DMEM with or without 10% BS or IGF-1 plus EGF. Cultures were incubated for different periods, lysed in 0.5% SDS or 1× TET lysis buffer, centrifuged, and the supernatants were assayed for radioactivity. Radioactivity of cells lysed in 0.5% SDS was used to measure total release; radioactivity of cells that received medium with serum was used to estimate spontaneous release. Percent of specific ^{125}I release was calculated as follows: % specific release = [(experimental release − spontaneous release)/(total release − spontaneous release)] × 100.

RESULTS

Kinetics of Cell Loss in Serum-Free Medium. Fig. 1 shows that the decrease in viable quiescent BALB/c 3T3 cells after replacement of 10% BS-containing growth medium with serum-free medium follows approximately first-order kinetics until the establishment of a new steady-state value ≈5.0 hr after the beginning of serum deprivation (56). In most experiments one-half of the cells become nonviable in ≈1 hr. The average steady-state level reached by 5 hr is maintained for at least 15–20 hr, and it is commonly in the 10% range, although the level can be as low as 5 and as high as 30% (1, 56). Scher *et al.* (21) also described approximately first-order kinetics for the decline in cell number of density-arrested BALB/c 3T3 fibroblasts deprived of serum. However, the decline was much slower in the plasma-supplemented medium used by Scher *et al.* (21). A new steady-state level of ≈17% was reached in ≈5 days (21).

Short-Term (3- or 5-hr) Cell Survival-Promoting Activities of aFGF and bFGF. Fig. 2 *A* and *B* shows that both aFGF and bFGF are highly active in enhancing the survival of quiescent BALB/c 3T3 cells, with bFGF exhibiting somewhat greater activity per unit weight. The maximum short-term protective effects of bFGF, PDGF, and IGF-1 are comparable; however, the activity per unit weight of IGF-1 is lower than that of bFGF or PDGF (ref. 1 and present results).

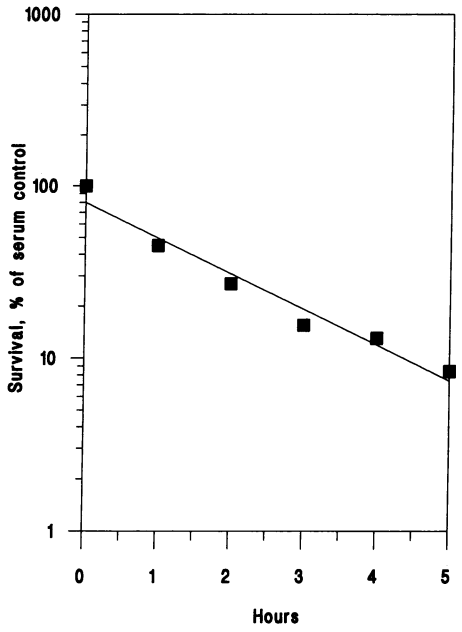

FIG. 1. The survival curve for density-inhibited quiescent BALB/c 3T3 mouse fibroblasts in serum-free medium follows approximately first-order kinetics. The results are the geometric means of six experiments; correlation coefficients ranged from 0.918 to 0.997 with a mean of 0.960. Mean neutral red uptake in 10% BS controls was 0.797 ± 0.148 OD unit. In a series of eight other experiments in which cell survival in serum-free medium was measured at 24 hr mean neutral red uptake was 9.6% (56); thus, by 5 hr a new steady state is reached.

Long-Term (20-hr) Cell Survival-Promoting Activities of aFGF and bFGF and Effects of Heparin. Table 1 shows that heparin at 100 ng/ml, but not at 10 ng/ml, greatly increased the long-term (20-hr) protective activity of aFGF without any protective effect itself. Heparin did not significantly alter the concentration-dependent effects of bFGF used at 2 and 10 ng/ml. Long-term survival in the presence of aFGF plus heparin or bFGF is not associated with S-phase entry of cells in the absence of other growth factors (data not shown).

Dependence of Cell Survival-Promoting Activities of aFGF, bFGF, and PDGF on mRNA and Protein Synthesis. We have investigated the effects of DRB, a selective inhibitor of

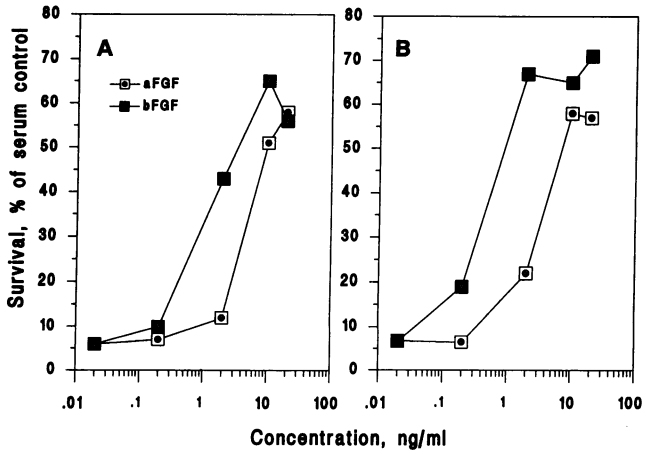

FIG. 2. aFGF and bFGF markedly enhance survival of serum-deprived quiescent BALB/c 3T3 cells. Growth factors were added in serum-free medium, and cultures were incubated for 3 (*A*) or 5 (*B*) hr before neutral red staining. Neutral red uptake in 10% BS controls was as follows: 0.867 ± 0.026 OD unit (*A*) and 0.992 ± 0.075 OD unit (*B*); in serum-free controls neutral red uptake was 4% (*A*) and 5% (*B*) of the respective 10% BS control.

Table 1. Heparin enhances the otherwise low long-term (20-hr) survival-promoting activity of aFGF and does not affect the high activity of bFGF

Agent	Concentration, ng/ml	Cell survival, % of BS control*		
			Heparin	
		No heparin	10 ng/ml	100 ng/ml
None		3	2	2
aFGF	2	13	16	48
aFGF	10	19	12	54
bFGF	2	30	22	39
bFGF	10	66	61	66

*Neutral red uptake in 10% BS controls was 0.993 OD unit.

messenger precursor RNA transcription, and of CHX, an inhibitor of protein synthesis, on the growth factor-enhanced survival of quiescent BALB/c 3T3 cells. Twenty or forty micromolar DRB decreased slightly the short-term (3-hr) survival of quiescent cells mediated by bFGF (20 ng/ml) or PDGF (5 ng/ml) and had a marginally greater effect on aFGF (20 ng/ml)-mediated survival (data not shown). In sharp contrast, CHX at 1 μg/ml decreased the aFGF- or bFGF-mediated survival by 75% and at 5 μg/ml by >90%, while having little effect on PDGF-mediated survival.

Results of long-term (20-hr) survival experiments with CHX (Table 2) further support the critical importance of protein synthesis in aFGF- or bFGF-mediated cell survival and the lack of such a role in PDGF-mediated survival. Results with DRB suggest that the role of mRNA synthesis in long-term survival of cells increases from PDGF to bFGF and again from bFGF to aFGF.

As reported (1), DRB and CHX themselves decrease survival of quiescent BALB/c 3T3 cells in serum-free medium in the absence of growth factors. Therefore, direct lethal effects of DRB and CHX could account for some cell loss when cultures are incubated with growth factor plus DRB or CHX; however, this effect may, at least in part, be counterbalanced by a protective effect of the growth factors either in serum-free medium or in serum against the lethal effects of inhibitors (22, 56). The finding of note is the very marked differential in the effects of CHX with respect to aFGF or bFGF on the one hand vs. PDGF on the other.

Kinetics of Blocking FGF-Mediated Cell Survival with CHX. We have investigated the following questions: (*i*) how soon after removal of serum from quiescent cultures aFGF- or bFGF-mediated survival becomes dependent on protein synthesis, (*ii*) how long a period of CHX treatment is required to maximally suppress aFGF- or bFGF-mediated survival. Groups of cultures were examined 1, 2, or 3 hr after serum removal and aFGF or bFGF addition at 20 ng/ml with or without CHX at 1 μg/ml. Table 3 shows that cell survival with either aFGF or bFGF is already affected by CHX within 1 hr of serum replacement with FGF and that decrease in cell survival is maximal within 2 hr.

Is the requirement for protein synthesis in FGF-mediated cell survival independent of the duration of FGF exposure? Serum-deprived quiescent cells again received FGF for 1, 2, or 3 hr, but CHX, when present, was present only during the last hr of cell exposure to aFGF or bFGF. Such 1-hr exposure of FGF-protected serum-deprived cultures to CHX leads to a ≈25% decrease in survival, regardless of time of CHX addition (data not shown). This result is consistent with the approximately first-order cell-death kinetics illustrated in Fig. 1, which show that within any given time interval within 5 hr after serum deprivation, an approximately constant fraction of the population enters the death process.

Effect of Staurosporine on the Cell Survival-Promoting Activity of FGF. We found in previous short-term (3-hr) experiments that 50 nM staurosporine markedly decreased the survival-promoting effect of 100 nM phorbol 12-myristate 13-acetate but not that of 0.75 or 2.9 mM 8-bromoadenosine 3′,5′-cyclic monophosphate (56), which is consistent with its mode of action as a protein kinase inhibitor with some selectivity for protein kinase C. Staurosporine did not significantly decrease or only slightly decreased the survival-promoting effect of PDGF, indicating that protein kinase C function is not critically required. This fact is not surprising, as PDGF also activates at least one other pathway—i.e., the cAMP–protein kinase A pathway—that can mediate cell survival.

In the present 3-hr experiments, 50 nM staurosporine had only a slight suppressive effect on the cell survival-enhancing activity of aFGF and bFGF (data not shown). This fact suggests that the diacylglycerol–protein kinase C signal-transduction pathway is not the only pathway through which FGFs could activate a protein synthesis-dependent cell-survival mechanism.

Morphology of Cells Undergoing the Death Process. Previous phase-contrast cinemicrographic observations by Scher *et al.* (21) revealed that a serum-deprived cell would undergo the death process from the first visible change, which was darkening of the cytoplasm, to complete disintegration within a time span of <30 min. In the course of this, the cell border began to ruffle, and the cell contracted by pinching off large vesicles. Further contraction gave rise to "mulberry-like" cell forms that continued to shed vesicular debris, until only a contracted nuclear body remained (21). As already emphasized, Scher *et al.* (21) followed the death process in cell populations incubated with 10% plasma, which delayed onset of cell deaths by ≈15 hr and greatly decreased the rate at which cell deaths occurred. In our experiments cell deaths begin occurring within the first 30 min after shifting the density-inhibited quiescent BALB/c 3T3 cells from serum-containing to serum- and growth factor-free medium.

To investigate changes in nuclear morphology and their relationship to plasma membrane vesiculation, cultures of

Table 2. Effect of RNA and protein synthesis inhibitors on long-term (20-hr) survival of cells mediated by aFGF and bFGF

Inhibitor	Cell survival					
	None	aFGF (20 ng/ml) + heparin*		bFGF (20 ng/ml)		PDGF (5 ng/ml)	
		% BS control†	% no inhibitor	% BS control†	% no inhibitor	% BS control†	% no inhibitor
None	6	72		74		34	
DRB (20 μM)	2	35	49	41	63	24	72
DRB (40 μM)	4	23	31	28	37	17	53
CHX (1 μg/ml)	2	19	25	18	19	31	93
CHX (5 μg/ml)	1	9	11	6	6	22	67

*Alone, heparin (100 μg/ml) had no effect on cell survival, which was 6% of the 10% BS control.
†Neutral red uptake in 10% BS control was 1.015 OD units. Results are the means of two experiments.

Table 3. Inhibition of protein synthesis rapidly decreases FGF-mediated cell survival

Hr	Decrease in survival with CHX (1 μg/ml), %: aFGF (20 ng/ml)	bFGF (20 ng/ml)
1	26	21
2	75	67
3	72	67

Viability of quiescent BALB/c 3T3 cells was determined 1, 2, or 3 hr after serum removal and addition of either aFGF plus CHX or bFGF plus CHX and expressed relative to cell survival in CHX-free samples. Neutral red uptake in triplicate 3-hr 10% BS controls was 0.714 ± 0.095 OD unit and in duplicate serum-free controls the uptake was 0.370 ± 0.069 OD unit at 1 hr, 0.212 ± 0.038 OD unit at 2 hr, and 0.0815 ± 0.012 OD unit at 3 hr or 52%, 30%, and 11% of 10% BS control, which agrees well with the results of Fig. 1. Without CHX, survival values at 1, 2, and 3 hr, expressed as % of 3-hr 10% BS control, were 55, 66, and 70% for aFGF and 62, 69, and 48% for bFGF, respectively.

density-inhibited quiescent BALB/c 3T3 cells that had been shifted to serum- and growth factor-free medium were stained for DNA with Hoechst dye 33258. Fig. 3 shows cultures incubated for 1 hr after medium change: phase-contrast photomicrographs of selected fields are on the left, and fluorescence photomicrographs of the same fields are on the right. The control cultures that received 10% BS (Fig. 3 *A* and *B*) display morphology typical for confluent BALB/c 3T3 cells. Chromatin is homogeneously distributed within the nuclei, each of which has several nucleoli.

Fig. 3 shows that nuclear morphology is not uniform in serum-deprived cells that appear in the early stages of contraction and show evidence of cytoplasmic blebbing at the plasma membrane (Fig. 3 *C* and *D*). As revealed by fluorescence microscopy, such cells may contain a nucleus displaying partially condensed chromatin (Fig. 3*D*, center) or a multi-lobed (Fig. 3*D*, below center) nucleus with condensed chromatin. The nucleus is deformed or appears to have disintegrated in cells in later stages of cytoplasmic blebbing and contraction (data not shown). Cells in similar stages of vesiculation and contraction sometimes display different nuclear morphologies.

Lack of DNA Cleavage at Internucleosomal Sites During the Death Process. DNA fragmentation was measured in the postnuclear supernatant of ^{125}I-labeled deoxyuridine-labeled quiescent BALB/c 3T3 cells incubated for up to 2 hr with or without serum or IGF-1 plus EGF. No significant differences were seen among cell samples incubated under different conditions. ^{125}I-labeled deoxyuridine release was <5% of total label in all cases, calculated as described. A series of experiments was also done in which DNA from nuclear and postnuclear fractions of quiescent cells incubated with or without growth factors was resolved in agarose gels and stained with ethidium bromide. No evidence of fragmentation of BALB/c 3T3 cell DNA into oligonucleosomal subunits was obtained, whereas such fragmentation was seen in P815 cells treated with valinomycin.

To summarize, analysis of DNA from cultures incubated without serum or growth factors failed to show any evidence of DNA cleavage into oligonucleosomal subunits of discrete sizes characteristic of cell death by the process of apoptosis.

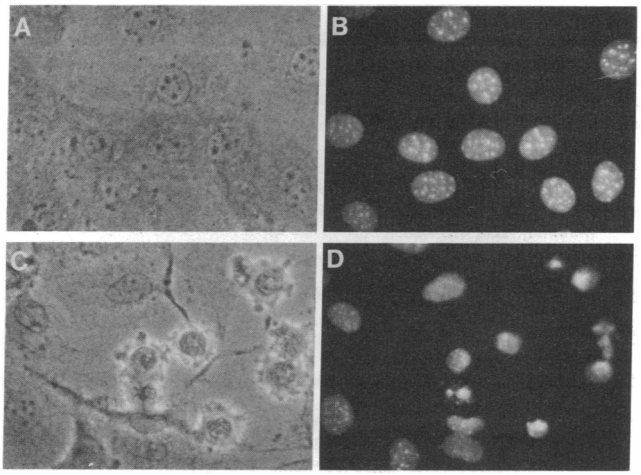

FIG. 3. Morphological changes in serum- and growth factor-deprived cells. Quiescent cultures of BALB/c 3T3 cells were stained with Hoechst 33258 dye 1 hr after medium change and photographed by phase-contrast (*Left*) or fluorescence (*Right*) optics. (*A* and *B*) Ten percent BS-containing medium. (*C* and *D*) Serum- and growth factor-free medium. (×220.)

DISCUSSION

Taken together our evidence indicates that aFGF and bFGF protect density-inhibited quiescent BALB/c 3T3 fibroblasts against cell death only via protein synthesis-dependent mechanisms, whereas PDGF can protect cells via mechanisms that either are or are not dependent on protein synthesis. bFGF is a highly active survival factor for quiescent BALB/c 3T3 cells. aFGF is somewhat less active and requires heparin for its long-term activity. Both the short-term (3-hr) and long-term (20-hr) effects of the FGFs critically depend on ongoing protein synthesis, whereas those of PDGF show little dependence (ref. 1 and present results). Previous findings in quiescent 3T3 cells showed that PDGF causes an accumulation of cAMP (23, 24) and that the cell-survival-enhancing action of the cAMP analog 8-bromoadenosine 3′,5′-cyclic monophosphate is largely independent of protein synthesis (56). These findings indicate that PDGF can protect quiescent cells against death through a protein synthesis-*independent* mechanism by elevating the cAMP level and thereby activating protein kinase A. bFGF, the survival-enhancing action of which requires protein synthesis, has no effect alone on basal cAMP synthesis, at least not in Chinese hamster fibroblasts (25), and thus the cAMP–protein kinase A pathway would not be open to bFGF.

The following evidence indicates that activation of the diacylglycerol–protein kinase C pathway is linked to one or more protein synthesis-*dependent* mechanisms, whereby aFGF, bFGF, and PDGF can protect quiescent cells against death. (*i*) PDGF (26–30) and FGF (29–31) activate protein kinase C. (*ii*) Enhancement of cell survival through protein kinase C activation by means of phorbol 12-myristate 13-acetate requires protein synthesis (56). However, our results indicate that stimulation of the protein kinase C pathway is not the only means of activating protein synthesis-dependent mechanism(s) through which the FGFs and PDGF can protect cells, as blocking of this pathway with staurosporine did not substantially decrease the survival-enhancing activities of these growth factors (ref. 56 and present results). That the mitogenic effects of FGFs may not be mediated via polyphosphoinositide breakdown and protein kinase C activation is of interest (31–33). Of possible relevance in connection with both cell protection against death and the mitogenic effect may be the demonstration of tyrosine phosphorylation of FGF receptor proteins of M_r 150,000 and M_r 130,000 (34) and of a M_r 90,000 protein (35). Taken together, the evidence clearly shows redundancy in the pathways mediating cell survival.

In the physiological mode of cell death or apoptosis (36), cells undergo well-defined degenerative changes that, in many systems, ultimately result in cleavage of the nuclear DNA into oligonucleosome chains and cell fragmentation (37–42). Apoptosis in nucleated eukaryotic cells is distin-

guishable on morphological and biochemical grounds from necrosis, which occurs from severe hypoxia, hyperthermia, cytocidal viral infection, exposure to a variety of toxins and respiratory poison, and complement attack (for review, see ref. 39). Necrosis can be viewed as a pathological response to a major perturbation in the cellular environment, whereas apoptosis appears to be, in many instances, a part of homeostatic regulation.

The regulation of physiological cell death appears complex and, moreover, the specific details vary from one system to another. In several systems inhibitors of macromolecular synthesis delay or prevent apoptosis (43–47); however, exceptions have been reported (50, 51). Furthermore, inhibition of mRNA or protein synthesis could itself cause apoptosis in the premyelocytic leukemia HL-60 cell line and to a varying degree in several other human cell lines (51). Thus, the fact that inhibition of macromolecular synthesis does not prevent death of quiescent BALB/c 3T3 cells deprived of cell growth factors (1) is not in and of itself sufficient to exclude the possibility that serum-deprived BALB/c 3T3 undergo apoptosis attended by DNA degradation into oligonucleosome chains.

Recent findings show that certain cell types can be protected against apoptosis by activation of protein kinase C (47, 52–55). We have evidence that protein kinase C activation provides one, but not the only, pathway for protecting serum-deprived quiescent BALB/c 3T3 cells against death (56).

However, we have found that serum-deprived BALB/c 3T3 cells usually do not undergo nuclear condensation and lobulation of the type commonly associated with apoptosis. We do observe chromatin condensation and fragmentation, but these changes appear secondary to cellular contraction, as vesicles form and are pinched off at the cell surface. We have not obtained any evidence of DNA cleavage into nucleosome-sized fragments and multiples thereof. Serum-deprived BALB/c 3T3 cells also do not display changes commonly associated with cell necrosis, such as swelling of cells before rupture. It is apparent that density-inhibited quiescent BALB/c 3T3 fibroblasts display an unusual death mode when incubated in serum- and growth factor-free medium. The available evidence is consistent with two hypotheses: (*i*) the past history of the cells—i.e., quiescence induced through density inhibition predisposes the cells to death, and (*ii*) after an initiating event, which may lead to ionic imbalance or drop in ATP level, death follows a rapid predetermined course. Further studies are needed to establish the precise mode of death of growth factor-deprived BALB/c 3T3 cells.

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