Autocrine Signals Enable Chondrocytes to Survive in Culture

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Abstract. We recently proposed that most mammalian cells other than blastomeres may be programmed to kill themselves unless continuously signaled by other cells not to. Many observations indicate that some mammalian cells are programmed in this way, but is it the case for most mammalian cells? As it is impractical to test all of the hundreds of types of mammalian cells, we have focused on two tissues—lens and cartilage—which each contain only a single cell type: if there are cells that do not require signals from other cells to avoid programmed cell death (PCD), lens epithelial cells and cartilage cells (chondrocytes) might be expected to be among them. We have previously shown that rat lens epithelial cells can survive in serum-free culture without signals from other cell types but seem to require signals from other lens epithelial cells to survive: without such signals they undergo PCD. We show here that the same is true for rat (and chick) chondrocytes. They can survive for weeks in culture at high cell density in the absence of other cell types, serum, or exogenous proteins or signaling molecules, but they die with the morphological features of apoptosis in these conditions at low cell density. Medium from high density cultures, FCS, or a combination of known growth factors, all support prolonged chondrocyte survival in low density cultures, as long as antioxidants are also present. Moreover, medium from high density chondrocyte cultures promotes the survival of lens epithelial cells in low density cultures and vice versa. Chondrocytes isolated from adult rats behave similarly to those isolated from developing rats. These findings support the hypothesis that most mammalian cells require signals from other cells to avoid PCD, although the signals can sometimes be provided by cells of the same type, at least in tissues that contain only one cell type.

MANY types of vertebrate cells require signals from other cells to survive, at least in culture. Developing neurons, for example, require neurotrophic factors (Hamburger and Levi-Montalcini, 1949; Korshing and Thoenen, 1983; Cowan et al., 1984; Levi-Montalcini, 1987; Purves, 1988; Barde, 1989; Oppenheim, 1991), myeloid cells require colony stimulating factors (Metcalf, 1989; Williams et al., 1990; Koury and Bondurant, 1990), endocrine-dependent cells require specific hormones (Kerr and Searle, 1973; Krypaniou and Issacs, 1988; Wyllie et al., 1973, 1980), developing oligodendrocytes and their precursor cells require specific growth factors and cytokines (Barres et al., 1992; Barres et al., 1993*b*), and so on. If deprived of survival factors, these cells seem to activate an intrinsic death program and kill themselves—a process called programmed cell death (PCD).[1] Cells that die in this way normally undergo a characteristic set of morphological changes referred to as apoptosis, where the cells shrink and often fragment (Kerr et al., 1972; Wyllie et al., 1980; Searle et al., 1982). Naturally occurring cell deaths in developing tissues (Glucksmann, 1951) are also thought to be examples of PCD (Saunders, 1966; Wyllie et al., 1980). As they can sometimes be prevented or postponed by treatment of the developing animal with exogenous survival factors (Hamburger et al., 1981; Hofer and Barde, 1988; Oppenheim et al., 1988; Barres et al., 1992, 1993*a*; Coles et al., 1993), it is likely that at least some of these deaths occur because the cells fail to get enough endogenous survival factors.

We recently proposed that most mammalian cells are programmed to kill themselves unless they are continuously signaled by other cells not to (Raff, 1992). Such "social" controls on cell survival would ensure that cells only survive when and where they are needed, just as similar social controls on cell proliferation are thought to ensure that cells only proliferate when new cells are needed (Baserga, 1985). But do most mammalian cells need signals from other cells to survive? If there are cells (other than blastomeres—Biggers et al., 1971) that can survive without signals from other cells, lens and cartilage cells might be expected to be among them, as both lens and cartilage are unusual in that they contain only a single cell type and are not innervated, vascularized,

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1. *Abbreviations used in this paper*: BSO, buthionine-sulfoximine; IGF, insulin-like growth factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCD, programmed cell death; T_3, triiodothyronine; Tm, melting point; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling.

or penetrated by lymphatic vessels (Goss, 1978; Fawcett, 1986). We have previously demonstrated that rat lens epithelial cells can survive for weeks in the absence of other cell types, serum, exogenous proteins, or non-protein signaling molecules if cultured at high cell density, but they undergo PCD if cultured in these conditions at low cell density; and culture medium from high density cultures promotes the survival of cells in low density cultures (Ishizaki et al., 1993). These results suggest that lens epithelial cells do not require signals from other cell types to survive but do require signals from other lens epithelial cells to survive, at least in culture.

Bruckner and his colleagues earlier reported similar observations on chick chondrocytes (Bruckner et al., 1989). They showed that these cells can survive for long periods in serum free culture without exogenous proteins if they are cultured at high density but die if cultured at low density, and they showed that conditioned medium from high density cultures promotes cell survival in low density cultures. They identified the active ingredients in the conditioned medium as low molecular mass compounds that could be replaced by antioxidants such as cysteine or dithioerythritol (Tschan et al., 1990). Here we confirm that both chick and rat chondrocytes require antioxidants to survive in low density cultures, and we show that they die by apoptosis in the absence of antioxidants. More important, however, we demonstrate that antioxidants are not sufficient on their own to protect chondrocytes from PCD in low density culture: other signals are also required and these can be provided by the addition of medium conditioned by high density chondrocyte cultures, FCS, or a combination of known growth factors; the survival factors in conditioned medium seem to be of high molecular mass (>10 kD). We also demonstrate that chondrocytes from adult rats behave similarly in these respects to chondrocytes from developing animals, and that medium from high density chondrocyte cultures also promotes the survival of lens epithelial cells in low density cultures and vice versa. We conclude that a cultured chondrocyte, isolated from either a developing or mature animal, requires signals from other cells to avoid PCD and these signals can be provided by other chondrocytes. These findings support the hypothesis that PCD is the default pathway for most mammalian cells (Raff, 1992; Raff et al., 1993).

Materials and Methods

Animals and Materials

Sprague-Dawley rats were obtained from the breeding colony of the University College London Animal Facility. Fertilized eggs were purchased from Ross Poultry Ltd. (Andover, UK). Recombinant human platelet-derived growth factor BB (PDGF), transforming growth factors α and $\beta 2$ (TGF-α and TGF-β), bFGF were purchased from Peprotech, Inc. (Rocky Hill, NJ). Insulin, triiodothyronine (T_3), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), HOECHST 33342, superoxide dismutase from beef liver, cysteine, cystine, glutathione (reduced or oxidized), buthionine-sulfoximine (BSO), cycloheximide, α-chymotrypsin, collagenase, soybean trypsin inhibitor, trypsin, BSA (highly purified, crystalline grade), poly-D-lysine, and staurosporine were purchased from Sigma Chemical Co. (St. Louis, MO). Catalase from bovine erythrocytes, terminal deoxynucleotidyl transferase from calf thymus, and biotinylated dUTP were purchased from Boehringer Mannheim GmbH (Germany). L-15 medium, FCS, high melting point (Tm) agarose, and low Tm agarose were purchased from GIBCO-BRL (Gaithersburg, MD). EDTA was purchased from BDH Laboratory Supplies, Merck, Ltd. (Poole, UK). DME and Ham's F-12 medium (F-12) were purchased from Flow Laboratories (Irvine, UK). Insulin-like growth factor 1 (IGF-1) was kindly provided by Dr. Mats Lake (Kabigen AB, Stockholm, Sweden). Ultrafiltration devices (Ultrafree-CL filters, 10,000 NMWL low-binding cellulose) and 0.22 μm filters (MillexGV) were purchased from Millipore Corp. (Bedford, MA).

Isolation of Chondrocytes

Postnatal rat chondrocytes were prepared according to Bruckner et al. (1989) with some modifications. Sterna from postnatal day 11 (P11) rats were rinsed in L-15, cleaned of muscle and connective tissue, and the distal part of the xiphoid process was cut off and collected in L-15. After the perichondrium was carefully removed using a dissecting microscope, the xiphoid processes were incubated in a 1:1 mixture of DME and F-12 (DME/F-12), containing 1 mg/ml of chymotrypsin and 2 mg/ml of collagenase, for 20 min at 37°C. They were washed with DME/F-12 to remove superficial cells released by the enzyme treatment, and were then incubated in DME/F-12 containing 2 mg/ml of collagenase for 2 h at 37°C. The tissue fragments were disrupted mechanically by repeated trituration using a Pasteur pipette, and the released cells were passed through a Nylon mesh (20 μm pore size), washed twice in DME/F-12 containing 0.5% BSA, and resuspended in an appropriate volume of either F-12 for higher cell density cultures ($\geq 10^6$ cells/ml) or F-12 containing 0.5% BSA for lower cell density cultures ($\leq 10^5$ cells/ml).

Chondrocytes from male adult rats (body weight 250 g) were prepared as described above for preparation of P11 chondrocytes, except that only the distal tip of the xiphoid process was used. Chick chondrocytes were prepared from the sterna of 17-d-old chick embryos as described above for preparation of rat chondrocytes.

Preparation of Rat Lens Epithelial Cells

Lens epithelial explant cultures were prepared from P11 rats according to McAvoy and Fernon (1984), with some modifications as previously described (Ishizaki et al., 1993). Lens explants in 35-mm tissue culture dishes were washed once with Ca^{2+}- and Mg^{2+}-free DME, and incubated with a mixture of trypsin (0.05%) and EDTA (0.025%) in Ca^{2+}- and Mg^{2+}-free DME for 8 min at 37°C. The incubation was stopped by the addition of 0.1% soybean trypsin inhibitor in DME/F-12, and the debris of the lens capsules was removed by filtering through a Nylon mesh. The cells were washed once in DME/F-12 containing 0.5% BSA and resuspended in an appropriate volume of F-12 containing 0.5% BSA.

Agarose Gel Cultures

Cells were cultured in agarose gels, a culture system where chondrocytes maintain their differentiated phenotype (Horwitz and Dorfman, 1970; Benya and Shaffer, 1982). The agarose gel cultures were set up as described by Benya and Schaffer (1982), with some modifications, either in Terasaki microwell plates (Nunc, Roskilde, Denmark), 96-well tissue culture plates (Falcon Labware), 35-mm tissue culture dishes (Falcon Labware), or in a slide flask (Nunc, Roskilde, Denmark). High Tm agarose (1% in water) was autoclaved for 20 min and used while above 90°C. The plastic culture surface was rapidly coated with agarose, and the excess was removed by aspiration. The culture plates, dishes, or flasks were placed on a level surface at room temperature until the agarose solidified; they were then transferred to a CO_2 incubator until used. Cell suspensions were prepared in low Tm agarose (2% in water), which was autoclaved for 20 min, and then cooled to 37°C before being mixed with an equal volume of 2× concentrated DME at 37°C to give a final concentration of 1% agarose in DME. We did not coat the wells of the Terasaki microwell plates with high Tm agarose because the low Tm agarose containing the suspended cells solidified so rapidly when added to the microwells that most of the cells did not reach the bottom.

Chondrocytes or trypsinized lens epithelial cells suspended in F-12 with or without 0.5% BSA were mixed with an equal volume of 1% agarose in DME at 37°C to give the appropriate cell densities in 0.5% agarose in DME/F-12. An appropriate volume (10 μl for wells of a Terasaki plate, 100 μl for wells of a 96-well tissue culture plate, 1.5 ml for a 35-mm tissue culture dish, and 750 μl for a slide flask) of the cell suspension was added to each well of the culture plate, or to the culture dish or flask. The culture vessel was held at 37°C in a CO_2 incubator for 15 min before the agarose was allowed to gel at 4°C for 15 min. After gelation, an equal volume of DME/F-12 was added and the culture vessel was returned to a CO_2 incubator.

Preparation of Conditioned Medium

Chondrocytes or lens epithelial cells were cultured in agarose gel either in 96-well tissue culture plates or in 35-mm tissue culture dishes as described above, at 10^6–10^7 cells/ml. To obtain conditioned medium from high density chondrocyte cultures, medium was removed and replaced by fresh DME/F12 medium every 2 d. The conditioned medium was immediately transferred to low density agarose gel cultures, which were fed with conditioned medium every 2 d. In experiments where conditioned medium from high density chondrocyte cultures was tested on low density lens cell cultures or vice versa, medium was removed from high density cultures and replaced by fresh medium every 24 h, and the low density agarose gel cultures were fed with the conditioned medium every 24 h.

Fractionation of Conditioned Medium by Ultrafiltration

1.5 ml of conditioned medium from high density chondrocyte cultures in a 35-mm tissue culture dish was poured into the filter cup of the Millipore ultrafiltration unit (molecular mass cutoff of 10 kD), and centrifuged at 3,000 *g* for 45 min (15 min × 3, with 5 min intervals) at room temperature. The concentrate (1.0 ml) and the filtrate (0.5 ml) were sterilized by passage through a 0.22-μm Millipore filter and immediately transferred to low density chondrocyte cultures.

Cell Survival Assays

We used Terasaki microwell plates for assaying cell survival, as each cell was clearly visible in the microwell, whereas this was not the case when cells were plated at low density ($\leq 10^5$ cells/ml) in 96-well plates. Cell survival was mainly assessed by the MTT assay, which measures mitochondrial dehydrogenase activity (Mosmann, 1983). MTT was dissolved in DME/F-12 at 5 mg/ml and sterilized by passage through a 0.22-μm Millipore filter. This stock solution was added (one part to 10 parts of medium) to each microwell of the Terasaki plate, and the plate was incubated at 37°C for 1 h. Viable cells with active mitochondria cleave the tetrazolium ring into a visible dark blue formazan reaction product, whereas dead cells remain uncolored. In high density cultures ($\geq 10^5$ cells/ml), 100 cells, dead and alive, were counted in an inverted Zeiss microscope and the fraction of live cells was determined. In low density cultures ($\leq 10^4$ cells/ml), the total numbers of live and dead cells were counted. Whereas cells did not generally divide in serum-free cultures, they did in the presence of 10% FCS, where colonies of cells (consisting of ≥4 cells) were frequently observed; we counted each such colony as one live cell.

In some cases, cell survival was assessed by staining cell nuclei with the vital DNA-binding dye HOECHST 33342. The stock solution (2 mg/ml, made in PBS and sterilized by passage through a 0.22-μm Millipore filter) was diluted 1:50 with DME/F-12, and added (one part to 10 parts of medium) to each microwell of the Terasaki plate, and the plate was incubated at 37°C for 30 min. The cells were counted in an inverted fluorescence microscope (Olympus, Tokyo, Japan). Dead cells were readily recognized, as they had a condensed or fragmented nucleus.

Terminal Deoxynucleotidyl Transferase-mediated dUTP-Biotin Nick End Labeling

In some cases, in situ DNA cleavage was assessed by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) technique, as described by Gavrieli et al. (1992), with some modifications. P11 rat chondrocytes were cultured in agarose gel in 96-well tissue culture plates as described above, at 10^4 cells/ml in DME/F-12 containing 1 mM cysteine, with or without 10% FCS. After 4 d in culture, the cells were fixed in 4% paraformaldehyde for 30 min at 4°C, washed extensively in 10 mM Tris-HCl, pH 8.0, and then permeabilized in 0.1% Triton X-100 in 10 mM Tris-HCl, pH 8.0, for 5 min at room temperature. After extensive washing in 10 mM Tris-HCl, pH 8.0, the cells were preincubated for 10 min at room temperature in the reaction buffer for terminal deoxynucleotidyl transferase supplied by Boehringer Mannheim GmbH (200 mM potassium cacodylate, 0.25 mg/ml BSA, 25 mM Tris-HCl, pH 6.6). The reaction was started by removing the preincubation buffer and adding the reaction mixture containing 500 U/ml terminal deoxynucleotidyl transferase, 2.5 mM $CoCl_2$ and 40 μM biotinylated dUTP. After 60 min at 37°C, the reaction was terminated by the addition of 300 mM NaCl and 30 mM sodium citrate. After 25 min at room temperature, cells were washed with PBS, and incubated with streptavidin fluorescein (diluted 1:100, Amersham, UK) for 60 min at room temperature in the dark. After extensive washing in PBS, the cells were examined in an inverted Olympus fluorescence microscope.

Time-lapse Video Recording

Chondrocytes isolated from P11 rats were plated at 10^5 cells/ml in agarose gel in a slide flask, as described above. The flask was placed on the stage of an inverted Zeiss microscope and maintained at 37°C. The cells were viewed using phase-contrast optics and time-lapse video recordings were made as described previously (Ishizaki et al., 1993).

Electron Microscopy of Staurosporine-treated Cells

Chondrocytes from P11 rats were plated at 2×10^7 cells/ml on glass coverslips that had been previously coated with poly-D-lysine (10 μg/ml). After 12 h in culture, the protein kinase inhibitor staurosporine (1 μM) was added and the cultures were maintained at 37°C for an additional 4 h, before they were fixed with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at room temperature overnight. After rinsing with veronal acetate buffer (pH 7.2), the cells were postfixed in 1% osmium tetroxide in the buffer for 2 h, and then stained with uranyl acetate, all at 4°C in the dark. The cells were then washed in the buffer, dehydrated through a graded series of alcohol, and embedded in Epon. After removing the glass coverslips, thin sections were cut on an LKB ultratome (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) parallel to the substratum. The sections were counterstained with lead citrate and uranyl acetate and examined using a Jeol electron microscope (model 100CX II) at 80 kV.

Results

Chondrocyte Survival in Protein-free Medium Is Cell-density Dependent

To study the survival requirements of chondrocytes in culture, chondrocytes prepared from the xiphoid process of the sternum of P11 rats were cultured in agarose gels at various cell densities in protein-free DME/F12 medium in Terasaki microwells. Cell survival was assessed by the MTT assay after various times. Viable cells converted the MTT into a dark blue reaction product (Fig. 1 *a*), while dead cells remained uncolored (Fig. 1 *b*). As reported previously for chick chondrocytes (Bruckner et al., 1989), survival of rat chondrocytes was strikingly cell-density dependent: when plated at $\geq 10^6$ cells/ml, most of the cells survived for many weeks, but when plated at $\leq 10^5$ cells/ml, most of the cells died within a few days (Fig. 2 *a*). When examined by phase contrast microscopy (Fig. 1, *c* and *d*) or fluorescence microscopy after staining with the DNA-binding dye HOECHST 33342 (Fig. 1, *e* and *f*), the dead cells in low density cultures had the typical morphological features of apoptosis (Fig. 1, *d* and *f*). In addition, in situ end-labeling using the TUNEL method (Gavrieli et al., 1992) revealed DNA fragmentation in the nucleus of the dead cells (Fig. 3). Time-lapse video recording also showed that the cells at low density died by apoptosis: after one or more episodes of surface blebbing, the cells shrank and stopped moving (Fig. 1, *g–i*).

As reported previously for chick chondrocytes (Tschan et al., 1990), the addition of cysteine to the culture medium greatly enhanced the survival of rat chondrocytes when cultured at $\leq 5 \times 10^5$ cells/ml, but, even in the presence of cysteine, when cells were plated at very low density ($\leq 10^4$ cells/ml), most of the cells died with the characteristic features of apoptosis within a few days (Fig. 2 *b*). Cystine also promoted survival, but to a lesser extent than cysteine, and as expected, the protective effect of both cysteine and cystine apparently depended on their intracellular conversion by

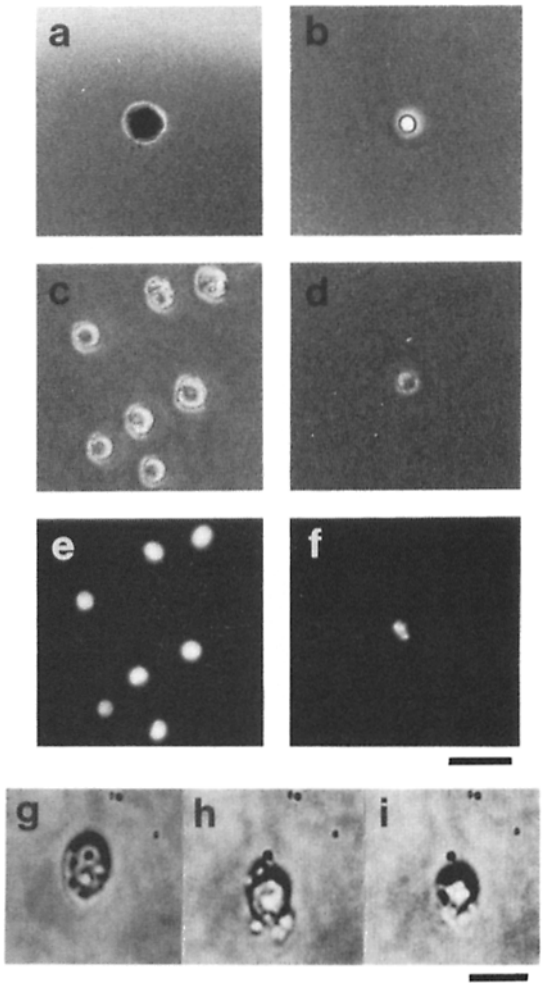

Figure 1. Apoptosis of chondrocytes in low density cultures. P11 rat chondrocytes were cultured in agarose in protein-free medium at high (*a*, *c*, and *e*) or low (*b*, *d*, *f*, *g*, *h*, and *i*) cell density and their viability was assessed by MTT assay (*a* and *b*), phase contrast microscopy (*c* and *d*), HOECHST 33342 staining and fluorescence microscopy (*e* and *f*), or time-lapse video recording (*g–i*). In *a–f*, the cells were cultured for 1–3 d in Terasaki microwells in the presence of cysteine: on the left, the cells were plated at high density (10^6 cells/ml) and are alive; on the right, the cells were plated at low density (10^4 cells/ml) and show the characteristics of apoptosis. In *g–i*, cells in a low density (10^5 cells/ml) culture were followed by time-lapse video recording: one cell is shown as it progressed from healthy (*g*, 98 h after plating), to active surface blebbing (*h*, 131 h after plating), to shrunken and immobile (*i*, 141 h after plating). Bars: (*a–f*) 20 μm; (*g–i*) 10 μm.

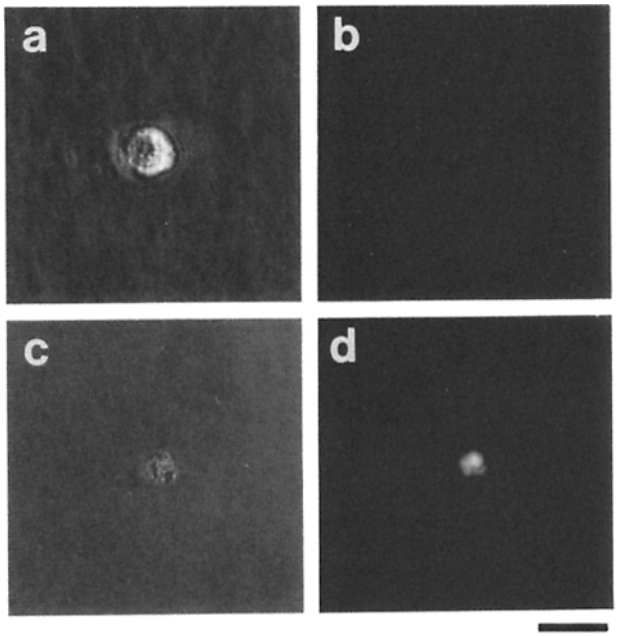

Figure 3. In situ DNA cleavage in chondrocytes in low density cultures, as revealed by the TUNEL technique. P11 rat chondrocytes were cultured at low cell density in agarose in DME/F-12, containing 1 mM cysteine, in the presence (*a* and *b*) or absence (*c* and *d*) of 10% FCS. After 4 d, the cells were fixed, permeabilized, and processed for TUNEL. They were observed by phase contrast (*a* and *c*) or fluorescence microscopy (*b* and *d*). Bar, 20 μm.

glutathione synthase to the antioxidant glutathione, as an inhibitor of glutathione synthase, BSO, substantially inhibited their survival-promoting activity (Fig. 4). Glutathione in reduced form (GSH) was as effective as cysteine, while its oxidized form (GSSG) was less effective; neither of their effects were affected by BSO (Fig. 4). Catalase or superoxide dismutase, used alone or together, did not enhance survival (Fig. 4).

To determine if chick chondrocytes can survive at low density in protein-free medium in the presence of cysteine, we prepared chondrocytes from the sternum of 17-d-old chick embryos and cultured them at various densities, as described above for rat chondrocytes. Although chick chondrocytes survived better than rat chondrocytes at low cell densities in the presence of cysteine, when plated at $\leqslant 10^4$ cells/ml, most of the cells died with the characteristic features of apoptosis within a few days (Fig. 5 *a*). These cell densities were

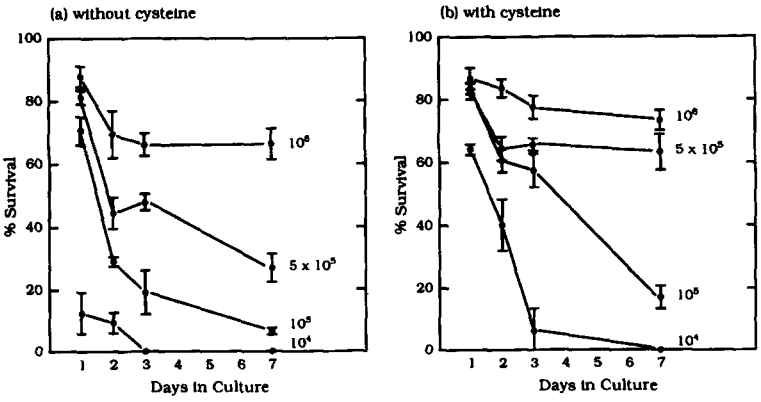

Figure 2. Cell-density dependence of rat chondrocyte survival in culture. P11 rat chondrocytes were cultured in agarose in DME/F-12 in the absence (*a*) or presence (*b*) of 1 mM cysteine at various cell densities in Terasaki microwells, and their viability was assessed by MTT assay after various times. In this and in the following figures, the results are expressed as means ± SD of four experiments. The survival at 1 h was 89 ± 3%.

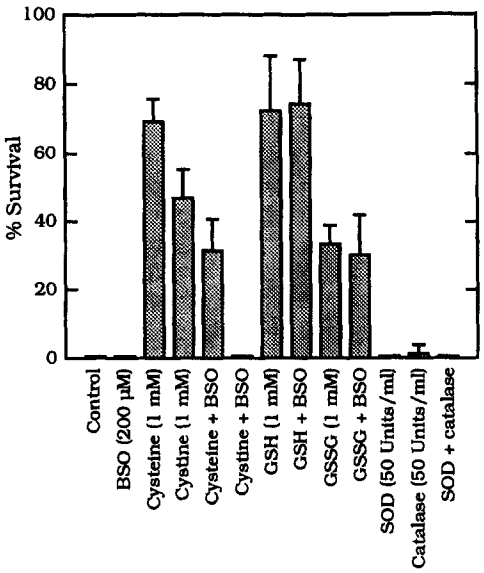

Figure 4. Effects of antioxidants on the survival of rat chondrocytes cultured at low density. P11 rat chondrocytes were cultured for 1 d in agarose in DME/F-12 at 10^4 cells/ml, and their viability was assessed by MTT assay. *BSO*, buthionine-sulfoximine; *GSH*, reduced glutathione; *GSSG*, oxidized glutathione; *SOD*, superoxide dismutase.

much lower than those tested by Bruckner and his colleagues (Bruckner et al., 1989; Tschan et al., 1990).

Chondrocytes Support One Another's Survival in Culture by Secreting Survival Factors That Are Not Antioxidants

The finding that chondrocytes survived at high density but not at low density in agarose gel cultures where the cells cannot make contact suggests that the cells in high density cultures promote one another's survival by secreting survival-promoting factors. Consistent with this suggestion, and as reported previously by Bruckner et al. (1989) for chick chondrocytes, culture medium from high density cultures of rat chondrocytes promoted the survival of rat chondrocytes in low density cultures; but unlike these workers, we found that the conditioned medium was only effective in the presence of cysteine (not shown, but see Fig. 5 *b*). When we tested conditioned medium from high density (10^7 cells/ml) chick chondrocyte cultures on the survival of chick chondrocytes, we found that it did not need cysteine to save chick chondrocytes cultured at 10^6 cells/ml, as reported by Bruckner et al. (1989), but it did need cysteine to save chick chondrocytes cultured at $\leq 10^5$ cells/ml (Fig. 5 *b*).

When conditioned medium from high density rat chondrocyte cultures was fractionated by ultrafiltration using a membrane with molecular mass cut-off of 10 kD, the survival-promoting activity was recovered in the concentrate and not in the filtrate (Fig. 6).

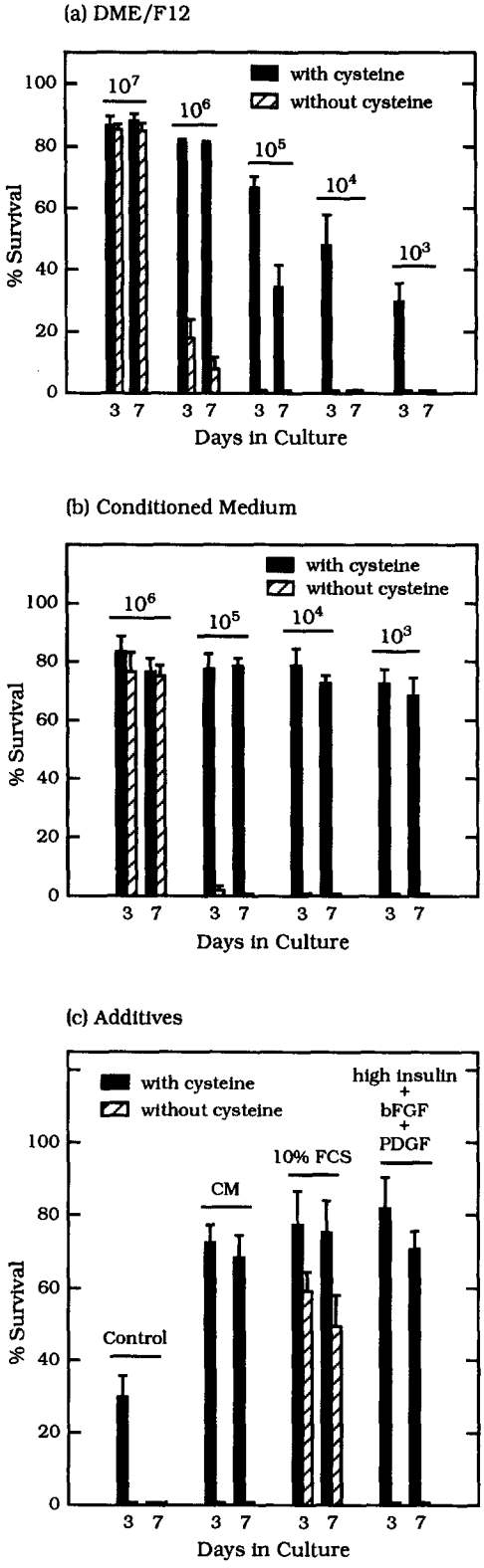

Figure 5. Survival of chick chondrocytes in culture. Embryonic chick chondrocytes were cultured in agarose in DME/F-12 at various cell densities in Terasaki microwells with or without 1 mM cysteine (*a*). The effects of conditioned medium from high density (10^7 cells/ml) chick chondrocyte cultures on the survival of chick chondrocytes at different cell densities are shown in *b*, while the effects of 10% FCS or the combination of high insulin (5 μg/ml), bFGF (10 ng/ml), and PDGF (10 ng/ml) on the survival of chick chondrocytes in low density (5×10^3 cells/ml) cultures are compared to the effect of conditioned medium (*CM*) from high density cultures in *c*. The medium was removed and replaced with fresh medium and additives every 2 d. Whereas very few cells divided in serum-free cultures, many divided in the presence of FCS, forming colonies of cells; each such colony was counted as a single live cell. MTT assays were performed after 3 and 7 d.

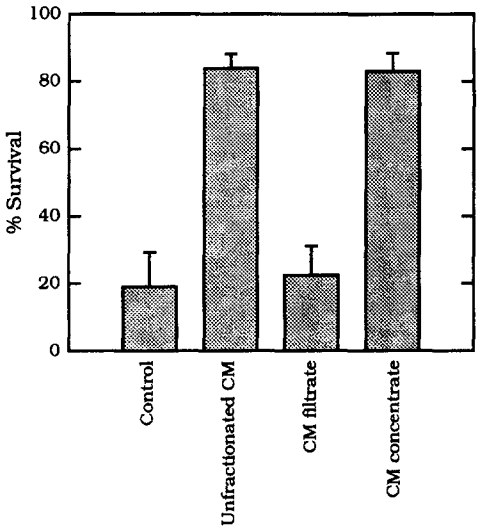

Figure 6. Ultrafiltration of conditioned medium. Conditioned medium (*CM*) from high density (10^6 cells/ml) rat chondrocyte cultures was fractionated by ultrafiltration using a filter that retained molecules larger than 10 kD. The concentrate and the filtrate were immediately tested for their ability to promote the survival of rat chondrocytes in low density (10^4 cells/ml) cultures in the presence of 1 mM cysteine. MTT assays were performed after 3 d.

FCS or a Combination of Known Growth Factors Promote the Survival of Chondrocytes in Low Density Cultures

As shown in Fig. 7, 10% FCS was as active as medium from high density chondrocyte cultures in promoting the survival of rat chondrocytes in low density culture. We also tested a variety of known growth factors alone and in combinations, including insulin, bFGF, PDGF, TGF-α, TGF-β, and T_3. Although only bFGF and high concentrations of insulin (5 μg/ml, sufficient to activate IGF-1 receptors) had activity on their own, the combination of high insulin, bFGF, and PDGF was as effective as FCS or conditioned medium from high density chondrocyte cultures in promoting survival (Fig. 7); the PDGF, in fact, added little, as survival in insulin and bFGF was almost as good as in insulin, bFGF and PDGF (not shown). TGF-α (10 ng/ml), TGF-β (10 ng/ml), and T_3 (40 ng/ml) had little effect (not shown). As for conditioned medium, the survival-promoting activity of the growth factors was absolutely dependent on the presence of cysteine, and even FCS was much less effective in the absence of cysteine (not shown, but see Figs. 5 *c* and 10). For chick chondrocytes as well, FCS or the combination of high insulin, bFGF, and PDGF was as effective as conditioned medium from high density cultures in promoting survival of cells in low density cultures, in the presence of cysteine (Fig. 5 *c*).

Culture Medium from High Density Chondrocyte Cultures Promotes the Survival of Lens Epithelial Cells in Low Density Cultures and Vice Versa

We found previously that medium from high density cultures of rat lens epithelial cells promoted the survival of lens epithelial cells in low density cultures, but in that case FCS and various combinations of known growth factors did not (Ishizaki et al., 1993). To determine the specificity of the conditioned media from high density chondrocyte or lens epithelial cell cultures, we tested them against the other cell type. As shown in Fig. 8 *a*, conditioned medium from high density lens cell cultures promoted the survival of chondrocytes in low density cultures just as effectively as did conditioned medium from high density chondrocyte cultures. Conditioned medium from high density chondrocyte cultures saved lens cells in low density cultures, but not as effectively as did conditioned medium from high density lens epithelial cell cultures (Fig. 8 *b*). As before, the conditioned media were not effective in the absence of cysteine (not shown).

Staurosporine Induces Chondrocytes to Undergo Apoptosis Even in the Presence of Cycloheximide

If a chondrocyte depends on signals from other chondrocytes for its survival, one might expect that if intracellular signaling pathways were blocked, even chondrocytes in high den-

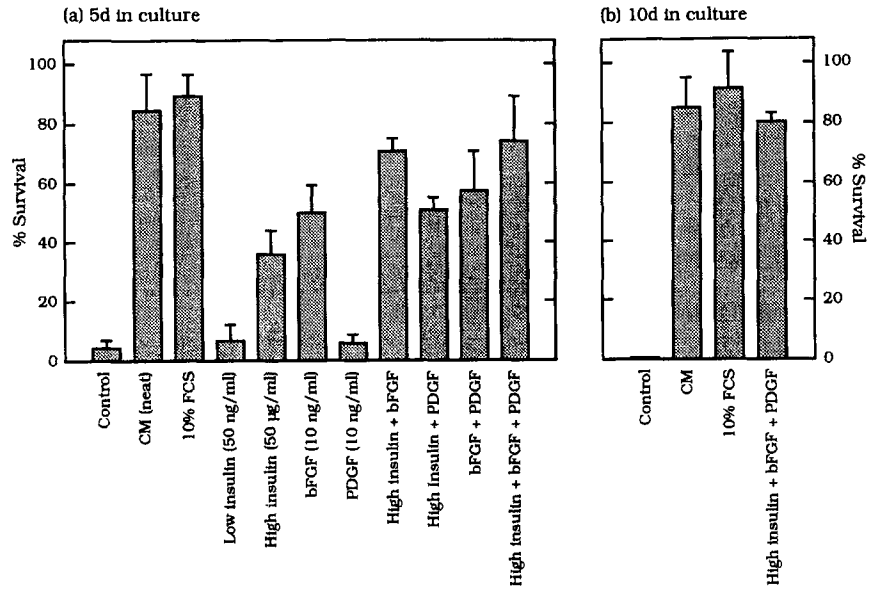

Figure 7. Effects of FCS and several growth factors on the survival of rat chondrocytes in low density culture. P11 rat chondrocytes were cultured at 10^4 cells/ml in agarose in DME/F-12 with 1 mM cysteine in Terasaki microwells in the presence or absence of FCS or growth factors. The medium was removed and replaced with fresh medium and additives every 2–3 d. MTT assays were performed after 5 (*a*) or 10 d (*b*). Whereas very few cells divided in serum-free cultures, many divided in the presence of FCS, forming colonies of cells; each such colony was counted as a single live cell.

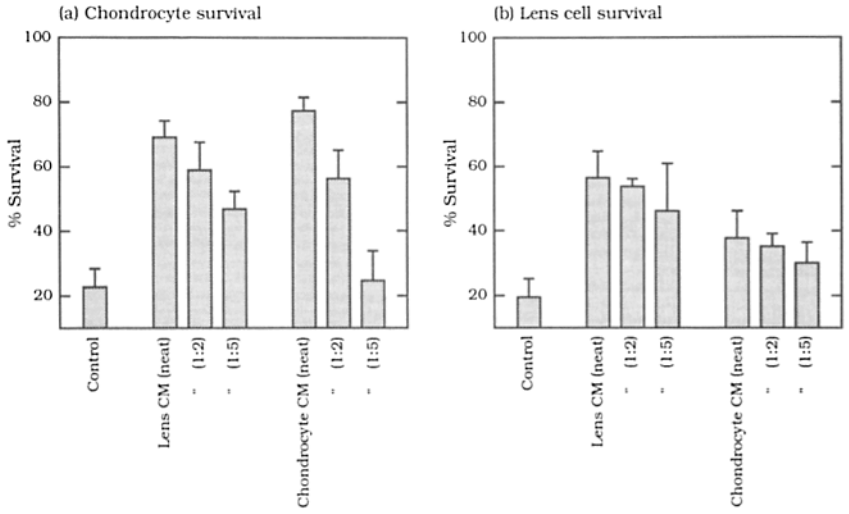

Figure 8. Conditioned medium from high density chondrocyte cultures promotes the survival of lens epithelial cells and vice versa. Conditioned media from high density (10^6 cells/ml) rat lens epithelial cell cultures and from high density (10^6 cells/ml) P11 rat chondrocyte cultures were tested at various dilutions for their ability to promote the survival of either P11 rat chondrocytes (*a*) or rat lens epithelial cells (*b*) in low density (10^4 cells/ml) cultures. The medium was removed and replaced by fresh conditioned medium at the appropriate dilution every day. MTT assays were performed after 3 d.

sity culture would undergo PCD. Treatment of high density cultures of rat chondrocytes with a high concentration (1 μM) of the broad specificity protein kinase inhibitor staurosporine induced almost all of the cells to die within less than 24 h with the morphological features of apoptosis (Fig. 9). Even when protein synthesis was inhibited by treatment with cycloheximide, staurosporine induced almost all chondrocytes in high density cultures to die by apoptosis, and the same was true for lens epithelial cells in high density culture (not shown).

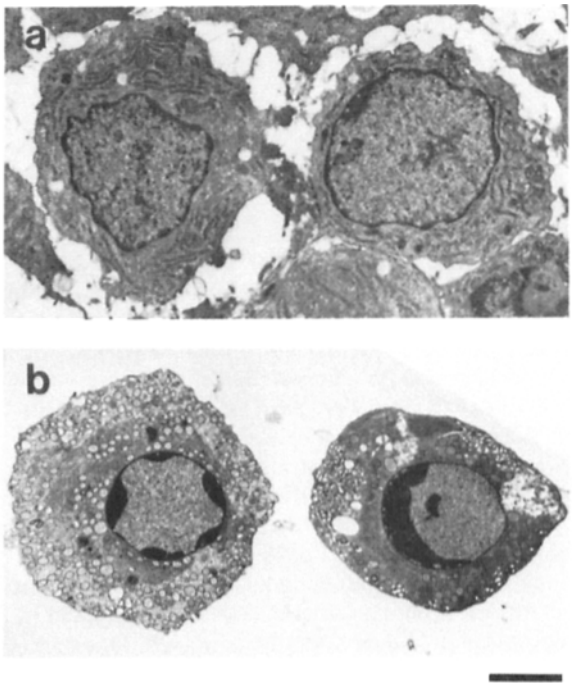

Figure 9. Electron micrographs of rat chondrocytes treated with staurosporine. P11 rat chondrocytes in high density (10^6 cells/ml) cultures were left untreated (*a*), or were treated with staurosporine (1 μM) (*b*) for 4 h. They were then fixed and processed for electron microscopy. The staurosporine-treated cells show the characteristic ultrastructural features of apoptosis. Bar, 25 μm.

Chondrocytes from Adult Rats Also Die by Apoptosis When Either Cultured at Low Density or Treated with Staurosporine

When chondrocytes prepared from the xiphoid process of the sternum of an adult rat were cultured in agarose gels in protein-free DME/F12 medium at low density (10^4 cells/ml), they behaved indistinguishably from chondrocytes cultured from P11 rats. Most of the cells died within 3 d with the morphological features of apoptosis, but they could be saved by conditioned medium from high density P11 chondrocyte cultures, by FCS, or by the combination of high insulin, bFGF and PDGF (Fig. 10), but only if cysteine was pres-

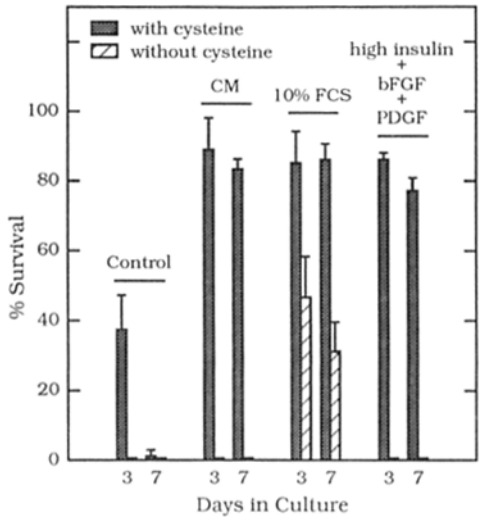

Figure 10. Survival of adult rat chondrocytes in culture. Adult rat chondrocytes were cultured in agarose gel in DME/F-12 with or without 1 mM cysteine at 10^4 cells/ml in Terasaki microwells. Some wells contained neat conditioned medium (*CM*) from high density (10^6 cells/ml) P11 rat chondrocyte cultures, 10% FCS, or a combination of high insulin (5 μg/ml), bFGF (10 ng/ml), and PDGF (10 ng/ml). The medium was removed and replaced by fresh medium and additives every 2 d. MTT assays were performed at 3 d and 7 d. Whereas very few cells divided in serum-free cultures, many divided in the presence of FCS, forming colonies of cells; each such colony was counted as a single live cell.

ent (not shown). When cells in 10% FCS were treated with 1 μM staurosporine, almost all of the cells died within 1 d with the morphological features of apoptosis (not shown).

Discussion

Our studies confirm the findings of Bruckner and his colleagues (1989) that chondrocytes do not require signals from other cell types to survive in culture: both chick and rat chondrocytes survive for weeks if cultured at high cell density in the absence of other cell types, serum, exogenous proteins, or non-protein signaling molecules. These findings are difficult to reconcile with the results of Quarto et al. (1992), who reported that chondrocytes isolated from chick tibia do not survive in high density cultures in the absence of insulin or thyroid hormone.

Our studies also confirm the findings of Bruckner and colleagues (1989) that chondrocytes require help from other chondrocytes to survive in culture: the cells die rapidly when cultured at low cell density, but can be saved by conditioned medium from high density chondrocyte cultures. Although the mode of chondrocyte death in low density cultures was not previously reported, we show here that the deaths have the characteristic morphological features of apoptosis (Kerr et al., 1972; Wyllie et al., 1980), suggesting that the cells die by active PCD.

Bruckner and colleagues (Tschan et al., 1990) found that the survival-promoting activity in conditioned medium from high density cultures of chick chondrocytes was associated with small molecules ($\leqslant$2 kD) and that it could be replaced by a variety of antioxidants, including cysteine or dithioerythritol. They concluded that the survival-promoting activity resided in a low molecular weight antioxidant rather than in growth factors, and, therefore, that chondrocyte survival does not require stimulation by growth factors. Although we have confirmed the crucial importance of antioxidants for the survival of chondrocytes in low density cultures ($\leqslant 10^5$ cells/ml for rat chondrocytes and $\leqslant 10^6$ cells for chick chondrocytes), we find that antioxidants are not enough: extracellular signaling molecules also seem to be required. This is suggested by our findings that (*a*) the survival-promoting activity in the conditioned medium from high density rat chondrocyte cultures collaborates with antioxidants such as cysteine and is associated with molecules that are larger than 10 kD, and (*b*), this activity is mimicked by either FCS or a combination of known growth factors.

The low cell density cultures of chick chondrocytes studied by Bruckner and his colleagues (Bruckner et al., 1989; Tschan et al., 1990) were more than 10-fold higher density than the low density cultures of chick chondrocytes that we have studied, which almost certainly explains the differences in our results. Thus we find that, while conditioned medium from high density chick chondrocyte cultures does not require antioxidants to promote the survival of chick chondrocytes cultured at 10^6 cells/ml, as reported by Bruckner et al. (1989), it does require antioxidants to promote the survival of chick chondrocytes cultured at $\leqslant 10^5$ cells/ml. We conclude that chondrocytes, like many other cell types, require signals from their neighbors in order to avoid PCD, at least in culture, that these signals do not function solely as antioxidants, and that chondrocytes themselves can secrete such signals. It seems likely that the requirements for antioxidants for chondrocytes and lens epithelial cells (Ishizaki et al., 1993) to survive in low density cultures reflects the fact that lens and cartilage are not vascularized and hence these cells are normally not exposed to oxygen concentrations as high as those present in culture (Tschan et al., 1990).

Of the growth factors that we tested, the combination of bFGF and a high concentration of insulin (high enough to activate IGF-1 receptors) was most effective at promoting chondrocyte survival in low density cultures. Chondrocytes have been shown to make IGF-1, IGF-2 (which also activates IGF-1 receptors), and bFGF in vitro (Demarquay et al., 1990, 1992; Hill and Han, 1991), and it seems likely that these growth factors promote chondrocyte survival in vivo. There are an increasing number of examples where several distinct extracellular signaling molecules have been shown to collaborate to promote cell survival in culture (for example, see Arakawa et al., 1990 and Barres et al., 1993*b*), presumably reflecting the advantages of combinatorial control (see below).

It is interesting that medium conditioned by high density cultures of lens epithelial cells promotes the survival of chondrocytes in low density cultures as well as of lens cells at low density, and medium conditioned by high density cultures of chondrocytes promotes the survival of lens epithelial cells in low density cultures as well as of chondrocytes at low density. This cross-talk was not predicted, as lens epithelial cells at low density, unlike chondrocytes at low density, could not be saved by any combination of the ten growth factors and cytokines that we previously tested (including high insulin and bFGF, which together save chondrocytes at low density) in the presence of antioxidants (Ishizaki et al., 1993). The results, therefore, suggest that chondrocytes produce one or more survival factors that are not among the ones we have tested on lens cells or chondrocytes. One theoretical advantage of having cells depend on signals from their neighbors for survival is that any cell that ends up in an abnormal location would fail to receive the survival signals it requires and would consequently die; because cells seem to require a combination of signals for sustained survival, at least in culture (Arakawa et al., 1990; Barres et al., 1993*b*), a relatively small selection of signaling molecules used in different combinations could specifically control the survival of many distinct cell types (Raff et al., 1993). It should not be surprising, therefore, that lens cells and chondrocytes share some survival factors, especially as these cell types are very unlikely ever to encounter each other's environment.

As previously shown for a number of cell types (Jacobson et al., 1993; Raff et al., 1993, and unpublished observations), including lens epithelial cells (Ishizaki et al., 1993), chondrocytes die with the characteristic features of apoptosis when treated with a high concentration (1 μM) of the broad-spectrum protein kinase inhibitor staurosporine. Moreover, this staurosporine-induced cell death, as well as the staurosporine-induced death of lens epithelial cells, does not seem to depend on new protein synthesis, as it is not inhibited by a high concentration of the protein synthesis inhibitor cycloheximide. Thus chondrocytes and lens epithelial cells, like many and perhaps most mammalian cells (Coles, H., K. Raff, M. Jacobson, and M. C. Raff, unpublished observations), seem to constitutively express all of the protein components required for programmed cell death. There is evidence that, during endochondrial ossification, terminal hypertrophic chondrocytes normally die in situ by apoptosis just before they are invaded by bone-forming tissue (Farnum

and Wilsman, 1987, 1989), but it is not known what activates the death program in these cells. Interestingly, hypertrophic chondrocytes have been reported to have lost the expression of bFGF receptors (Iwamoto et al., 1991).

We have shown that chondrocytes, like lens epithelial cells, do not require signals from other types of cells to survive in culture but do require signals from other cells of the same type. In tissues like lens and cartilage where there is only one cell type, it is not surprising that the survival signals act in this autocrine manner. In the great majority of tissues, however, there are many cell types and one might expect survival signals to act mainly in a paracrine manner, as has been shown in a number of cases (for example, Levi-Montalcini, 1987; Metcalf, 1989; Barres et al., 1992; Gaur et al., 1992).

If chondrocytes and lens cells require signals from their neighbors to avoid PCD, it seems very likely that most of our cells do, at least during development. As chondrocytes isolated from adult rats also seem to require signals from other chondrocytes to survive in culture, it seems likely that even in adults most cells need to be continuously signaled by other cells to avoid PCD.

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