Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 4784–4788, July 1986
Cell Biology

Evidence for soluble factors regulating cell death and cell proliferation in primary cultures of rabbit endometrial cells grown on collagen

(apoptosis/cell death factor/cell proliferation factor)

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Communicated by David M. Prescott, March 10, 1986

ABSTRACT **Primary cultures of rabbit endometrial cells grown on collagen substrates exhibit cyclic changes in DNA content throughout extended periods of culture. These cycles are characterized by periods of significant increases and decreases in the DNA content of the cultures or number of cells present, yet through the entire duration of culture there is no net change in the total DNA. The rates of cell proliferation and cell death change through time in culture with the same periodicity as the changes in DNA. Neither changes in the rate of cell proliferation nor the rate of cell death alone are sufficient to account for the changes in DNA. Rather, there appears to be a feedback mechanism operating between cell proliferation and cell death such that when one increases, the other increases concomitantly in order to maintain a homeostasis in total culture mass. This homeostasis appears to be mediated by a soluble cell proliferation factor (CPF) and a cell death factor (CDF) produced by the cells. CPF and CDF may be obtained from either conditioned media or cultured cell extracts. These biological activities are heat and trypsin sensitive. The major mode of cell death in these cultures appears to be apoptosis or programmed cell death, characteristic of renewing epithelia. The data suggest that this tissue culture model system represents a renewing cell population containing stem cells and their progeny, whose total growth is strictly regulated by CPF and CDF. As such, it provides a model system in which to study homeostasis and how it may be altered in hyperplasia and neoplasia, as well as its regulation by hormones.**

Previous studies from this laboratory have provided evidence for the existence of at least two subpopulations of epithelial cells in the rabbit endometrium. Each of these subpopulations is characterized by its location in the epithelium (gland vs. lumen), its cell-cycle stage (cycling vs. noncycling), and its response to estrogen and progesterone (1–6).

It appears that there are stem cells in glandular epithelium. Upon dividing, the progeny migrate toward the lumen where they are lost by death or sloughing. Estrogen can increase the proliferation of stem cells, the migration of daughter cells, and the lifetime of these cells in the lumen (5). Progesterone, on the other hand, can promote the division of the daughter cells located in luminal epithelium, the formation of new glands, and subsequent differentiation of the cells into nonproliferating cells that produce uteroglobin (1–6).

Similar subpopulations of cells have been demonstrated in primary cultures of rabbit endometrial cells in chemically defined media (7–15). Moreover, these studies have shown that progesterone can lead to an inhibition of the estrogenic proliferative effect in culture by stimulating the production of an inhibitory factor by the cycling cells (12). A similar inhibitory activity is produced in high-density cultures (13). These data suggest a complex interaction among cells that effects a control of proliferation and that can be modulated by steroid hormones.

Thus, four intrinsic regulatory mechanisms for control of uterine epithelium growth have been proposed (6, 15). These include cell proliferation, cell migration, cell loss, and terminal differentiation, which may be influenced by the ovarian steroids.

We adopted the use of collagen substrates for culture of the endometrial cells in order to define more fully these intrinsic regulatory mechanisms of growth control in a simplified *in vitro* system. Collagen substrates permit the formation of three-dimensional structures similar to endometrial glands, an impossibility when cells are grown on plastic. The data reported here indicate that some of the above-mentioned regulatory mechanisms appear to be operational in this model. More importantly, we provide evidence that cell proliferation and cell death of the rabbit endometrial cells may be regulated by a feedback mechanism mediated by a soluble cell proliferation factor (CPF) and a cell death factor (CDF) produced by the cells.

MATERIALS AND METHODS

Cell Culture Conditions. Cells used in these experiments were obtained from rabbit endometrium and were cultured in serum-free chemically defined media as described (7).

Collagen-coated dishes were prepared as described (14) using 1 ml of collagen per dish and cells were plated at 125,000 cells per dish in 2 ml of medium. The day of plating was referred to as day 1. On day 3 of culture, the gels were detached from the dish with a sterile pipette tip and were allowed to float in the medium for the remainder of the culture period. Media were changed every 24 or 48 hr as required for individual experiments, always at the same time of day. To harvest the cells, the collagen gels were transferred to borosilicate glass centrifuge tubes (12 × 75 mm; Fisher) containing 1.0 ml of 0.3% collagenase (Type IA, Sigma) in Hanks' balanced salt solution. The gels were incubated at 37°C for 1 hr or until the collagen was digested, then centrifuged at 700 × *g* (4°C for 10 min). The pellet was resuspended in 1.0 ml of cold saline, recentrifuged, and the resultant cellular pellet was hydrolyzed with 0.5 ml of 0.5 M NaOH. This lysate was left at 4°C overnight, then neutralized with an equal vol of 0.5 M HCl in preparation for DNA determination by the Hoechst dye-binding technique as described (14). Gels lacking cells were prepared for DNA

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Abbreviations: CPF, cell proliferation factor; CDF, cell death factor; MI, mitotic index; DI, death index; LDH, lactic dehydrogenase.

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assay in the same manner to control for background fluorescence in the DNA assay.

DNA synthesis was measured by incubation with [*methyl*-^{3}H]thymidine (specific activity, 62 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear). Cells were labeled by adding 1 μCi of [^{3}H]thymidine per 35-mm dish in 2 ml of medium for 24 hr. The cells were harvested from the collagen gels as described above, hydrolyzed with 0.5 ml of 0.5 M NaOH, and neutralized with an equal vol of 0.5 M HCl. Dishes containing collagen gels but no cells were incubated with [^{3}H]thymidine-containing medium and were treated in the same manner to control for possible nonspecific binding of [^{3}H]thymidine using the technique described below. Cells from parallel dishes were prepared for DNA assay to enable expression of data as cpm incorporated per μg of DNA. The DNA from the dishes incubated with [^{3}H]thymidine was collected by vacuum filtration through mixed cellulose/acetate-cellulose/nitrate filters (25-mm diameter, 0.45-μm pore size; Millipore HAWP) in a 12-well Millipore filter apparatus. The cell lysate was poured into the well, the tube was washed two times with 2 ml of 4× SSC (0.6 M NaCl/0.06 M trisodium citrate), then the filters were washed three times with 4× SSC under vacuum. The radioactivity on the filters was counted in scintillation vials in 7.5 ml of Aquasol-II (New England Nuclear). Several experiments using DNase have demonstrated that the label recovered by this technique is specifically that incorporated into DNA (data not shown).

Cell death was estimated by determining lactic dehydrogenase (LDH) activity in culture medium (Sigma kit 340-UV). Cells cultured on collagen gels received fresh medium daily. Every 24 hr, media were removed from each of five dishes for determination of LDH. The DNA from these same dishes was assayed and cell death was expressed as units of LDH activity per μg of DNA.

The cultures were prepared for light microscopy by fixing the collagen gels in 10% formalin. They were dehydrated, embedded in paraffin, sectioned, and the sections were stained with hematoxylin and eosin for quantitation of mitotic index (MI; number of mitoses × 100 per total number of cells on the section) and death index (DI; number of dead cells × 100 per total number of cells on the section). The number of cells counted for any given day ranged from 1250 to 4500. These quantitations were done blinded.

Conditioned Media Experiments. Cells were cultured on collagen at 125,000 cells per 35-mm dish in 2 ml of control medium. On day 2, media on all dishes were changed and fresh control medium was added. Beginning on day 3, conditioned media were removed every 24 hr from all dishes, pooled in sterile 50-ml Corning tubes, and centrifuged at 700 × *g* for 10 min to remove particulate matter. The supernatants were transferred to sterile 50-ml tubes and kept frozen at −20°C until assayed. Biological effects were determined by measuring the change in DNA content of the cultures incubated with conditioned media compared to control cultures.

Preparation of Biological Activity in Cell Homogenates. Cells were plated at 125,000 cells per dish on collagen substrates and medium was changed daily. The cells from each of five dishes were harvested from the collagen gels daily using collagenase; the cell pellet was frozen until preparation of the extract. To prepare the extracts used in the experiments reported here, the pellets were resuspended in 1 ml of control medium and frozen/thawed three times on ethanol/dry ice. The volume of the lysate was brought up to 5 ml with control medium and was transferred to nitrocellulose centrifuge tubes. (In subsequent experiments, the extracts were prepared in sterile phosphate-buffered saline, which yielded similar results.) The lysates were centrifuged at 105,000 × *g* at 4°C for 70 min. The supernatants were sterilized through Millipore filters (Millex-GS; 0.22 μm pore size). Aliquots were frozen in 0.5-ml volumes in sterile capped plastic tubes, and they were thawed as needed.

Heat and Trypsin Sensitivity of Biological Activity. Cell extracts were prepared as described. An aliquot of the undiluted extract was heated to 70°C in a water bath for 20 min and then cooled to 37°C. Another aliquot was trypsinized for 1 hr at 37°C with trypsin (0.1 mg/ml) (Sigma) in saline. The reaction was stopped by adding trypsin inhibitor (0.2 mg/ml; ICN). Biological activity was determined by measuring DNA content of cultures incubated with untreated, heated, or trypsinized extract compared to control.

Statistical significance was evaluated throughout the paper by using the Student's *t* test.

RESULTS

Growth of Cells on Collagen. Cells grown on collagen gels formed epithelioid structures with gland-like crypts (Fig. 1*A*). Dying cells were easily identified (Fig. 1*B*).

Primary cultures of rabbit endometrial cells grown on collagen exhibited significant cyclic changes in total DNA

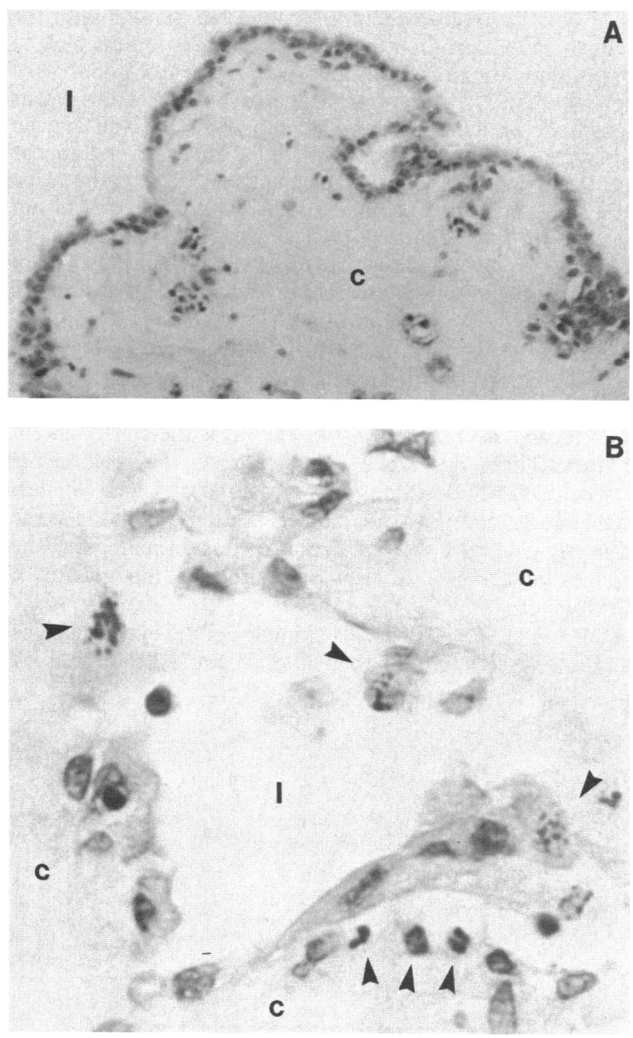

FIG. 1. Light microscopy of endometrial cells cultured on collagen gels. Cells were cultured on collagen gels as described, and the gels were detached on day 3. (*A*) Representative section of cells cultured on collagen gels, consisting of simple cuboidal epithelial cells and gland- or crypt-like structures. l, Lumen at the cell media interface; c, collagen gel. Hematoxylin and eosin. (90×.) (*B*) Higher magnification of a section of one of the gland-like structures formed by the cells, showing some cells at various stages of apoptosis (arrowheads). l, Lumen; c, collagen gel. Hematoxylin and eosin. (370×.)

content for extended periods of time (Fig. 2). Yet, throughout the entire period of culture there is no net change in the DNA concentration (slope = 0.01385 by regression analysis of all points). Control experiments indicated that cell number changed in parallel with the measured changes in DNA content on the cultures. Cells lost from the collagen gels during the measured decreases in DNA were not found in the culture medium, nor were they observed to be attached to the plastic dish after the collagen gel was detached (data not shown). Thus, the loss of DNA in the cultures appears to be due to cell death.

Changes in the Rate of Cell Proliferation and Rate of Cell Death. The changes in the DNA content and cell number in these cultures reflect changes in the net mass of the cultures. Both cell proliferation and cell death contribute to the net mass of a given tissue. It is conceivable that in this tissue culture system either only one of these parameters may change while the other remains constant, or both the rate of cell proliferation and the rate of cell death may change.

Cell proliferation (determined at daily intervals after a 24-hr pulse of [^{3}H]thymidine) changed as the amount of DNA in the cultures changed (Fig. 3). Cell death (measured by LDH activity in the medium) changed in parallel with the changes in cell proliferation. These changes appear closely correlated with the changes measured in the DNA content of the cultures. That is, when a decrease in total DNA was measured, an increase in cell death was observed and an increase in DNA synthesis occurred. Thus, it would appear that there is some feedback mechanism operating between cell proliferation and cell death. It is unclear whether or not the changes in cell death are the cause or result of the changes in cell proliferation. However, it is evident that the two are interrelated and both contribute to the changes measured in the DNA content of the cultures.

These observations are further substantiated by histological evidence. When dead cells were quantitated in sections of collagen cultures prepared at 24-hr intervals, the percentage of dead cells (DI) (Fig. 4) increased as the DNA content of the cultures decreased. This parallels the relationship between DNA and cell death as measured by LDH. Similarly, the MI quantitated in histological sections increased as the DI increased and the DNA decreased, again mimicking the changes in cell proliferation measured by the uptake of [^{3}H]thymidine.

CPF and CDF Activities. The kinetics of the cyclic changes in the DNA content of the cultures could be altered by changing the medium, suggesting that we were removing some positive or negative effector from the cultures when

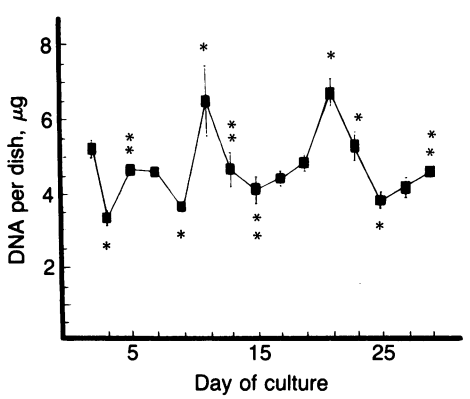

FIG. 2. Long-term culture of cells on collagen gels. Cells were plated at 125,000 cells per 35-mm collagen-coated dish in 2 ml of control medium. Media were changed every 48 hr. Collagen gels were detached from the dish on day 3. Cells from five dishes were removed from culture on days 2 and 3, and every 48 hr thereafter until day 29. Each point is the average μg of DNA per dish (n = 5) ± SEM. *P = <0.001; **P = <0.01 when compared to previous data point by using Student's t test.

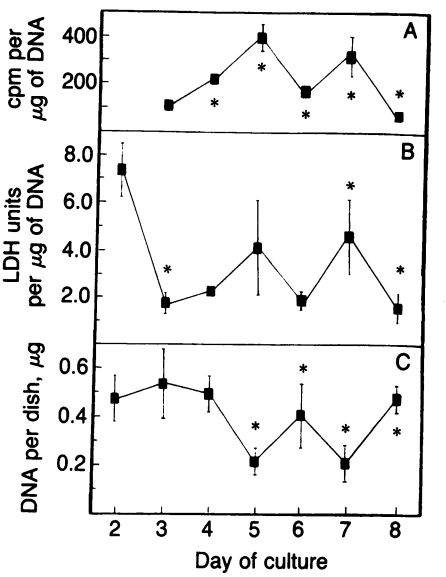

FIG. 3. Change in the rate of incorporation of [^{3}H]thymidine and LDH activity in culture media compared to change in DNA content of the cultures. Cells were cultured on collagen as described in Fig. 2. Media were changed daily and collagen gels were detached on day 3. Each day, cells were harvested from five dishes for determination of DNA. The media from these same five dishes were saved for determination of LDH activity. Beginning on day 2, each of five dishes plus 1 no-cell control were given 1 μCi of [^{3}H]thymidine, removed from culture 24 hr later, and the cells were harvested for determination of incorporation of [^{3}H]thymidine into DNA as described. (*A*) Average cpm of [^{3}H]thymidine incorporated per μg of DNA ± SEM (n = 5 minus 1 no cell). (*B*) Average units of LDH activity present in media per μg of DNA ± SEM (n = 5). (*C*) Average μg of DNA per dish (n = 5) ± SEM. *P = <0.02 when compared to previous data point.

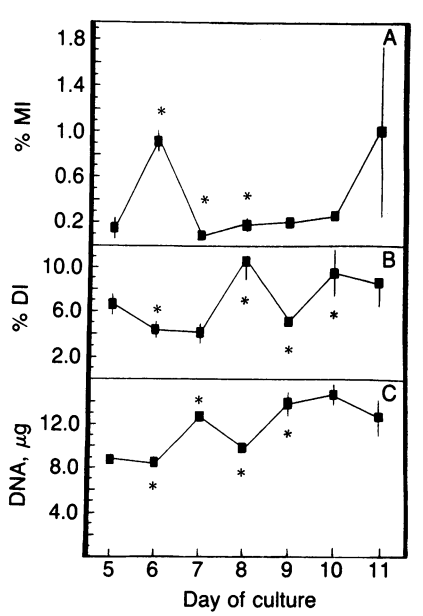

FIG. 4. Change in MI and DI compared to change in DNA content of the cultures. Culture conditions were as described in Fig. 3. Each day, five dishes were removed from culture for DNA determination. Two other cultures were removed daily, processed for light microscopy and stained with hematoxylin and eosin. MI and DI were quantitated as described. Histological sections from cultures on days 2–4 were not adequate to make these determinations. (*A*) MI, average percent of total cells in mitosis ± SEM. (*B*) Average percent of dead cells ± SEM. (*C*) Average μg of DNA per dish (n = 5) ± SEM. Statistical significance is expressed as in Fig. 3.

they were given fresh medium. To test this hypothesis, we undertook conditioned medium experiments in which media collected from high or low points in DNA content of given cultures were added to other cultures to assay for biological activity.

In one such experiment (Fig. 5), media that had been collected from a peak in the DNA content of given cultures stimulated an increased DNA content in the cultures on which it was assayed. This effect was observed when the conditioned media were left on the test cultures for 24 hr. After 48 hr with the conditioned media, there was a significant loss of DNA from the treated cultures ($P < 0.01$ compared to cultures with conditioned media for 24 hr), resulting in even less DNA than control cultures and suggesting a feedback between increasing and decreasing DNA. It was possible that the cultures, having been stimulated to grow with the addition of conditioned medium for the first 24 hr, responded to a negative-feedback mechanism with increased cell death in the following 24 hr.

A similar feedback was observed when conditioned media collected from a low point in the DNA content of given cultures were tested for biological activity (Fig. 6). After 24 hr with this conditioned media, there was significantly less DNA in the cultures on which it was assayed. There was in fact a significant decrease of DNA in 24 hr with the conditioned media compared to the amount of DNA present on day 3 when the conditioned media had been added ($P < 0.01$). After 48 hr, although the DNA in the treated cultures was still significantly less than control, there was significantly more DNA on these dishes after 48 hr with the conditioned media than in those having received this conditioned media for 24 hr ($P < 0.001$ compared to cultures having received conditioned media for 24 hr).

Heat and Trypsin Sensitivities of the Biological Activities. The heat and trypsin sensitivities of the cell proliferation and cell death activities were tested with cell extracts. As in Fig. 7, the extract added from day 3 to day 4 resulted in an increase in DNA content, which was completely abolished by heating or trypsinizing the extract prior to addition. When this same extract was added to cultures from day 4 to day 5, a loss of DNA was observed. Again, this activity was heat and trypsin sensitive.

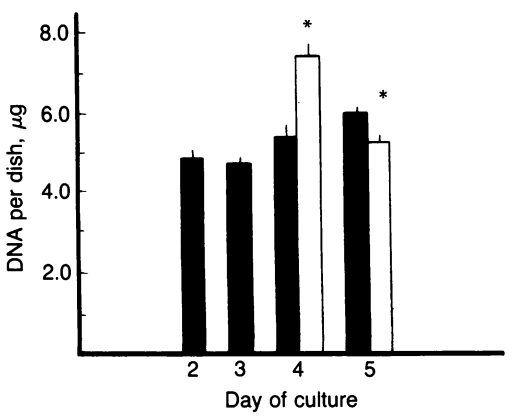

FIG. 5. Demonstration of growth factor activity in conditioned media. Culture conditions were as described in Fig. 2. Conditioned media that had been collected from a peak in the DNA content of other cultures was added at a dilution of 1:1 in control media on day 3, incubated with the cultures for 24 or 48 hr, and removed on day 4 or 5, respectively. Each bar represents the average DNA content per dish ($n = 5$) ± SEM. Solid bars, DNA in control cultures; open bars, DNA content in cultures having received conditioned media. *$P = <0.001$ when compared to controls on the same day by using the Student's t test.

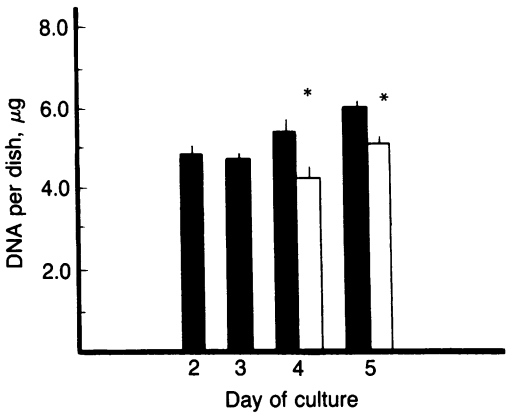

FIG. 6. Demonstration of death factor activity in conditioned media. Culture conditions and conditioned media additions were as described in Fig. 5. Data and statistical significance are also expressed as in that figure. The conditioned media used here, however, had been collected from a low point in the DNA content of other cultures.

DISCUSSION

The kinetics of the cyclic changes measured in the DNA content of the cultures on collagen gels suggest the presence of a heterogeneous interactive population of cells in support of previous experimental data and hypotheses (1–16). For cell loss to occur in the cultures, yet for proliferative ability to be maintained, there must be a subpopulation of stem cells present that continually replenishes itself and provides progeny capable of differentiation, senescence, and/or death (daughter cells). Cell proliferation and cell death are dynamic properties of this system, and both change continually throughout the culture period. The size of the population at any time depends on the balance between both cell death and cell proliferation.

The necessity for a balance between cell proliferation and cell death in maintaining the size of adult cell populations is a long held tenet in biology. It is equally well-recognized that changes in either one of these parameters may lead to changes in the other, such that homeostasis is reattained. This has classically been shown by wounding experiments in epidermis or partial hepatectomy. Tissue-specific inhibitors

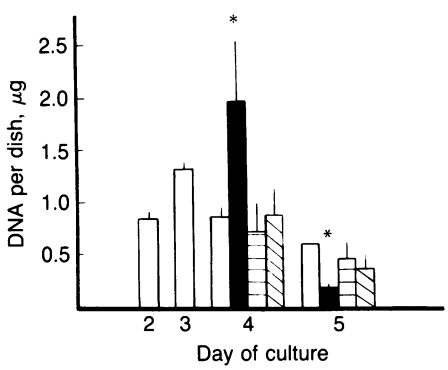

FIG. 7. Heat and trypsin sensitivity of biological activities. Cells were cultured at 125,000 cells per 35-mm collagen-coated dish. On day 3, and again on day 4, media were changed. Cultures were given control media (open bars) or 1% cell extract that had either received no treatment (solid bars), been heated to 70°C for 20 min (diagonal stripes), or treated with trypsin (0.1 mg/ml, 37°C, 1 hr) followed by trypsin inhibitor (0.2 mg/ml) (horizontal stripes). Control, untreated extract and heated extract received trypsin inhibitor alone. Data are expressed as average μg of DNA per dish ($n = 5$) ± SEM in each group. *$P = <0.001$ when compared to control cultures on the same day by using the Student's t test.

of mitosis, chalones, were proposed to be mediators of the feedback regulation of proliferation for these and other tissues (reviewed in refs. 17–19). However, in physiological situations, such as in renewing cell populations, cell death is as important a contributor to the overall size as is cell proliferation. The homeostasis we observe in our system suggests that cell death may be as actively and precisely regulated as cell proliferation. This regulation appears to be an intrinsic property of rabbit endometrial cells. The data suggest that both CPF and CDF are continually produced in culture and that they exert their effects on different target-cell subpopulations. The biological activity observed, therefore, depends on the amount of each factor produced and the proportion of each target-cell population present in the cultures. The mode of cell death observed in this system is significant. We predominantly see apoptosis as opposed to necrosis. While the latter is primarily seen as a response to noxious stimuli, apoptosis is a programmed cell death observed in a variety of physiological and pathological situations. It has a prime role in morphogenesis in embryos (20), functions in renewal of adult cell populations (21, 22), is implicated in atrophy of endocrine-dependent tissues upon removal of trophic hormones (23, 24), and also may be observed in tumor-cell populations (25). Evidence for apoptosis being genetically regulated comes from studies in the nematode *Caenorhabditis elegans*. Mutants have been developed that are defective in initiation of cell death or in phagocytosis of dead cells (26–28). Morphological distinctions between apoptosis and necrosis have been summarized elsewhere (29, 30) and will not be treated at length here. Morphological and biochemical changes in apoptosis have been characterized in thymocytes (31). The morphology observed in two-thirds of the dead cells in the collagen gel cultures is consistent with those changes observed in apoptosis.

Based on the data reported here and the other literature discussed, it would appear that our tissue-culture model system exhibits a homeostasis analogous to that observed in the intact tissue. Most importantly, we have identified soluble factors of a protein nature, CPF and CDF, which mediate this homeostasis. We now have preliminary evidence that these two biological activities may be isolated from rabbit uteri and separated by column chromatography (unpublished work). Thus far, these two activities are defined solely according to their effects on net DNA in the cultures. Upon purification of the factors, it will be possible to determine their exact roles in cell proliferation and cell death and thus to define how steady-state growth is maintained in rabbit endometrium. It is likely that alterations in the production of or response to the CPF or CDF may be involved in physiological processes or in the development of hyperplasia or neoplasia. The rabbit endometrium is a steroid-responsive tissue, which is under a relatively constant estrogen stimulation since the animal is an induced ovulator. Estrogen is linked to the unusually high incidence of spontaneous endometrial hyperplasia and neoplasia in this animal (32) and in humans (33). The proliferative effects of estrogens on the endometrium may be mediated by production of CPF or inhibition of CDF, or both. Excess estrogen stimulation may lead to hyperplasia and neoplasia by causing an inbalance between CPF and CDF. Progesterone, on the other hand, is often useful in the treatment of endometrial cancer (34). Progesterone is observed to inhibit the proliferative effect of estrogen on glandular epithelium in rabbit endometrium (2), and in tissue culture progesterone induces the production of a factor inhibiting the proliferative effect of estrogen on quiescent cells (12), which may or may not be related to the factors reported here. Progesterone may have effects on the production of or response to CPF and CDF antagonistic to those of estrogen, explaining its effectiveness as treatment for cancer by restoring a balance between cell proliferation and cell death. With our experimental model system, we may easily test these hypotheses and obtain a better understanding of the pathogenesis of diseases involving derangements of growth such as cancer. It is also possible that similar factor-mediated homeostasis may be operational in other tissues and species.

We would like to thank Ms. R. Lieberman, Ms. C. Williams, and Dr. D. Orlicky for their help and stimulating discussions, and Ms. J. Harness for critical review of the manuscript. This work was supported in part by National Institutes of Health Grants CA-09157 and CA-25365.

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