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SERUM-FREE GROWTH OF ADULT HUMAN PROSTATIC EPITHELIAL CELLS

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SUMMARY

Proliferation of adult human prostatic epithelial cells in serum-free medium occurs upon the addition of cholera toxin, epidermal growth factor, pituitary extract, and hydrocortisone to basal medium PFMR-4A. Insulin and selenium enhance proliferation and permit growth at lower cell densities. Reducing the level of calcium in the medium dramatically alters morphology and also seems to increase proliferation. Mortal strains of cells derived from normal central or peripheral zone, benign hyperplasia, or cancer respond similarly to growth factors and calcium, but two populations of cancer cells which have been long-lived and may be immortal lines behave differently. GKC-CA cells require serum proteins or high levels of pituitary extract for optimal growth, and neither GKC-CA cells or cells of another cancer line, WB-CA, proliferate well in medium containing reduced levels of calcium. These observations may, however, be a reflection of attachment phenomena rather than of growth responses per se. Growth of cells in serum-free medium has allowed definitive studies of the effects of androgens, and regardless of cell type no response to androgens of prostate epithelial cells under any experimental conditions has been seen.

Key words: serum-free; prostate; epithelial; androgens.

INTRODUCTION

The prostate is a gland that is prone to becoming diseased and malignant (6,17) and consequently there is much interest in characterizing the properties and functions of prostatic epithelial cells. Lack of adequate culture systems has hindered studies in this area, but recently we demonstrated the ability to serially subculture adult human prostatic epithelial cells in a reproducible manner. Using cells isolated from normal central or peripheral zones of the prostate (18), from benign hyperplastic nodules, or from adenocarcinomas we succeeded in passaging primary cultures into secondary and tertiary cultures by the addition of cholera toxin to the growth medium (21). PFMR-4 (13) was chosen as the basal medium because it supported the best growth compared to numerous other media tested. Supplementation with a high level of serum was required in this system. Less density dependency of growth and a lowered requirement for serum were achieved by optimization of the basal medium (23). The concentrations of seven components were changed to make a modified medium, PFMR-4A, and factors and hormones were tested for beneficial effects on growth. As a result, better growth occurred in PFMR-4A supplemented with only 5% dialyzed serum, cholera toxin, epidermal growth factor (EGF), bovine pituitary extract (BPE), and hydrocortisone than in the original growth medium (PRMR-4 supplemented with 20% whole serum, cholera toxin, and EGF).

Our finding that hydrocortisone was very stimulatory to the growth of all types of prostatic epithelial cells (normal, benign hyperplastic, and malignant) led us to reevaluate the amount of serum supplementation required, because hycrocortisone often produces a serum-sparing effect (1,20). We found that growth improved with less serum and in fact was quite good without any serum at all. In this report we describe our analysis of the interactions of factors added to serum-free medium and their effects on the growth of prostatic epithelial cells.

MATERIALS AND METHODS

Media and supplements. PFMR-4A (22,23) was prepared in our laboratory from chemicals purchased from Sigma (St. Louis, MO). This medium is a modification of PFMR-4 (13) and contains 10-fold levels of isoleucine, leucine, threonine, glutamine, inositol, thymidine, and methionine. Except where indicated in experimental procedures, the trace element stock was omitted. Dialyzed serum (25) was prepared from whole fetal bovine serum purchased from Flow (McLean, VA). Steroid-free dialyzed serum was prepared according to the method of Page and Parker (19). Cholera toxin (List Biologicals, Campbell, CA), EGF, insulin, and endothelial cell growth supplement (Collaborative Research, Waltham, MA) were reconstituted and stored according to the suppliers' instructions. Bovine pituitary extract was purchased from the laboratory of Dr. Richard Ham (Department of MCD

TABLE 1

CLASSIFICATION OF CELL CULTURES

Cell Strains	Patient Age	Histology	Classification
RPS	77	cancer/BPH	mixed
WRB	71	nodular hyperplasia	mixed
VH-CA	66	adenocarcinoma	cancer
KGK-PZ	38	no BPH or cancer	normal peripheral
GKC-CA	64	adenocarcinoma	cancer
PS-CZ	41	no BPH or cancer	normal central
JBDG	69	nodular hyperplasia	mixed
WB-CA	55	prostatic metastasis	cancer
JLC-BPH	80	nodular hyperplasia	BPH
GEH	68	cancer/BPH	mixed

Biology, University of Colorado, Boulder CO 80309). Phosphoethanolamine and selenium (Sigma) were added to the medium from 1000× stocks. Hydrocortisone (Sigma) was dissolved in 100% ETOH and added to the medium from a 10 000× stock. Gentamycin (United States Biochemical Corp., Cleveland, OH) was added to serial cultures at a final concentration of 200 μg/ml and to medium for growth assays at 100 μg/ml. Testosterone and dihydrotestosterone (Sigma) were prepared as 1000× stocks in 100% ETOH. Tissue culture dishes coated with a 1:4 dilution of collagen (Collagen Corp., Palo Alto, CA) were routinely used.

Cells. The cells used in these studies are listed in Table 1. Epithelial acini were released from tissues by collagenase digestion (21,22). Primary and subcultures were maintained in PFMR-4A supplemented with 1 or 5% dialyzed serum (dFBS), 10 ng/ml cholera toxin, 10 ng/ml EGF, 100 μg/ml BPE (or 10 μg/ml BPE plus 0.1 m*M* phosphoethanolamine), and 1 μg/ml hydrocortisone.

When cells were isolated from tissues dissected from whole prostates, the histology of adjacent tissue sections was determined by Dr. John McNeal (Division of Urology, Stanford Medical Center, Stanford, CA 94305). Cell cultures were classified as normal central or peripheral zone if no hyperplasia or cancer was observed in the adjacent tissue sections. If the majority of the cells in the adjacent sections were characterized as benign prostatic hyperplasia (BPH) or cancer, then the cultures were classified accordingly. Cultures obtained from transurethral resection chips were classified as "mixed" based on the pathology report. All cultures were maintained at 37° C in a 95% air:5% CO_2 humidified incubator.

Growth assays. Collagen-coated dishes containing experimental media were inoculated with 1×10^3 to 5×10^4 cells/60-mm dish. After 14 d of incubation, cells were fixed and stained (21). Relative amounts of growth were determined visually, or the total area of the surface of the dish covered by cells was measured with an Artek colony counter in the laboratory of Dr. Richard Ham (28).

RESULTS

Growth in serum-free medium. Using PFMR-4A supplemented with cholera toxin, EGF, BPE, and hydrocortisone, we examined the proliferative response of cells to different levels of serum. Supplementation with 1% dFBS produced better growth than with 5% dFBS (Fig. 1 *A*), and growth without any serum at all was almost as good as with 1% dFBS if the cell density was high enough (Fig. 1 *B*).

Further assays were performed to determine which of the supplemental growth factors were necessary for

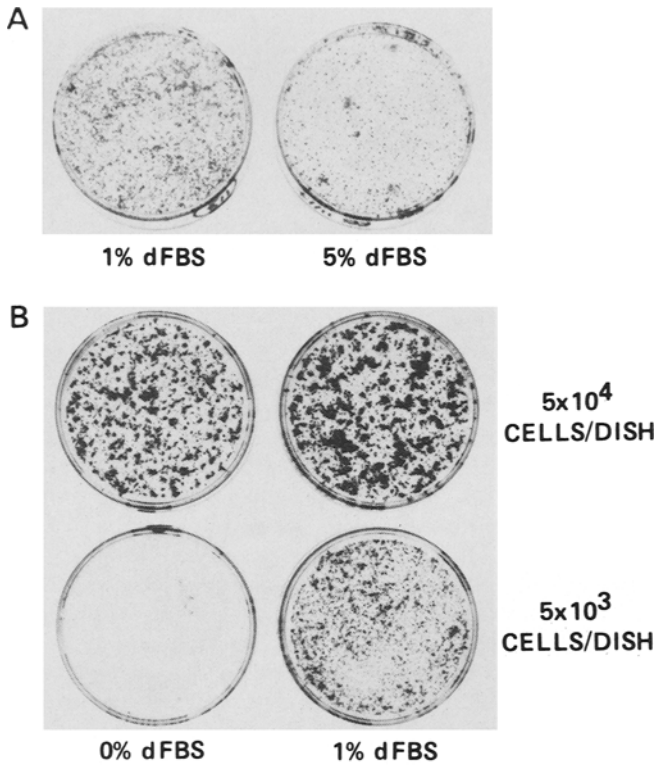

FIG. 1. Growth responses to serum. *A*, cells of strain RPS were inoculated into PFMR-4A supplemented with 10 ng/ml cholera toxin, 10 ng/ml EGF, 100 μg/ml BPE, 1 μg/ml hydrocortisone, and 1 or 5% dFBS (2.5×10^4 cells/60-mm dish). *B*, dishes containing PFMR-4A, cholera toxin, EGF, BPE, hydrocortisone, and no serum or 1% dFBS were inoculated with 5×10^4 or 5×10^3 cells/dish of strain WRB.

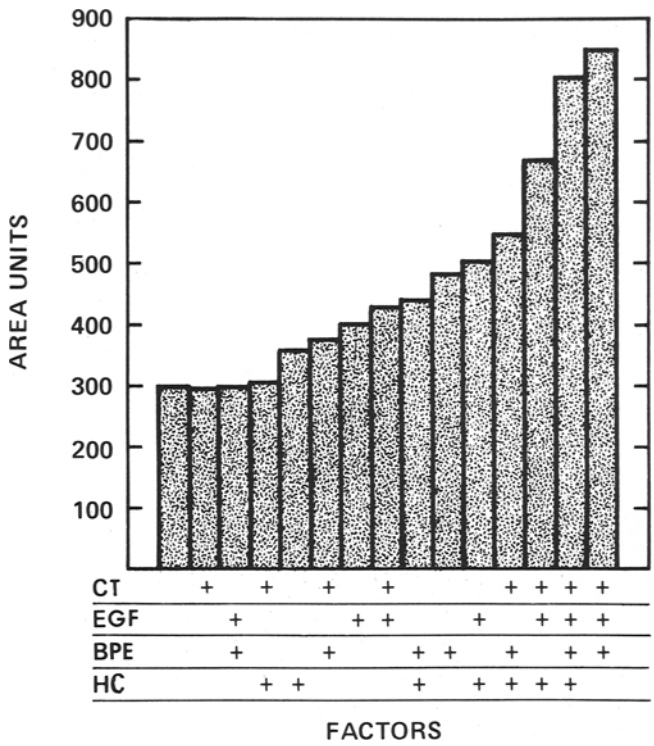

FIG. 2. Proliferation in response to growth factors in serum-free medium. JLC-BPH cells were inoculated at 5×10^4 cells/dish into PFMR-4A with no supplements or with combinations of cholera toxin, EGF, BPE, and hydrocortisone. On Day 14, cultures were stained and the surface area of the dishes covered by cells was determined by use of a colony counter and expressed in area units. *CT* = 10 ng/ml cholera toxin; *EGF* = 10 ng/ml epidermal growth factor; *BPE* = 100 μg/ml bovine pituitary extract; *HC* = 1 μg/ml hydrocortisone.

proliferation in serum-free medium (Fig. 2). The addition of each factor alone resulted in only minimal stimulation, but growth improved with combinations of two or more factors.

Bovine pituitary extract is another source besides serum of undefined growth factors in the medium. The addition of phosphoethanolamine or ethanolamine sometimes reduces the amount of BPE required for optimal growth (2) and this is the case for prostate cells. With the addition of 0.1 m*M* phosphoethanolamine, growth in medium supplemented with only 10 μg/ml BPE is equivalent to that in medium supplemented with 100 μg/ml BPE without phosphoethanolamine (Fig. 3 *A*). In some systems, endothelial cell growth supplement (ECGS) can replace BPE (14), but the addition of up to 150 μg/ml of ECGS to serum-free medium did not replace the stimulatory effects of BPE, either with or without phosphoethanolamine (Fig. 3 *B*). In this system, ECGS apparently cannot replace BPE.

Optimization of growth in serum-free medium. Usually a "cocktail" of growth factors and hormones is required to support optimal growth in serum-free medium, and insulin in particular is stimulatory to almost all cells tested (1). Prostate epithelial cells are no exception. At high cell densities a slight improvement in growth upon the addition of insulin is noted (Fig. 4 *A*), but at lower cell densities the stimulatory effect is dramatic (Fig. 4 *B*).

The addition of selenium and other trace elements often becomes critical for growth as the level of serum supplementation is decreased (16). The original formulation of PFMR-4 (13) included a stock containing seven trace elements, but we omitted these in our formulations (21,22) because growth in media supplemented with serum was not diminished by their absence. The addition of a stock solution containing the seven trace elements to serum-free PFMR-4A was toxic, so we examined the effect of selenium alone. The addition of selenium to PFMR-4A supplemented with cholera toxin, EGF, BPE, phosphoethanolamine, hydrocortisone, and insulin is definitely beneficial at levels ranging from 3×10^{-9} to 3×10^{-7} *M*, and the higher concentration of selenium allows almost "clonal" growth to occur in dishes inoculated with only 1×10^3 cells (Fig. 5).

Effects of calcium. Altering the level of extracellular calcium in the growth medium can have dramatic effects on the growth and differentiation of epithelial cells (2,8,11). Generally, increased proliferation occurs with decreased calcium and, alternatively, differentiation is enhanced by increased calcium. A change in morphology is often noted as calcium is decreased, and the population changes from closely packed cells with a "cobblestone" pattern to cells with wide spaces between them. In standard PFMR-4A, the concentration of calcium is approximately 9.2×10^4 *M*, and in this medium containing a fairly high level of calcium, prostate cells display a typical epithelial-like morphology of tightly

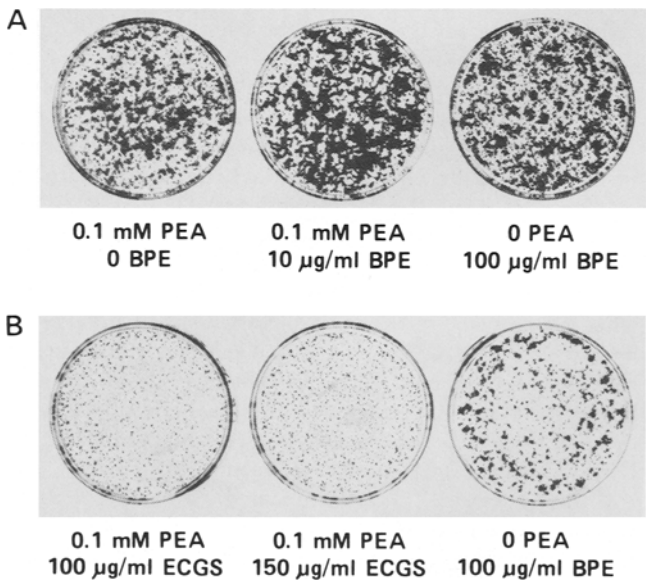

FIG. 3. Replacement of portion of pituitary extract by phosphoethanolamine but not by endothelial cell growth supplement. *A*, PFMR-4A was supplemented with 1% dFBS, 10 ng/ml cholera toxin, 10 ng/ml EGF, and 1 μg/ml hydrocortisone. Phosphoethanolamine (PEA) and BPE were added as indicated (5×10^4 cells/dish of strain WRB). *B*, PEA, BPE and ECGS were added as indicated to PRMR-4A supplemented with cholera toxin, EGF and hydrocortisone (5×10^4 cells/dish of strain VH-CA).

adherent, closely packed cells. As calcium is lowered, the cells take on a different appearance and become flattened and separate.

The dramatic change in morphology makes it difficult to compare relative growth by visual methods in response to different concentrations of calcium (Fig. 6). Therefore, cells (JLC-BPH) were inoculated into PFMR-4A containing 9.2×10^{-4} *M* calcium or 9.2×10^{-5} *M* calcium and supplemented with 1% dFBS, cholera toxin, EGF, BPE, and hydrocortisone. After 2 wk, the total number of cells was determined and the population cultured in the medium with less calcium contained 25% more cells. It seems that lower levels of calcium increase proliferation of prostatic epithelial cells as well as alter their morphology.

Anomalous responses of cancer cell lines. The previously described experiments were all undertaken with mortal cell strains derived from normal, BPH, or cancer tissues, and the results were generally equivalent regardless of the type of cell used. From these experiments, the following general conclusions were drawn: (a) prostatic epithelial cells can grow well in serum-free medium; (b) cholera toxin, EGF, BPE, hydrocortisone, and insulin each stimulate growth and act synergistically; and (c) lowering the concentration of calcium changes morphology and promotes growth.

When we examined the growth responses of two cancer strains which have been passaged for many months and may be immortal lines, a different picture emerged. The two lines, GKC-CA and WB-CA, express prostatic acid phosphatase and keratins but differ in several respects from the mortal cell strains that we have cultured. The morphologies of GKC-CA and WB-CA differ considerably from the common morphology exhibited by all of the mortal cell strains, even those derived from cancer tissue. The cells are very rounded and loosely attached and often detach from the substatum while retaining viability. This aspect of the cells' behavior may explain the results we obtained in our studies of the growth of GKC-CA and WB-CA cells in serum-free medium. GKC-CA cells grew very poorly in serum-free medium compared to medium supplemented with serum, even at high cell densities (Fig. 7). This requirement for protein supplementation could be met by using high levels of BPE, or serum (Fig. 7). WB-CA cells on the other hand, grow as well in serum-free medium as in serum-supplemented medium only if relatively high levels of calcium are present. If calcium is decreased to 9.2×10^{-5} *M*, then serum-free growth is decreased compared to proliferation in medium with serum (not shown).

The responses of GKC-CA and WB-CA cells to serum and calcium may be more related to attachment than to proliferation. Inasmuch as calcium and serum proteins are involved in attachment, perhaps deficiencies exacerbate the precarious attachment of these cells and the actual growth responses are not reflected. In fact, the initial plating efficiency of GKC-CA cells in serum-free medium is approximately 10% less than in serum-supplemented medium, and the same is true for WB-CA cells in serum-free vs. serum-supplemented medium with low calcium. It is our impression that, in addition, the cells tend to detach from the substratum during the course of the experiment in serum-free or low-calcium medium.

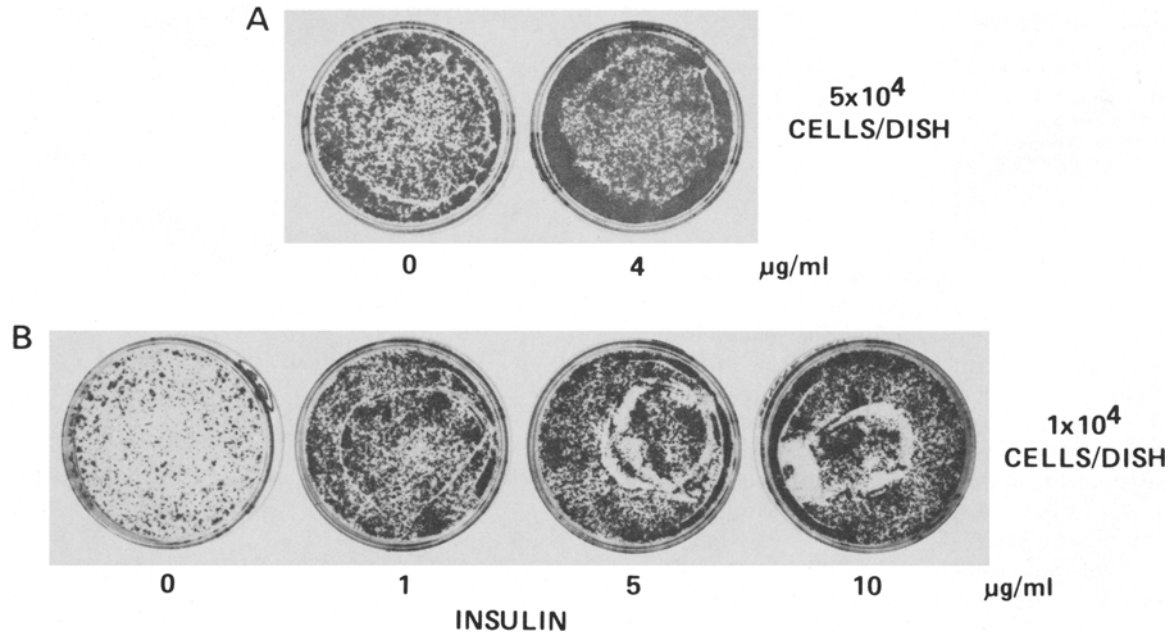

FIG. 4. Effect of insulin on cell growth. *A*, cells of strain PS-CZ were inoculated into PFMR-4A supplemented with 10 ng/ml cholera toxin, 10 ng/ml EGF, 10 μg/ml BPE, 0.1 m*M* phosphoethanolamine, and 1 μg/ml hydrocortisone. No insulin or 4 μg/ml of insulin was added (5×10^4 cells/dish). *B*, insulin was added to PFMR-4A supplemented with cholera toxin, EGF, 100 μg/ml BPE, and hydrocortisone as indicated (1×10^4 cells/dish of strain VH-CA).

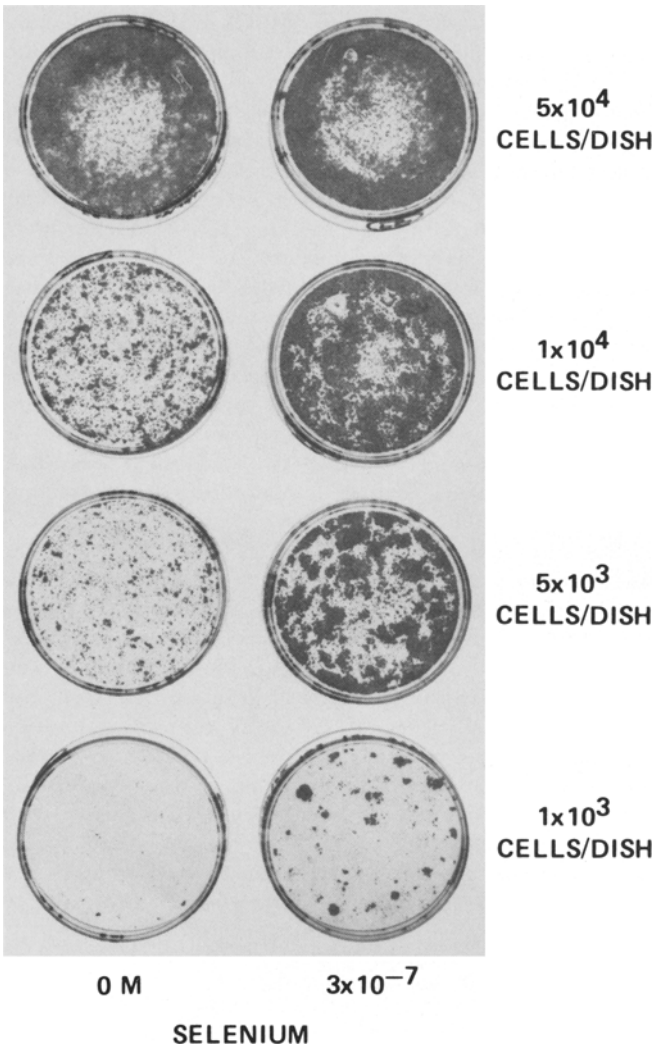

FIG. 5. The addition of selenium to serum-free medium. Selenium as indicated was added to PFMR-4A supplemented with 10 ng/ml cholera toxin, 10 ng/ml EGF, 10 μg/ml BPE, 0.1 m*M* phosphoethanolamine, 1 μg/ml hydrocortisone, and 4 μg/ml insulin. Cells of strain PS-CZ were inoculated at 1×10^3 to 5×10^4 cells/dish.

Lack of response to androgens. One of the reasons for achieving growth of prostatic epithelial cells in serum-free medium is to examine the effects of androgens without the complicated interactions of undefined factors. Previous attempts to demonstrate growth responses to androgens in serum-containing media were unsuccessful (unpublished results). Even the cocultivation of prostatic epithelial cells with fibroblasts from the prostate or foreskin, which presumably have androgen receptors (3), did not elicit a growth response to testosterone or dihydrotestosterone (Fig. 8).

The addition of testosterone or dihydrotestosterone to serum-free medium is also without effect on prostatic epithelial cells, except for toxicity at high levels (Fig. 9). Estradiol by itself is not stimulatory and does not act synergistically to promote an effect of androgens (not shown).

Although primary cultures are initiated and maintained in serum-containing medium, it is possible that there is a deficiency of androgens and that androgen-independent cells are selected. This seems unlikely because cells of strain GEH, initiated and maintained in medium supplemented with 1 μg/ml dihydrotestosterone, did not exhibit a growth response to androgens on subsequent testing. Lack of androgens in the culture medium also does not explain the poor growth of GKC-CA in serum-free medium because the addition of dihydrotestosterone does not improve growth (not shown).

DISCUSSION

Many of the factors required for optimal growth of adult human prostatic epithelial cells in serum-free medium are common to other culture systems, although each may exert different effects depending on the cell type. Raising the level of intracellular cAMP by factors such as cholera toxin, for example, can have opposite effects on different cell types (15). Large amounts of cholera toxin were required for successful subculture of prostatic epithelial cells in medium containing 20% serum (21). In medium containing little or no serum, cholera toxin by itself elicits only a minimal growth response, but it acts synergistically with other factors to promote proliferation, as do many combinations of growth factors and hormones (24).

In many cases the effects of a given factor on diverse cell types may be similar. Lechner and Kaighn (12) noted that EGF increased the plating efficiency and size of clones of normal neonatal prostate cells growing in

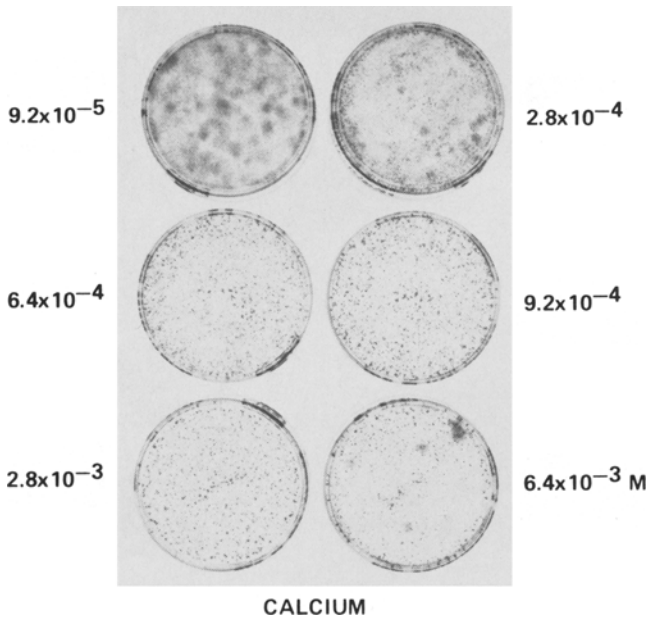

FIG. 6. Alteration of morphology by calcium. PFMR-4A was made with levels of calcium as indicated, and was supplemented with 10 ng/ml cholera toxin, 10 ng/ml EGF, 100 μg/ml BPE, and 1 μg/ml hydrocortisone. Cells of strain KGK-PZ were inoculated at 5×10^4/dish.

serum-deficient medium, and we also find that EGF enhances the growth of adult prostate cells in serum-free medium. In our system as well as in that of Lechner and Kaighn, hydrocortisone potentiates the activity of EGF; together, the two act synergistically to reduce the requirement for serum, in our case allowing some degree of serum-free growth which is further enhanced by the addition of other factors. Nevertheless, although most adult and neonatal prostatic epithelial cells respond similarly to EGF and hydrocortisone, they differ in other respects because adult cells cannot be reproducibly subcultured in the system developed for neonatal cells.

The response of prostatic epithelial cells to extracellular calcium is also typical of other types of epithelial cells. Only tissue-specific markers, prostate-specific antigen (29), and prostatic acid phosphatase (4) have been identified, so it is difficult to characterize the status of terminal differentiation of prostate cells, but lowering the level of calcium has a dramatic effect on morphology and seems to somewhat increase proliferation of mortal cell strains. The response to calcium of immortal lines of cancer cells seems to differ. Lechner and Kaighn (11) reported that cancer cells of line PC-3 (9) can proliferate with less calcium in the medium than can normal cells, but that the addition of EGF lowers the requirement for calcium by normal cells to that of cancer cells. We did not test the response of normal cells to calcium in the absence of EGF, but in its presence our two cancer lines, GKC-CA and WB-CA, required more calcium for optimal growth than did any of the normal, BPH, or cancer strains tested. This may reflect an attachment problem in medium with low levels of calcium or it may reflect an intrinsic difference in the growth response of immortal lines as compared to mortal strains. The development of immortal lines of prostate cancer cells seems to be a rate event, but the immortal lines that we have studied (GKC-CA, WB-CA, and PC3) all have certain similarities in that they were derived from highly malignant primary tumors or metastases and exhibit a rather loosely attached and rounded morphology in culture, which differs from the morphology of cancer cells that do not develop into immortal lines. Therefore these cells may also have similar growth requirement which differ from those of the mortal cells.

Fairly high cell densities are required for proliferation of prostate cells to grow in serum-free medium, although this may be partially overcome by the additions of insulin and selenium to the culture system. Similarly, cells of the established lines PC-3 and DU 145 (27) can grow in factor-free PFMR-4 only at high cell densities (10). This requirement could not be overcome by the addition of defined hormones or factors, although it could be overcome by the use of conditioned medium or cell feeder layers, indicating perhaps a requirement for autologous growth factors.

Of particular interest to the study of the prostate are the effects of androgens. Prostate cancer patients often respond initially to androgen deprivation, but eventually relapse, presumably due to the growth of malignant cells that have become androgen-independent. Investigators have attempted to document androgen sensitivity of prostate cells in culture (30) but the results have not been reproducible. We cannot demonstrate a dependency on androgens for growth in vitro of normal or malignant prostate cells. This does not seem to be the result of selection for androgen-independent cells in primary

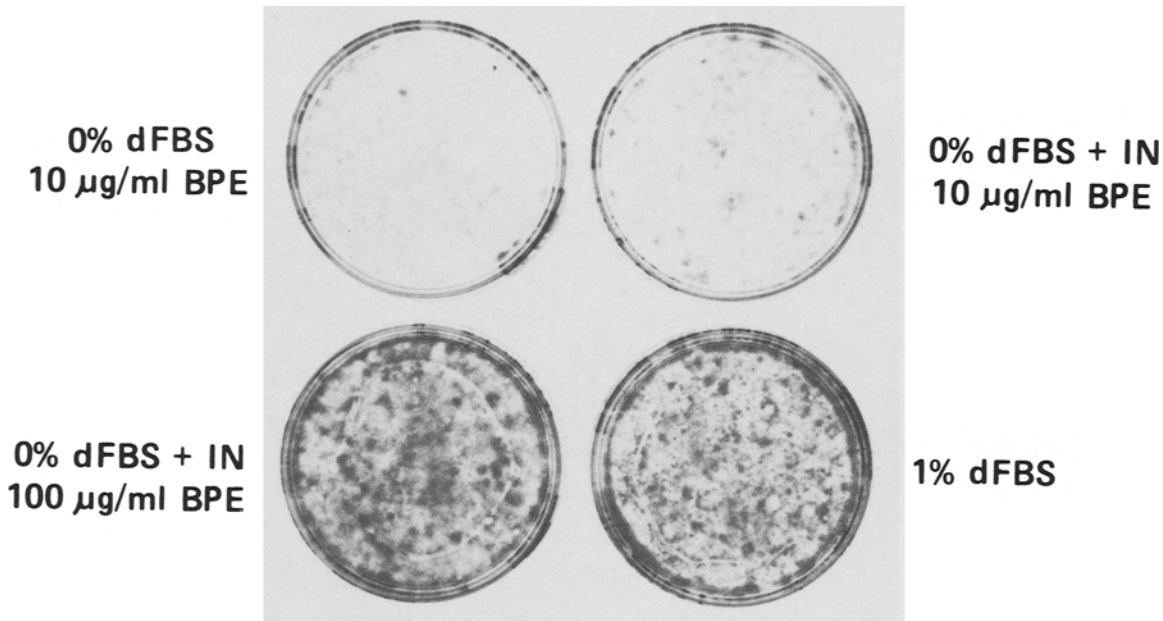

FIG. 7. Requirement of cancer cells for undefined protein supplements. Bovine pituitary extract, insulin (IN), and serum were added as indicated to PFMR-4A supplemented with 10 ng/ml cholera toxin, 10 ng/ml EGF, 0.1 m*M* phosphoethanolamine, and 1 µg/ml hydrocortisone. Cells of strain GKC-CA were inoculated at 1×10^4 cells/dish.

culture, inasmuch as the initiation of primary cultures in medium supplemented with dihydrotestosterone does not produce cells that respond to androgens.

It was conceivable that the effects of androgens were masked in our experiments performed with serum-supplemented media, yet even in serum-free medium we do not see any stimulation of growth by the addition of testosterone or dihydrotestosterone. It has been suggested that it may in fact be the stromal cells of the prostate that possess androgen receptors and mediate the effects of androgens through stromal-epithelial interactions. That the stroma can indeed exert a potent inducing effect on prostatic epithelium has been demonstrated by Cunha and colleagues (5), whose experiments with hybrid tissue formed by urogenital mesenchyme and prostatic epithelium showed that the epithelium was induced to differentiate in a prostate-specific manner by the mesenchyme. Androgen-insensitive stroma was incapable of inducing this differentiation.

In an effort to induce an effect of androgens, we cocultivated feeder layers of foreskin fibroblast cells with prostate epithelial cells, because fibroblasts of the genital skin have been shown to possess androgen receptors (3). Others have shown a fibroblast-mediated effect of testosterone in tissue culture, in which testicular peritubular cells secrete a protein under androgen control that modulates Sertoli cell functions (26). The growth of prostatic epithelial cells was generally inhibited by the presence of a fibroblast feeder layer, but growth with or without androgens was equivalent. Similarly, feeder layers of fibroblast cells derived from the prostate itself were incapable of inducing a response of the epithelial cells to androgens.

It may be that the primary effects of androgens on prostate cells are not growth-related. Giles and Cousins (7) showed that dihydrotestosterone altered zinc metabolism in PC-3 cells. As more markers of differentiation of prostate epithelial cells become available it will be

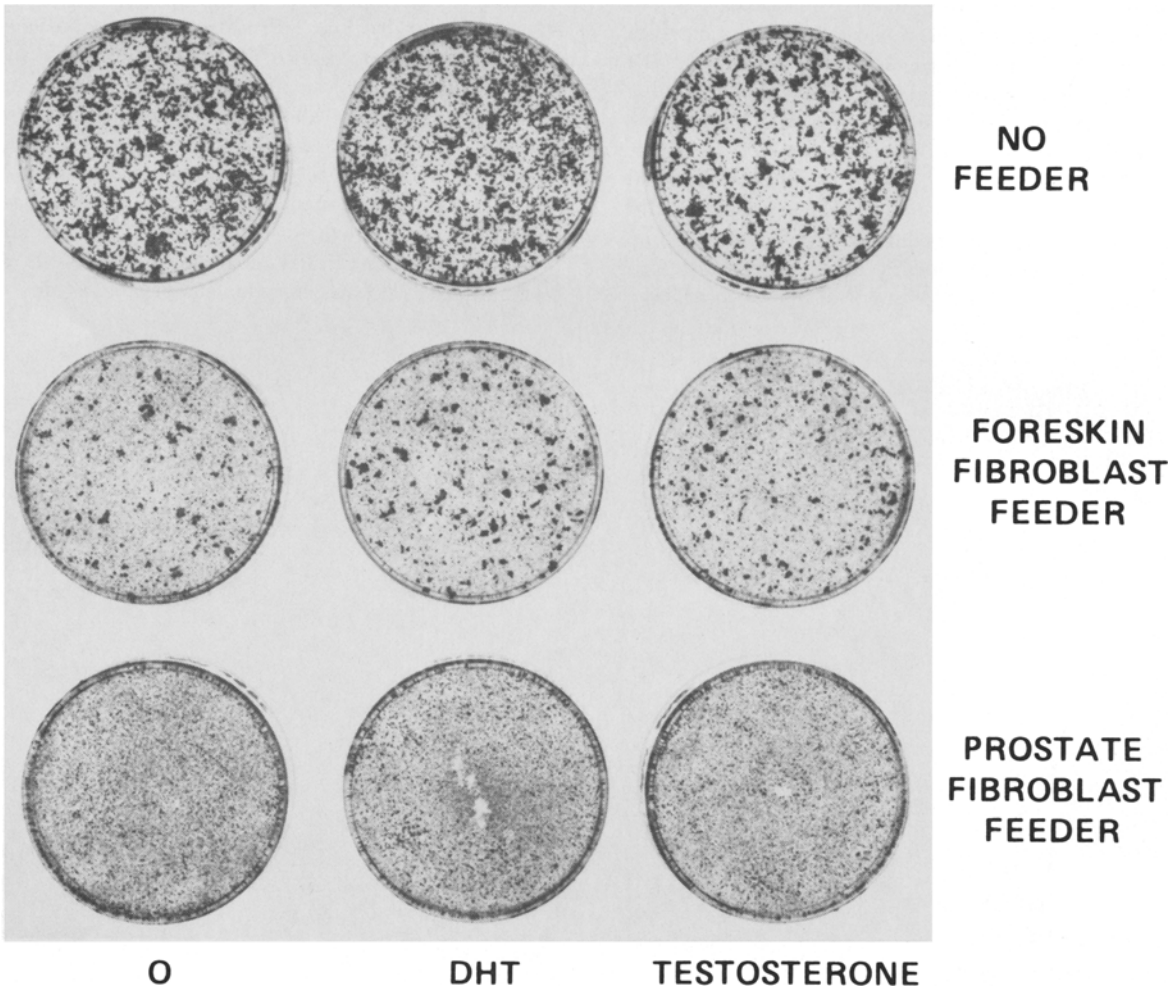

FIG. 8. Fibroblast feeder layers and effects on response of epithelial cells to androgens. Dishes containing irradiated feeder layers of fibroblasts isolated from foreskin or prostate tissue were inoculated with 5×10^4 cells/dish of strain JBDG using PFMR-4A supplemented with 1% steroid-free dFBS, 10 ng/ml cholera toxin, 10 ng/ml EGF, 100 μg/ml BPE, and 1 μg/ml hydrocortisone. Testosterone or dihydrotestosterone (DHT) were added at 0 or 1 μg/ml.

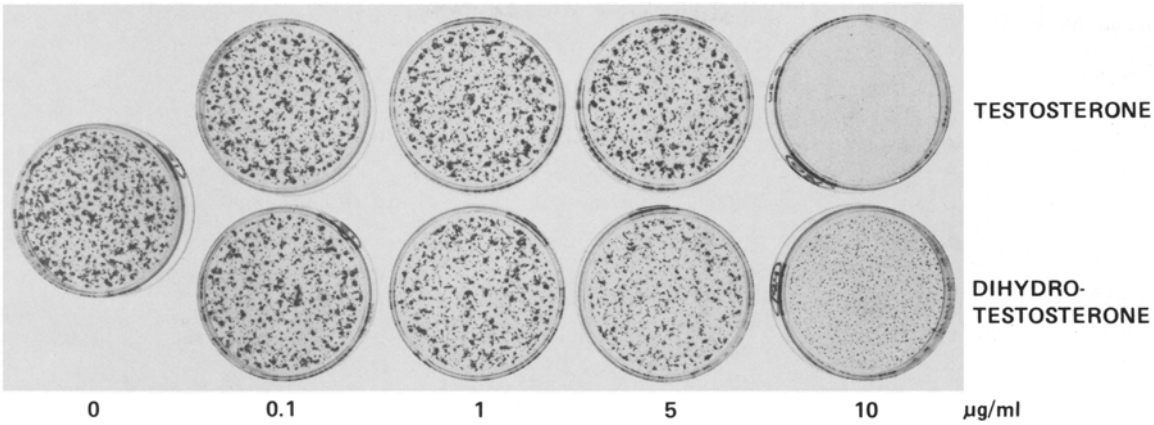

FIG. 9. Addition of androgens to culture media. Cells of strain WRB were inoculated at 5 × 10^4 cells/dish into PFMR-4A supplemented with 10 ng/ml cholera toxin, 10 ng/ml EGF, 100 μg/ml BPE, and 1 μg/ml hydrocortisone. Testosterone or dihydrotestosterone were added as indicated and relative growth was compared after 14 d.

possible to study the effects of androgens on cellular differentiation as well as growth in serum-free medium. The development of a serum-free culture system in which all types of adult human prostatic epithelial cells proliferate (PFMR-4A supplemented with cholera toxin, EGF, pituitary extract, phosphoethanolamine, hydrocortisone, insulin, and selenium) will facilitate such experiments.

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