Serial Culture of Single Adult Human Prostatic Epithelial Cells in Serum-free Medium Containing Low Calcium and a New Growth Factor from Bovine Brain

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ABSTRACT

Primary cultures of epithelial cells from human prostate acini proliferate in defined medium. However, the limited availability of human tissue and the lack of knowledge of the conditions required for clonal growth and serial culture of epithelial cells have limited progress in the study of human prostatic cell biology. Here we report conditions that permit the proliferation of single epithelial cells from normal, benign hyperplastic, and carcinomatous prostate through three to four serial passages, which represents at least seven to nine cumulative doublings of the cell populations. Primary cultures were prepared from prostatic acini. Monolayers resulting from the outgrowth of epithelial cells from acini were harvested and dissociated into suspensions of single cells which gave rise to discrete colonies in subsequent culture. The requirements for successful serial culture were (a) a low calcium concentration, (b) the presence of a growth factor that is concentrated in bovine neural tissue, (c) detachment of the epithelial cells with collagenase, and (d) harvest of cells before the cell concentration reached 6000 cells/cm² of culture surface. Suspensions of single cells were successfully stored between subcultures in 10% dimethylsulfoxide with 5% fetal bovine serum and revived after storage for up to 2 months in liquid nitrogen.

INTRODUCTION

Prostatic disease, either benign hyperplasia or carcinoma, afflicts the majority of men over the age of 60 years (1) and increases in incidence and severity with age. The cause of these diseases is unclear, partly because of the lack of a satisfactory model system. The culture of human prostatic epithelial cells in defined medium is a potentially important means of elucidating the fundamental cellular events leading to hyperplasia and to the development of carcinoma. A defined medium has been devised for primary cultures of acini from adult human prostates (2) and a serum-free medium has been made for dissociated epithelial cells from rat prostate (3). Human adult prostatic cells in culture offer some advantages over rat cells as a model for human cells from rat prostate (3). Human adult prostatic cells in culture proliferate in defined medium. However, the limited availability of isolated epithelial cells would significantly increase the material available for study from a single specimen of prostatic tissue.

Conditions for the development of cell lines both from human prostatic carcinoma (4, 5) and from neonatal prostates (6) have been reported. However, the most relevant material for the study of benign hyperplasia and the initiation of carcinogenesis is human adult prostate. Adult prostate epithelium in our hands did not survive the conditions worked out by Kaighn et al. (4), Lechner et al. (6), and Chaproniere² and has proved more difficult to subculture than rat cells. Peehl and Stamey (7) recently reported that a medium containing 20% FBS and high levels of cholera toxin supported serial culture of aggregates of normal, hyperplastic, and carcinoma-derived adult human prostatic epithelial cells and suggested that the nutrient medium might be important to their results. We report here the serial culture of dissociated adult prostatic epithelial cells using the serum-free medium previously reported for epithelial cells from the rat ventral prostate (3). The relatively low calcium concentration of the basal nutrient medium WAJC 404 and the presence of bovine hypothalamus extract (BHE) which contains a new prostate epithelial cell growth factor appear to be the most important improvements. This medium also supported the development of single, human prostatic epithelial cells into colonies at each passage.

MATERIALS AND METHODS

Growth Medium. The growth medium consisted of nutrient medium WAJC 404 (3) containing EGF (10 ng/ml; Collaborative Research, Waltham, MA), zinc-stabilized insulin (2.5 µg/ml; Sigma Chemical Co., St. Louis, MO), cholera toxin (10 ng/ml; List Biological Laboratories, Campbell, CA), 1 µM dexamethasone (Sigma), and BHE (20 µg/ml) (8, 9).

Bovine Hypothalamus Extract. This was prepared by the same method described for pituitary in Ref. 8. The active factor was further purified by ion exchange chromatography and gel filtration as described in Ref. 9.

Buffered Salt Solution. BSS consisted of 30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid hydroxide (pH 7.6), 4.0 mM glucose, 2.0 mM KCl, 12 mM NaCl, 1.0 mM KH₂PO₄, and 3.3 µM phenol red.

Human Prostatic Tissue. Human prostatic tissue was obtained from the Comprehensive Cancer Center, Birmingham, AL. Benign hyperplastic prostate was obtained by transurethral resection during surgery for urethral obstruction. Three specimens of normal prostate were obtained, one from autopsy (donor age, 53 years) and two from cystectomies. One patient had bladder carcinoma (age, 62 years) and one had ulceration of

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² D. M. Chaproniere, unpublished results.

³ The abbreviations used are: FBS, fetal bovine serum; BHE, bovine hypothalamus extract; EGF, epidermal growth factor; BSS, balanced salt solution; PDL, population-doubling level.
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the bladder (age 46 years). Eight specimens were from the prostates of patients diagnosed as having benign prostatic hyperplasia and three were from patients with prostatic carcinoma. Specimens were obtained by transurethral resection or prostatectomy. Pathology reports were obtained for all tissue specimens.

Isolation of Prostatic Acini. Tissue was minced, washed with BSS, and placed on a reciprocating shaker for 24 h at 37°C in collagenase (200 IU/ml; No. 4196 CLS; Worthington, Cooper Biomedical, Malvern, MA) in BSS containing 5% FBS. The digest was triturated by repeated pipetting with 10 µl pipette after 18, 23, and 24 h, then diluted with BSS, and centrifuged at 100 x g for 15 min. The pellet was suspended in BSS, again collected by centrifugation, and then resuspended in RPMI 1640 nutrient medium (GIBCO, Grand Island, NY). After 15 min at room temperature, the distinct acini settled and the supernatant containing single cells and small aggregates was removed. The acini were resuspended in growth medium and inoculated into T-75 tissue culture flasks (Corning Glassworks, NY). Cultures were incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. From 3 to 6 days later, the outgrowth of primary epithelial cells was subcultured for serial passage or used for experiments described in the text.

Serial Culture. Cells were detached from the culture flasks by shaking on a reciprocating shaker in 2.5 ml of collagenase at 675 lU/ml in BSS at 37°C. Cells completely detached after about 1.25 h. The detached cells were diluted with 10 ml of BSS, collected by centrifugation, washed by resuspension in BSS, and again collected by centrifugation. The cell pellet was suspended in growth medium and the cells were counted in a hemocytometer.

To initiate secondary cultures from single cells, 5 x 10⁴ cells were inoculated into T-75 flasks containing growth medium. The medium was changed every 2 to 3 days. Cells were subcultured before cell density reached 4 to 6 x 10⁶/flask.

Storage of Cultured Cells. Aliquots of cell suspensions harvested by the procedure described above were resuspended in 10% dimethyl sulfoxide and 5% FBS or bovine serum albumin (3 mg/ml) in medium WAJC 404 at approximately 10⁶ cells/ml and transferred to ampules which were then held for 1 h at 4°C. The ampules were then transferred to a Union Carbide type BF-5 freezing unit for at least 4 h before storage in liquid nitrogen. For subsequent culture the ampules were quickly thawed in water and the suspension was diluted into growth medium lacking dexamethasone and transferred to culture vessels.

Cloning Efficiency. Cloning efficiency was estimated using 24-well Linbro multiwell plates (Flow Laboratories, McLean, VA). Lines of four wells were seeded with 2000, 1500, 1000, 500, 250, and 100 cells/well in 1 ml growth medium. After 5 days of incubation, the culture was fixed with methanol and stained with Giemsa, and the number of colonies containing more than eight cells were scored and plotted against the initial cell concentration. The plot was linear up to the cell concentration lacking dexamethasone and transferred to culture vessels.

RESULTS

Serial Culture of Epithelial Cells in Serum-free Medium

Primary cultures of prostatic acini were established and the resultant epithelial sheet detached, dissociated into a single cell suspension, inoculated into new flasks, and serially cultured as described in "Materials and Methods." Epithelial cells from normal or hyperplastic and carcinomatous prostates proliferated at an exponential rate through three to four serial passages for a total of seven to nine cumulative doublings of the cell population (Figures 1 and 2). No correction for cloning efficiency at each subculture was included in the calculation. Cloning efficiency was determined in separate experiments as described in "Materials

Fig. 1. Cumulative PDLs of epithelial cells from specimens of normal (C, O), hyperplastic (▲), and carcinomatous (□) prostates from the first subculture until the cessation of proliferation. Acini were isolated and the cells were passaged as described in "Materials and Methods." At each subculture the cells were harvested and counted in a hemocytometer and the number of PDL since the last subculture was calculated. Subcultures were made at various intervals and the PDL was plotted against the time after the initiation of primary cultures. Cell proliferation exhibited an exponential pattern until it ceased abruptly at passage 3 or 4, which represented a PDL of 9.

Fig. 2. Cumulative population doublings of epithelial cells from a normal prostate (donor age, 46 years). PDL was determined as described in Fig. 1. Inset table, cloning efficiency at each serial subculture. Cloning efficiency was determined as described in "Materials and Methods." PDL at each passage is indicated by different symbols, P₀, time between initiation of culture and the first subculture (primary culture); ▲, passage 1; O, passage 2; □, passage 3; □, passage 4.
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and Methods" and was less than 10% at each subculture after the first (Fig. 2). Figure 3 shows the rate of proliferation of epithelial cells at various cumulative doubling levels (PDL) of the cell population. The minimum population-doubling time was approximately 2 days up to PDL 3 and then slowed to 3 days. Cells from each of the first three subcultures multiplied exponentially until the maximum density was obtained, when they began to slough into the medium. Cells from the fourth passage (seeded at PDL 8) multiplied exponentially for 7 days and then ceased abruptly. After PDL 9, cell number did not increase significantly.

Subcultured cells were stored frozen in 5% FBS in medium WAJC 404 in liquid nitrogen. Upon thawing, cells multiplied with a cloning efficiency of approximately 3% which was similar to that of subcultured cells that had never been frozen. The viability of cells frozen in bovine serum albumin (3 mg/ml) in the absence of serum was approximately one-half that of cells frozen in FBS.

Key Conditions Required for Serial Culture

Both the calcium ion concentration and the presence of BHE (or bovine brain or pituitary extracts) and the use of collagenase for cell detachment were key factors in the serial culture of prostatic epithelial cells. Harvest of cells in the exponential phase of growth also significantly improved the yield of cells that were capable of division in serial culture.

Low Calcium Concentration. When nutrient medium RPMI 1640 was used for primary cultures, the epithelial cells grew out of the acini as sheets of tightly apposed cells (Fig. 4c). In dense areas the epithelial sheet formed two layers of cells (2). The intact cell sheet could be detached by treatment with collagenase but could not be dissociated into single cells. The detached cell sheets attached poorly when transferred to fresh culture flasks and did not proliferate. Exposure to trypsin detached and dissociated the primary cell sheet into single cells which reattached but did not multiply in secondary culture. In contrast, the growth of epithelial cells in both primary and subsequent cultures in nutrient medium WAJC 404 resulted in a single layer of epithelial cells with few apparent cell-to-cell associations (Fig. 4d). Cells readily detached upon treatment with collagenase and dissociated into single cells and a few small aggregates (Fig. 4a). The multiplication of the epithelial cells was optimal above a calcium concentration of 0.12 mM (Fig. 5). Extensive association between cells occurred only at concentrations above 0.3 mM. Therefore, differences in calcium ion concentration appear to account for a major part of the differences in adhesion between cells in RPMI 1640 (0.4 mM Ca²⁺) and WAJC 404 (0.13 mM Ca²⁺).

Bovine Brain Extracts. These are a concentrated source of an apparently novel growth factor for normal rat prostatic epithelial cells (3). BHE was also essential for optimal proliferation of dissociated human cells in serial culture (Figs. 6 and 7). A partially purified sample of the active growth factor for rat cells stimulated optimal proliferation of human cells at a protein concentration that was one-hundredth that of crude BHE (Fig. 6).

Collagenase. Collagenase and trypsin were compared for efficiency of cell detachment and dissociation and their effect on subsequent clonal growth and serial culture. Detachment of epithelial cells by trypsin was incomplete. Cells from primary cultures and early subcultures of normal and hyperplastic prostates ceased to multiply after exposure to trypsin. Collagenase gave the best yield of proliferating epithelial cells at all passages tested.

Cell Density. Serially cultivated epithelial cells rarely reached a density greater than 6000 cells/cm² of culture surface (4 to 5 x 10⁵ cells/75-cm² flask) before cells began to enlarge and slough into the culture medium. Proliferation rate and cloning efficiency in subsequent culture declined significantly if cells were harvested beyond this stage.

Hormone Requirements for the Optimal Proliferation of Subcultured Cells in Serum-free Medium

The set of growth factors and hormones which stimulated the proliferation of rat prostatic epithelial cells (3) were tested on human prostatic epithelium separately and in combination. Epithelial cells from normal, hyperplastic, and carcinomatous prostates showed similar responses at all passage levels. A typical response of second passage normal epithelial cells is shown in Fig. 7. BHE was the most important growth stimulant at all passage levels and, like EGF and insulin, was an active mitogen both alone and in combination with other factors. The activities of BHE, EGF, and insulin were additive. Cholera toxin and dexamethasone had little effect alone but enhanced the activity of EGF and insulin.

DISCUSSION

We report here methods for the serial culture in serum-free medium of dissociated epithelial cells derived from isolated acini of human adult normal, hyperplastic, and carcinomatous prostates. In this medium the sole undefined component was a partially purified extract of bovine brain. The epithelial cells underwent a minimum population increase of seven to nine generations. Since no correction was made for cloning efficiency of less than 10% at each subculture, the number of actual generations of each cell in the population was probably at least 10-fold higher.

Key factors that promote successful serial passage were: (a) low calcium ion concentration; (b) the presence of an apparently novel neural tissue-derived prostate epithelial cell growth factor;
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Fig. 4. a, phase-contrast micrographs of a cell suspension resulting from the dissociation with collagenase of a primary culture of epithelium from a prostatic carcinoma. Similar suspensions were obtained from cultures derived from normal and hyperplastic tissue. Cell suspensions were diluted and used for clonal growth or further serial culture. x 100. b, stained colonies derived from single normal epithelial cells after 5 days in growth medium. x 40. c, d, primary cultures of epithelial cells from carcinoma after 4 days in medium RPMI 1640 (c) and in WAJC 404 (d), with the same hormonal supplement used in growth medium. Phase-contrast, x 100.

(c) the use of collagenase to detach the epithelial cells for subculture; and (d) subculture before a maximum cell density was attained. A low calcium concentration in the medium appeared to reduce cell-to-cell associations and facilitated the dissociation of cells with collagenase rather than trypsin, which irreversibly damaged the cells. Nutrient medium WAJC 404 (3) was developed to support the selective and optimal proliferation of rat prostatic epithelial cells in primary culture. The results presented here show that the medium was also an improved basal medium for human prostatic epithelial cells. This suggests that there is little species specificity of nutrient medium requirements between rodent and human prostatic epithelial cells. Medium WAJC 404 in the presence of zinc-stabilized insulin (5 µg/ml), EGF (10 ng/ml), cholera toxin (10 ng/ml), 1 µM dexamethasone, and BHE (20 µg/ml) supports the serial passage and clonal proliferation of dissociated, human prostatic epithelial cells and suppresses the proliferation of fibroblasts.

The growth-stimulatory activity of bovine brain extracts for epithelial cells from normal rat prostate resides in an apparently novel polypeptide that is heat, acid, trypsin, and mercaptoethanol labile with a pI of 4 to 6 and molecular weight of about 22,000 (9). The results here suggest that the same factor is active for human prostatic epithelial cells and was probably the deficient factor in previous attempts to culture single, dissociated prostatic epithelial cells. Further purification and characterization of the growth factor is in progress and will be reported elsewhere.5

5 W. L. McKeehan, P. S. Adams, and D. Fast, manuscript in preparation.
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Trypsin detached epithelial cells incompletely. Although the detached cells reattached to new culture vessels, they did not multiply. Previous attempts by one author to use the nonenzymatic methods described by Lechner et al. (6) in conjunction with a medium consisting of RPMI 1640 with 10% fetal bovine serum resulted in a similar inability of cells to proliferate. Nonenzymatic methods were not investigated under the conditions described in this paper since collagenase yielded satisfactory results.

Neonatal (6) and carcinomatous (4, 5) human prostatic epithelium have been successfully subcultured and cell lines have been derived from these cultures. However, nonmalignant adult epithelium has proven more difficult. Recently Peehl and Stamey (7) reported the successful subculture of adult human prostatic epithelial cells in medium containing 20% serum. They found cholera toxin to be the most important growth factor for serial culture under these conditions. We found cholera toxin stimulated cell proliferation in the serum-free conditions reported here but that its importance was secondary to brain-derived prostate growth factor, insulin, and EGF. Like dexamethasone, cholera toxin stimulated proliferation only in the presence of these factors. The results reported here differ significantly from those of the above authors and others in that we have established conditions for serial and clonal culture of single, dissociated nonmalignant adult human prostatic epithelial cells in serum-free medium. Although partially purified brain-derived growth factor is still chemically undefined, results thus far suggest that the use of more purified preparations of brain-derived growth factor supports serial passage and clonal growth of prostatic epithelial cells.

It is unclear whether the maximum proliferative life span of human prostatic epithelial cells is expressed under the conditions reported here. Further passage may be made possible by the discovery of yet unidentified factors. Nevertheless, the results presented here for the serial culture of dissociated human prostatic epithelial cells in serum-free and near-defined medium, together with clonal proliferation, increase severalfold the quantity of cells available from single specimens of human prostate. Successful storage of cells in liquid nitrogen permits their utilization over a longer period. These two factors significantly enhance the utility of cultured prostatic epithelium as a model system for the study of the control of growth and function of the normal, hyperplastic, and carcinomatous prostate.

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