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# Isolation, in vitro Cultivation, and Electron Microscopy of Normal and Malignant Prostatic Epithelial Cells from the Copenhagen Rat

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Summary. Procedures are described for the isolation and cultivation of normal rat prostatic epithelial cells. The techniques, which involve collagenase digestion and Ficoll purification of the epithelial population, are efficient, inexpensive, and produce pure monolayers. Included is a scanning and transmission electron microscopic study comparing cells isolated in vitro to rat prostatic epithelial cells in situ. Further ultrastructural comparisons are made to a malignant cell line, the Dunning R3327H Copenhagen rat prostatic adenocarcinoma.

Key words: Tissue culture - Prostatic epithelial cells - Prostatic adenocarcinoma - Scanning electron microscopy - Cell surface.

## INTRODUCTION

Despite the high incidence of benign and malignant prostatic disease, relatively little information is available concerning the fundamental biological mechanisms of the prostate at the cellular level. Much of the present understanding of prostatic tumours has been derived from clinical observations and studies on chemically induced tumours in experimental animals (6, 14, 26, 27). Only limited success has been achieved in establishing in vitro cultures of prostate from both human and animal tissues (7, 15, 21, 32, 36, 40, 43, 44, 46, 50, 51). Successful cultures of pure prostatic epithelial cells have mainly utilized neoplastic and hyperplastic human tissues. In vitro cultivation of truly normal epithelial cells has been hindered, due to the severely limited availability of viable, non-diseased human prostate (7). Since no animal is known in which the incidence of spontaneous prostatic adenocarcinoma approaches that of man, few prostatic adenocarcinoma cell lines are generally available for comparative studies with normal cells.

An effective tissue culture system would greatly facilitate analysing both the interactions between normal and malignant cells, and the actions of specific aetiological compounds, chemotherapeutic agents, and hormones. In addition, certain immunological and biochemical properties are determined most easily in vitro. Tissue culture systems are also valuable in studying the dynamics of cell surfaces and receptors, since plasma membranes are directly exposed on cells maintained within a more fully controlled environment.

The difficulties experienced by laboratories in maintaining prostatic tissues in vitro reflect not only problems inherent in the separation of viable, normal cells (15, 32, 36, 40), but also specific nutritional and hormonal requirements of cells (4). Other problems encountered in tissue culture of prostate have included positive identification of cells (15, 48), passage to secondary culture (21, 22, 45), and the establishment of conditions for the selective enrichment of epithelial populations (31, 32, 36). In most explants, cells with a higher growth rate, fibroblasts, soon dominate the culture population, even on short-term culture.

The purpose of this manuscript is to describe procedures for purification and monolayer cultivation of normal prostatic epithelial cells from the Copenhagen rat, which we have found to be efficient and lead to essentially pure cultures of epithelial cells in a short time. The usefulness of the techniques is demonstrated by a comparative scanning and transmission electron microscopic study of normal acinar cells and a malignant cell line, the Dunning R3327H rat prostatic adenocarcinoma (41, 42).

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## MATERIALS AND METHODS

Preparation of pure epithelial monolayers from the rat prostate depends upon sequential enzymic digestions of the gland to remove most of the stroma and produce a monodisperse suspension of viable cells. The preparation can be further separated into epithelial and mesenchymal populations by isopyknic centrifugation in polysucrose. The techniques we employed were modified from work reported by Pretlow and others (15, 31, 32, 46, 47) on the isolation of human malignant cells.

### Reagents and Solutions

The growth medium used throughout the experiments was Dulbecco's modification of Eagle's minimal essential medium (Flow Labs), supplemented with testosterone (Sigma, 1 μg/ml), insulin (Sigma, $10^{-3}$ to $10^{-4}$ U/ml), and the polyamines, spermine and spermidine (Calbiochem, 1 drop of a 25 μg/ml solution added to each ml of growth medium). Calf serum (Gibco) and rat serum (obtained from blood of the organ donor and processed during tissue dissociation procedures) were added to the growth medium either alone or in combination, and the type and concentration of serum in the final nutrient mixture was dependent upon the stage of the isolation procedure. Conditioned medium, added in some steps, was prepared by exposing subconfluent monolayers of normal epithelial cells to growth medium for three days.

Tissue dissociation enzymes were stored as frozen aliquots which were thawed and diluted as needed. The concentration of stock collagenase (Worthington, Type I) was 1% w/v in Hank's balanced salt solution (HBSS), diluted to a working concentration of 0.1% in growth medium containing 2% calf serum. Divalent cation chelating agent ethylene-diamine-tetraacetate (EDTA) (Fisher) was stored as a .25% solution w/v in calcium and magnesium free HBSS (cmfHBSS) and diluted to .025% for use. Trypsin (Sigma, crude) was made up as a 2.5% w/v solution and diluted to a working strength of 0.25% in cmfHBSS.

For cell separations, Ficoll (Sigma polysucrose, MW 400,000) was dissolved in serum-free growth medium several hours before use at a concentration of 6% w/v.

All solutions to which cells were exposed contained penicillin (Sigma, 60 μg/ml), streptomycin (Sigma, 100 μg/ml), and Fungizone (Gibco, amphotericin B, 2.5 μg/ml), and were sterilized by membrane filtration (Gelman metricel GA-8, 0.20 micrometer pore size).

### Isolation of Cells

Copenhagen rats and the Dunning R3327H tumour cell line were provided by Dr. David Lubaroff of the Urology Department, University of Iowa. To obtain normal cells, right and left dorsolateral prostates were surgically removed under ether anaesthesia from adult male rats weighing 80-400 grams and placed in growth medium at 37°C. Simultaneously, blood was drawn from the heart as a source of serum for plating the cells. Prostates of each animal were minced (1 mm cubes) and incubated in 7 cc of 0.1% collagenase solution for 2-4 hours at 37°C in a 15 cc centrifuge tube. Gentle pipetting of tissue pieces every 30 minutes facilitated digestion of the stroma by the enzyme. After the digestion was complete and the acini were freed from the stroma (monitored with the dissecting microscope), the acini were allowed to settle for 2 min., and the supernatant was aspirated and replaced by fresh growth medium with 2% calf serum. The acini were then rinsed twice in cmfHBSS, each time allowing them to settle for 2 min. before aspirating the supernatant. At this time, acinar explants were made by placing a few acini from each tube beneath sterile coverslips held in place with stopcock grease in dishes filled with medium containing 10% calf serum, 5% rat serum, and 5% conditioned medium. The remaining acini in each tube were resuspended in 7 cc .025% EDTA solution. After 5 min., chelating solution was replaced by 7 cc of 0.25% trypsin for 20 min. Breakdown of the acini into a monodisperse cell suspension was aided initially by gentle pipetting, and during the last 5 min. of the time period by passing the solution through a 22-26 gauge needle. After 20 min. a few drops of calf serum were added to stop enzymatic activity, and any acini remaining intact were allowed to settle to the bottom of the tube. The cell suspension (supernatant) was transferred to a clean tube, pelleted by centrifugation, washed twice, and resuspended in 2 cc of growth medium with 10% calf serum.

To further purify the epithelial preparation, 2 cc suspended cells were placed on top of 8 cc Ficoll in a 15 cc centrifuge tube and spun at 80-100 xG for 4 min. After centrifugation, the top layer, containing fibroblasts and cell debris, was removed and the remaining solution centrifuged at 180 xG for 5 min. The pellet, consisting of epithelial cells, was washed and resuspended in growth medium containing 10% calf serum, 5% rat serum, and 5% conditioned medium.

Viability was tested by trypan blue exclusion (.08%), and the cells were seeded in 60 mm plastic dishes (Falcon) at a density of $10^6$ cells/ml of above medium and incubated at 37°C in 5% $CO_2$.

### Maintenance and Subpassage of Cultures

After 4 days, when the cells had attached and begun to spread, the plating medium was aspirated from the culture dish along with non-attached

cells and debris. Cultures were maintained in growth medium with 15% calf serum changed every 3-4 days until monolayers were confluent, about 2-3 weeks. Endogenous acid phosphatase activity was demonstrated on cell cultures by adapting techniques for staining histological sections (1).

For subpassing, monolayers were removed from the dishes by either one of two methods. Cells either were soaked in 1.5 mM EDTA in cmfHBSS for 15 min. and scraped from the dish with a rubber policeman, or were exposed to .025% EDTA for 3 min. followed by 10-15 min. in 0.25% trypsin. The cell suspension from each dish was pelleted, washed, resuspended in growth medium, and seeded into two or three dishes containing medium with 10% calf serum, 5% rat serum, and 5% conditioned medium. After 3-4 days, plating medium was again replaced by growth medium with 15% calf serum.

## Scanning and Transmission Electron Microscopy

Specimens of prostate were taken for electron microscopy at the time of surgery, after collagenase digestion, 3 days post-plating, at approximately 50% confluency, and when cultures were 100% confluent. In vivo tissue samples were processed by sequential immersion of 1 mm tissue blocks in fixative solutions, while isolated acini were handled by gentle centrifugation and fixation of the pellet. For sampling of tissue cultures, cells were grown on discs cut with scissors from Aclar plastic (Allied Chemicals), which could be handled with forceps.

In the following fixation schedule, time intervals refer to preparation of in vitro monolayers. For in vivo tissue blocks, all timed steps were extended to three times as long, to ensure adequate penetration of the fluids. Specimens were rinsed in HBSS and fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer with 0.1 M sucrose for 20 min. at 37°C. Following two buffer changes of 2 min. each, cells were post-fixed in 1% osmium tetroxide in buffer for 5 min., rinsed twice in buffer, and exposed for 5 min. to a 1% saturated solution of thiocarbohydrazide (TCH) (Eastman) in double distilled water filtered through Whatman number one qualitative paper. The specimens were then rinsed twice in DDW, treated with osmium again for 5 min., and rinsed twice in DDW. The additional osmium bound to the specimen by the ligand TCH helps prevent charging artifact and increases resolution in the scanning microscope, in addition to enhancing the contrast of thin sections in the transmission microscope (16). Tissues were rapidly dehydrated at 5 min. intervals through a series of graded ethanols, ranging in concentration from 50% to absolute. After three changes of absolute ethanol, specimens for SEM were critical-point dried in $CO_2$, mounted on stubs with copper tape or silver paint, coated with 150 Å of gold-palladium in a Hummer vacuum evaporator, and viewed in a Cambridge Stereoscan S-4 scanning electron microscope. Specimens for TEM were immersed in two changes of propylene oxide for 5 min. each, infiltrated for 4 hrs. with a mixture of 50% Epon-50% propylene oxide, and embedded in Epon for thin sectioning. Grids were stained with uranyl acetate for 1 min. and lead citrate for 1 min. prior to examination with a JEM 100B transmission electron microscope.

Some of tissue blocks embedded for TEM were thin-sectioned and then processed for SEM by a technique of Epon removal (10, 56). This technique has the advantage that one is able to view the same acini, and indeed the same cells, in both the TEM and SEM, as well as the light microscope (1 μm sections stained with toluidine blue).

# RESULTS

## Morphology of Normal Cells in Vivo

The morphology of normal prostatic acinar cells fixed in situ prior to collagenase treatment is illustrated in Figures 1-4. By TEM, the columnar cells exhibit polarisation of organelles typical of secretory epithelia and identical to that established for dorsolateral prostate of other strains of rat (9) (Fig. 1). Readily identifiable above the basally located nucleus (NU) are a zone consisting of parallel cisternae of rough endoplasmic reticulum (ER), a Golgi zone (GC), and an apical zone (AZ). Figure 2 is a scanning electron micrograph of the acinar cells illustrated in Figure 1 after removal of the embedding medium and critical point drying. The sequential processing of specimens through both TEM and SEM provided instructive views of the apical surfaces and interiors of cells not appreciable by TEM alone, especially when considering surface-related phenomena such as microvilli and secretion. Most of the dome-shaped apical cell surfaces were covered more or less homogeneously with stubby microvilli interrupted by areas of bare surface membrane with no apparent pattern to the distibution (Figs. 2 and 4). Some cells possessed longer microvilli near the cell borders which were in contact with similar structures on neighbouring cells (Fig. 4). A very small portion of the cells had longer microvilli over their entire apical surface (Fig. 6). These cells, however, were present in discrete focal patches throughout the acini. No totally bare or ruffled cells were seen. Also present were smooth-surfaced protuberances or saccules extending from the apex of many cells (Figs. 2 and 4). The structures ranged from spheroidal to ovoid in shape and are probably related to the process of apo-

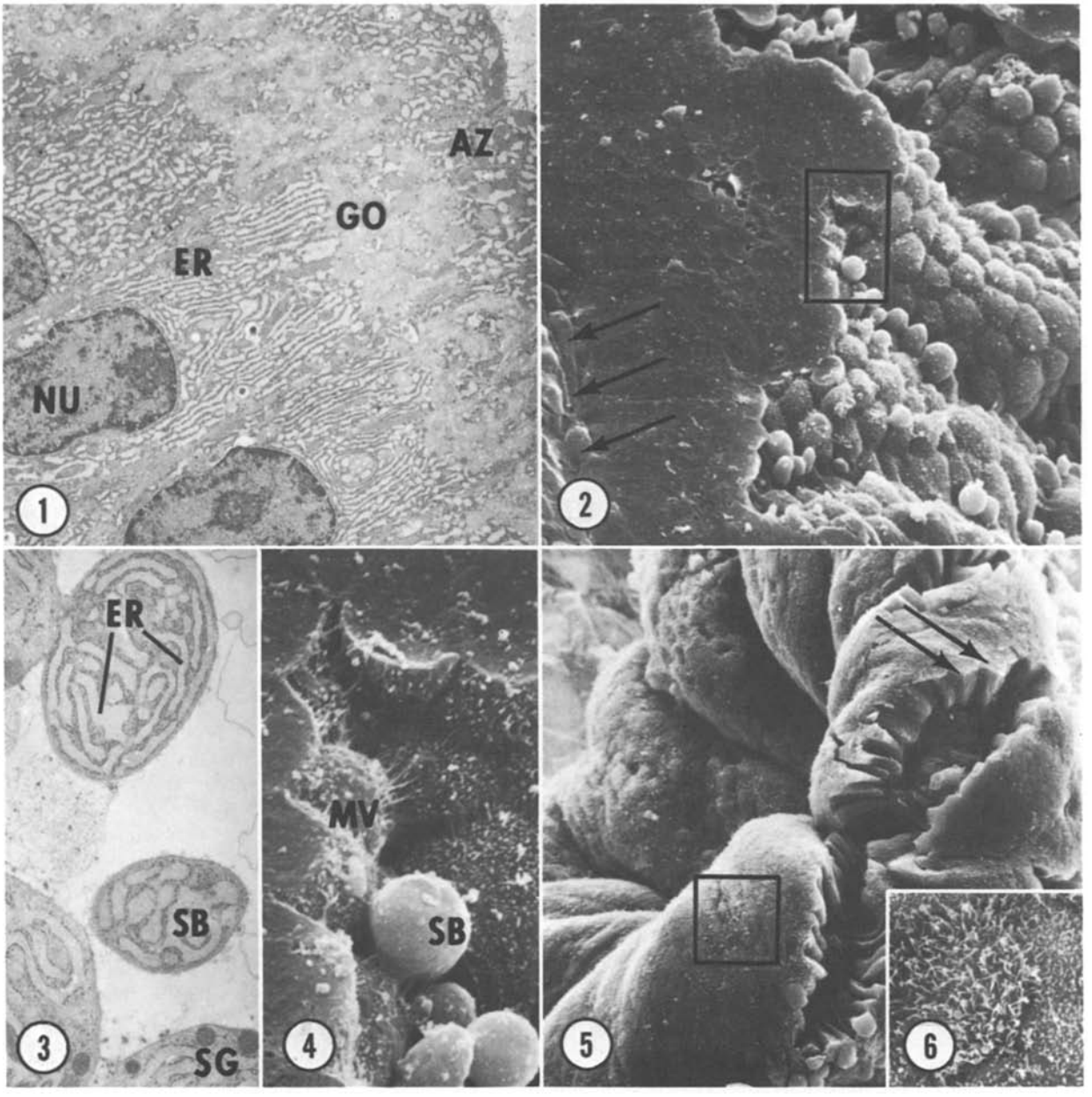


Fig. 1. TEM of rat prostatic acinar cells in situ demonstrating columnar shape and stratification of organelles, 4000X. Nucleus (NU), endoplasmic reticulum (ER), Golgi zone (GO), apical zone (AZ)

Fig. 2. SEM of same prostatic acinus depicted in Figure 1, after Epon removal, 1500X. Note rounded cell apices and homogeneous distribution of stubby microvilli. Area enclosed is enlarged in Figures 3 and 4

Fig. 3. TEM of area enclosed in Figure 2, showing apocrine secretion bodies (SB), which contain dilated cisternae of endoplasmic reticulum (ER), 5600X. Also present at cell apex are merocrine secretion granules (SG)

Fig. 4. SEM of same acinar cells depicted in Figure 3, 6375X. Secretion bodies (SB) can be identified from the rest of the cell apices by the distinctive smooth surface. Surrounding cells possess both long and short microvilli

Fig. 5. SEM of single prostatic acinus after collagenase digestion, 500X. Columnar cell shape is well-preserved after treatment (arrows). Area enclosed is enlarged in Figure 6

Fig. 6. SEM of acinar cell surfaces after collagenase treatment, 4375X. Both long and short microvilli are unchanged

crine secretion that has been suggested to occur in prostate (20, 23). By TEM some of the secretory bodies (SB) appeared to be detached from the cell. They were readily identifiable by their lack of surface microvilli, and the presence of dilated ER cisternae and polysomes (Fig. 3). Merocrine secretion granules (SG) were excluded from the saccular structures, whereas they were present in abundance in the cortical cytoplasm proper (Fig. 3), staining densely osmiophilic.

## Tissue Culture

A single prostatic acinus after digestion with collagenase is shown in Figure 5. When removed from the supportive stroma, these delicate structures usually turned inside-out. The acinar cells suffered no other apparent morphological changes after collagenase treatment. They retained their columnar shape (arrows, Fig. 5), as well as the normal distribution of microvilli and other surface structures (Fig. 6).

When isolated acini were plated as explants, an epithelial sheet spread radially from the acini for about 1-2 mm on the culture dish. After about two weeks, spread of the monolayers halted, while most of the cells in the explant remained with the mass of the acini. These small monolayers of tissue proved to be unsatisfactory for investigation, since they produced only a minimal amount of tissue that was difficult to subpassage and often contained fibroblasts at the leading edge of the sheet.

Acini that were isolated with collagenase, dispersed with trypsin, and purified in Ficoll produced monolayers much more rapidly and with greater efficiency than the acinar explant technique. In addition, this method produced a far greater number of cells which were easily subpassaged up to nine times over a five month period. The purity of the epithelial population approached 100% in many instances, and we were able to produce viable monolayers from every rat in the last 30 sacrificed by this method, compared to a 5% success rate using acinar explants.

Figure 7 illustrates a four day old culture of adult epithelial cells which had a 95% viability when tested with trypan blue before plating, and was greater than 99.5% epithelial as determined by staining for prostatic acid-phosphatase (Figs. 9 and 10). Four days after plating the cells were polygonal in shape and arranged in islands or small groups; isolated cells were rarely seen. Noticeable by phase microscopy were 1-2 prominent nucleoli and abundant perinuclear granules (Fig. 7). Few mitotic figures were seen in the prostatic cultures, especially from older animals, and the cells appeared to come to confluency mainly by extensive spreading, rather than by cell division. As illustrated in Figure 8, after two weeks in culture, the cells became extensively spread on the substratum and more stellate in appearance, due to numerous cytoplasmic extensions. Coincident with the extensive spreading, the number of perinuclear granules detectable by phase microscopy became greatly reduced.

Figures 9 and 10 are representative of two week cultures stained for prostatic acid phosphatase. Most of the cells show heavy concentrations of acid phosphatase-positive granules in the perinuclear region (Fig. 9), but significant numbers of granules also appear spread throughout the cytoplasm and processes of the cell (Fig. 10). Reaction product was distributed similarly for both unfixed cells as those fixed 5 hours in 10% formalin. Control fibroblast populations showed little reactivity. Virtually all acid phosphatase activity in epithelial cells was tartrate inhibitable.

The extremely flattened nature of the cells as well as other morphological changes induced by growth in vitro are more easily visualized in the SEM (Fig. 11). Most noticeable is the loss of microvilli and secretory bodies from the dorsum of the interphase cell. Most surface specializations were confined to the periphery of the cell, where there were found clusters of microvilli, ruffles, and small spherical excressences, some of which were identified as merocrine secretion granules by TEM. Also present at the cell periphery were long filopodia (FP), which extended from the cell to the substratum (Fig. 11) and the dorsum of neighbouring cells (Fig. 12). Many terminated in bulbous swellings, and similar sized enlargements were seen along their length. Often the areas of contact between cells had a scalloped appearance (Fig. 12), and bridges of cytoplasm by which the cells were in contact contained numerous filaments and desmosomes (Fig. 14). The surface of many of the cells in vitro had parallel arrays of shallow undulations not appreciable by any other means than SEM (Fig. 11). These correspond to the intracellular locations of bundles of microfilaments, which permeated the cytoplasm of the cell in directions mainly parallel to the substratum; the appearance of such structures in the cells after a period of in vitro growth marked one of the major ultrastructural differences between the in vivo and in vitro specimens. By TEM, the microfilaments of the cytoplasm comprised at least two distinct subpopulations; bundles of larger 10 nm filaments enmeshed the centrally placed nucleus (Fig. 13), while others spread radially throughout the cell, especially near its base where it contacted the substratum (Fig. 16). The bundles were common at the cell periphery where they often ran parallel to the cell border and were indistinguishable from the filaments associated with desmosomes (Fig. 14). Smaller 5-7 nm filaments were distributed in the cortical cytoplasm, where they were arranged in a lattice-like meshwork subjacent to the plasma-

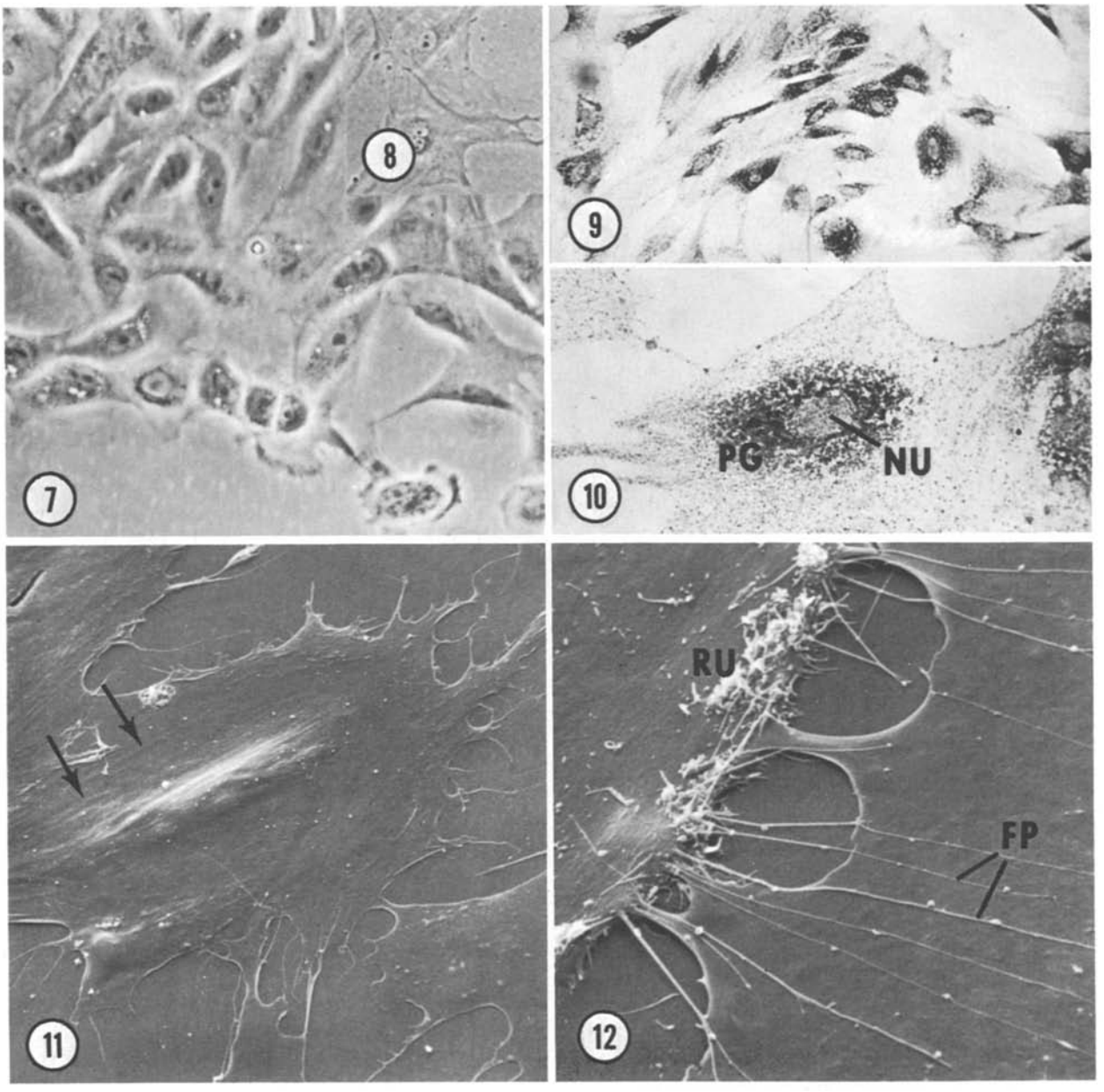


Fig. 7. Phase contract micrograph of four day old cultures of adult cells (>99.5%) isolated by collagenase-Ficoll technique, 1000X. Polygonal shape and perinuclear granules are characteristic of these cells

Fig. 8. Phase contrast micrograph of culture shown in Figure 7, but after two weeks in vitro, 250X. Cells have become extensively spread on substratum and perinuclear granules are not seen

Fig. 9. Bright field micrograph of two week cultures stained for prostatic acid phosphatase, 275X. Note heavy staining in elongate cells as well as polygonal. Control fibroblast cultures failed to stain positively

Fig. 10. Brightfield micrograph of two week cultures stained for prostatic acid phosphatase, 1075X. Concentration of acid phosphatase-positive granules (PG) is greatest around the nucleus (NU), although granules also appear in abundance throughout the cytoplasm, cell edges, and processes. Acid phosphatase activity was present after 5 hrs incubation in 10% formalin, and was completely inhibited by tartaric acid

Fig. 11. SEM of two week culture of adult acinar cells, 800X. Rounded central area overlies nucleus, while the rest of the cell is extremely flattened, measuring 4-5 micrometers in thickness by 120 micrometers in diameter. Subtle ridges on dorsum of cell (arrows) mark intracellular location of stress fibers

Fig. 12. SEM of cell-to-cell contacts in vitro, 2050X. Many cell borders exhibited a scalloped morphology. Surface activity in normal cells, such as microvilli and ruffles (RU), was confined to the cell periphery. Also present were long gilopodia (FP) which traversed both the substratum and dorsum of neighboring cells. These structures were missing from malignant cell cultures

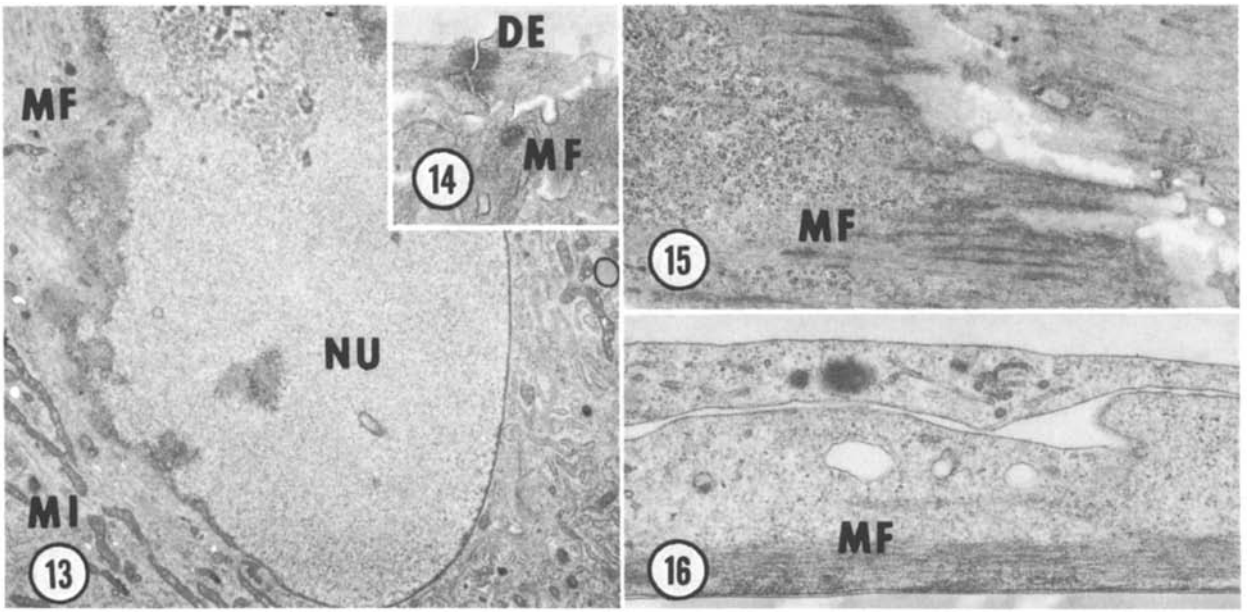


Fig. 13. TEM of same cell cultures as shown in Figures 7-10, 5675X. Endoplasmic reticulum has become vesicular and lacks secretory product. Mitochondria (MI) are long and branched, and the euchromatic nucleus (NU) is surrounded by abundant microfilaments (MF)

Fig. 14. TEM of cell periphery showing heavy concentrations of microfilaments (MF) associated with numerous desmosomes (DE), 14,000X

Fig. 15. TEM of en face section of acinar cell, 12,000X. Numerous bundles of 10 nm filaments termed stress fibers or cables permeate the cytoplasm and enmesh the nucleus

Fig. 16. TEM of perpendicular section through normal cells, illustrating the preferential location of cables near substratum and points of cell anchorage, 23,125X

lemma. Other morphological consequences of in vitro growth were reminiscent of castration effects on cells in vivo, such as regression of the endoplasmic reticulum, which after two weeks in vitro appeared to become depleted of secretory product and assumed small vesicular profiles. There was also a distinct reduction in the number of structures able to be identified as merocrine secretion granules, coincident with an increase in dense bodies and lipid droplets, which were pleomorphic, osmiophilic, and occurred in large numbers both perinuclearly and throughout the cytoplasm. The structures were interpreted as those staining positively for acid phosphatase.

## R3327H Prostatic Adenocarcinoma Cell Line

Cells from a spontaneous adenocarcinoma of the dorsolateral prostate in an aged Copenhagen rat are shown by phase microscopy in Figure 17. When plated under identical conditions as the normal cells, they were similar in speed of attachment and morphology for the first 48 hours in vitro (cf. Figs. 17 and 7). They grew as islands of cells with 24 nucleoli per cell; single cells formed islands within a few days and were usually confluent in one week. A modest number of perinuclear granules were noted by phase microscopy, and the cells stained intensely for prostatic acid phosphatase. In contrast to the normal cells, which became extensively spread after several days in vitro, the R3327H cells remained as slightly rounded polygonal cells and did not flatten with age in vitro. When the islands of cells reached confluency, multiple cell layers did not form as with most transformed cell lines. Rather, after 7-10 days in vitro, focal areas of the monolayer detached from the substratum. Fluid accumulated in the pockets between the cells and dish such that dome-shaped blisters of cells were formed. After a few days, the blisters progressed to form spheroidal aggregates of cells, which remained attached to the underlying cells (Figs. 18 and 19). The unusual spheroidal aggregates were cyst-like in appearance; they were comprised of a single layer of cells surrounding a hollow, fluid-filled space.

Viewed by SEM, the surfaces of the rounded malignant cells exhibited many more surface specializations than their normal counterparts in vitro. The most prominent surface features were

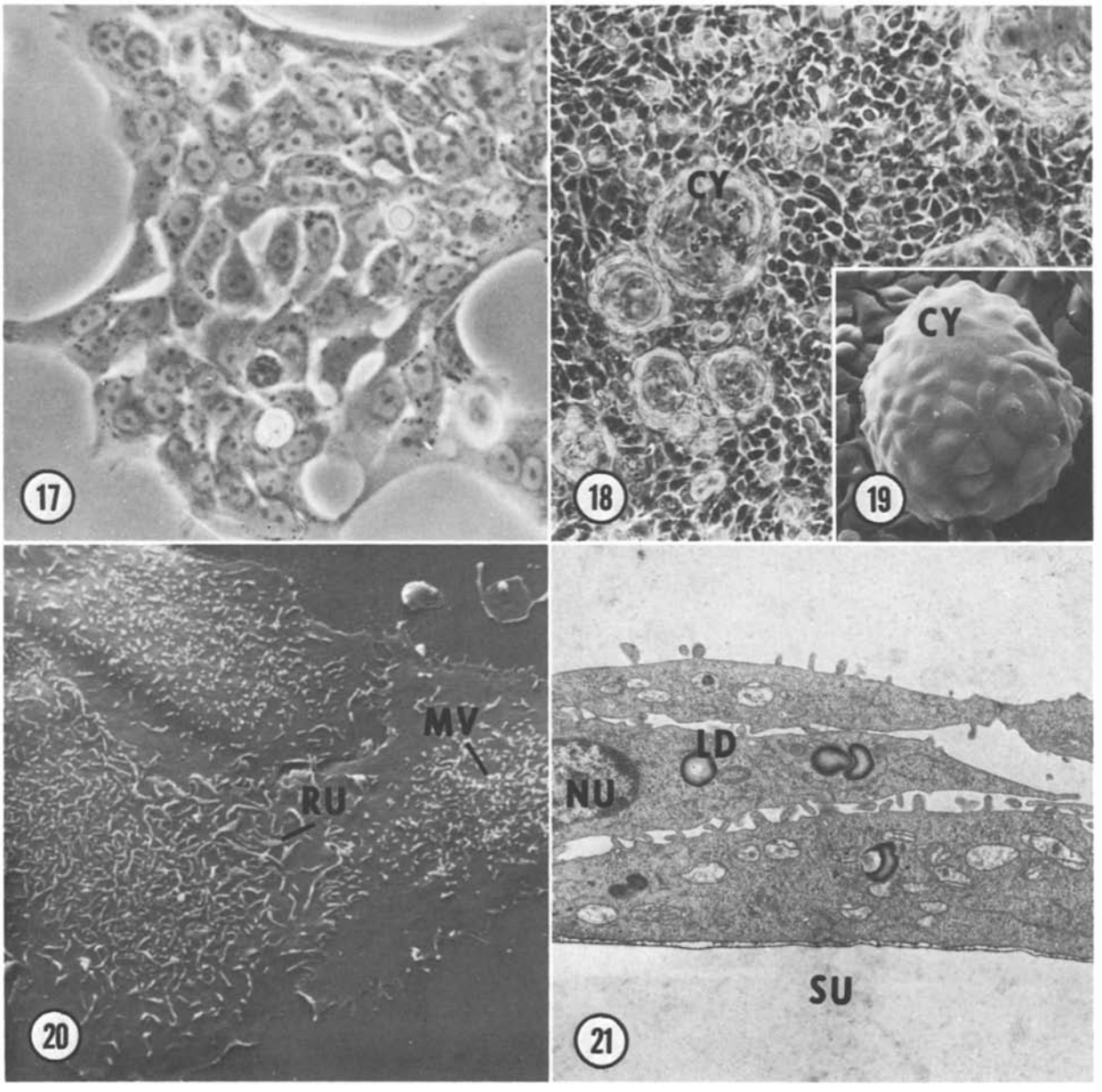


Fig. 17. Phase contrast micrograph of R3327H rat prostatic adenocarcinoma cells in vitro, 900X. Note similarities in size, shape, and nuclear configuration to early cultures of normal cells (Fig. 7)

Fig. 18. Phase contrast micrograph of confluent R3327H cells, 350X. Hollow, cyst-like structures (CY) develop after a few days at confluency

Fig. 19. SEM of a cyst (CY) on confluent R3327H culture, 750X. Fluid-filled balls are comprised of a single layer of cells and attached to the underlying monolayer by a "stalk" of cells. Surfaces are the same as those on the culture dish (shown in Fig. 20)

Fig. 20. SEM of R3327H cells in vitro, 2550X. Cells are dome-shaped and covered with a variable distribution of both short microvilli (MV) and microplicae or ruffles (RU). Cells characteristically have an apron of cytoplasm around periphery that is essentially free of surface adornments

Fig. 21. TEM of R3327H cell in vitro, 9000X. Cells possess euchromatic nuclei (NU), numerous lipid droplets (LD), free polyribosomes, and collapsed ER profiles. Mitochondria have a bizzare cristae and large irregular granules (see also Fig. 25). Microfilament bundles (cables, stress fibers) are totally lacking. Substratum (SU)

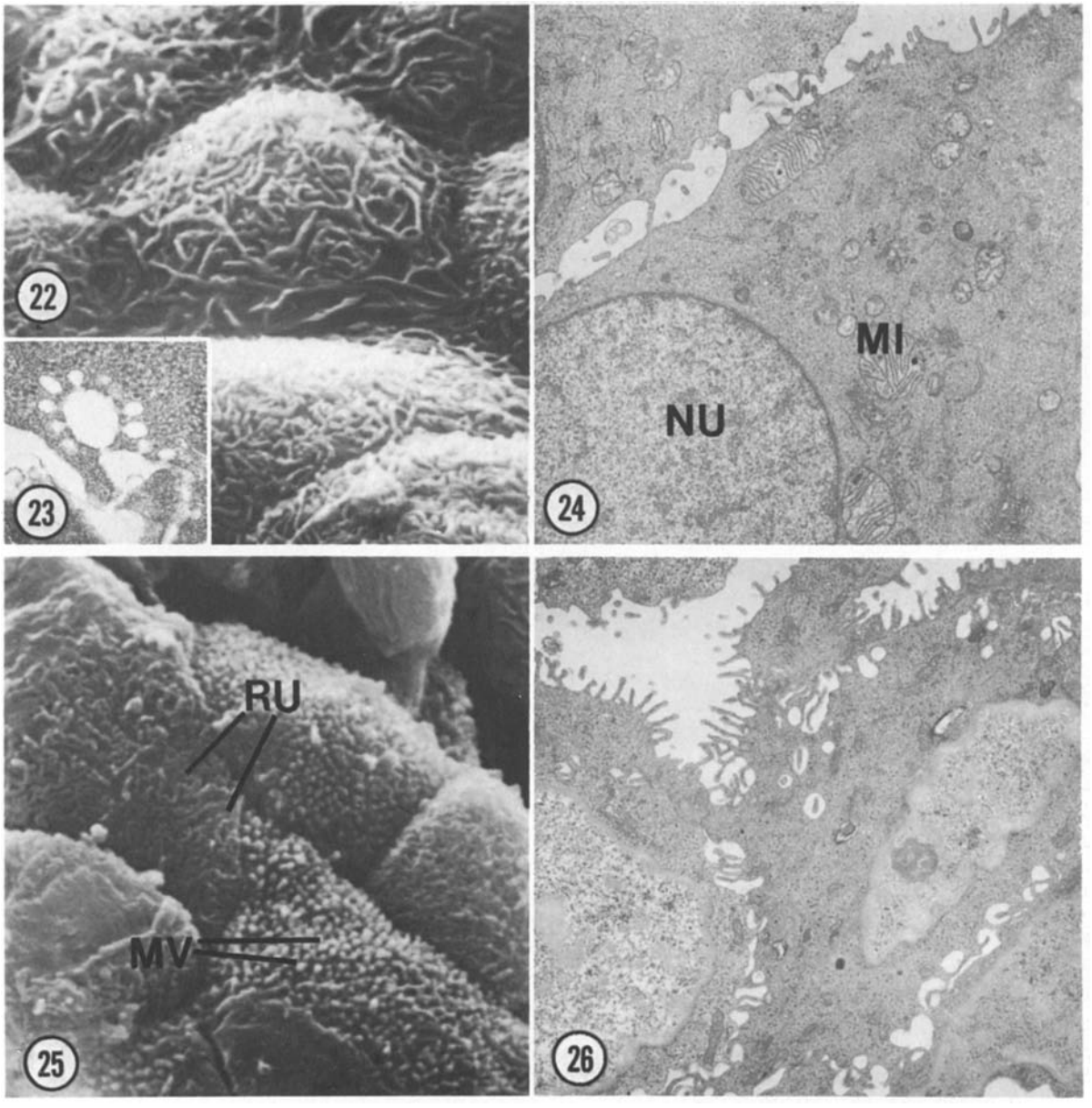


Fig. 22. SEM of R3327H cells at high density, 5500X. Cells exhibit extensive arrays of ruffles, some of which are the location of cup-shaped invaginations (arrows)

Fig. 23. TEM of R3327H cell periphery illustrating peculiar vesicular profiles associated with invaginations in surface ruffles, 53,400X

Fig. 24. TEM of en face section of R3327H cells in vitro, 6825X. Note lack of stress fibers and perinuclear microfilaments. Nucleus (NU); mitochondrion (MI)

Fig. 25. SEM of tumour formed by injecting R3327H cells subcutaneously into the axillary region of a male Copenhagen rat, 5025X. Cells grow in cords and acini, and surface configuration is identical to that grown in vitro. Ruffles (RU); microvilli (MV)

Fig. 26. TEM of tumour depicted in Figure 26, 7500X. Cells have similar ultrastructural features with the exception of changes in nuclear shape and staining characteristics of internal nuclear lamina

ruffles and stubby microvilli (Fig. 20). Some cells possessed either microvilli or ruffles alone, while others had patches of both microvilli and ruffles, with areas of apparent transitional forms interspersed. Subconfluent R3327H cells did not form slender extensions (filopodia) from the periphery of the cell, which in normal cell cultures contacted the substratum and dorsum of neighbouring cells. The cells also differed from normal in that most displayed smooth membranes around the circumference (Fig. 20). Further differences in surface membrane between normal and malignant cells were implied by the observation that the adenocarcinoma cells were more easily dislodged from both Aclar and polystyrene substrate during critical point drying of the cultures for SEM.

By transmission microscopy, interphase cell cytoplasm contained an abundance of free polyribosomes and occasional profiles of collapsed endoplasmic reticulum (Figs. 21 and 24). Osmiophilic lipid droplets and dense bodies were found in the vicinity of the euchromatic nucleus. Mitochondria assured a vesicular shape with incomplete, distorted cristae and large irregular grannules. The most obvious morphological deviation was the lack of large bundles of microfilaments in the malignant cells, which in normal cells formed one of the major organelles. Small inconspicuous microfilaments (7 nm diameter) were observed in the cortical cytoplasm, mainly in areas associated with numerous desmosomes.

At high densities in vitro, the adenocarcinoma cells were heavily populated with ruffles and microvilli (Fig. 22). In addition, small, cup-shaped invaginations developed in association with many of the larger microplicae. In thin section, these pockets were the locus of peculiar vesicular profiles continuous with the cell surface and consisting of a larger central vesicle surrounded by and connected to a halo of smaller vesicles (Fig. 23).

When R3327H cells were injected subcutaneously into the axillary region of syngenic rats, tumours developed within several weeks. In this type of in vivo environment, the tumour cells formed an adenocarcinomatous pattern of growth, characterised by arrangement into acinus-like structures and cords of cells. The surfaces of these cells possessed the same distribution of morphological components as did cells grown in vitro; cells were covered with microvilli and ruffles (Fig. 25). By TEM the cells exhibited no ultrastructural differences from those in vitro with the exception of the nucleus. In those grown subcutaneously, a peripheral nuclear zone stained much less intensely than in cells grown in vitro; nucleoli remained densely staining (Fig. 26).

## DISCUSSION

### Tissue Culture

Normal prostatic tissue has proven remarkably difficult to grow in vitro (7, 46). Although recent successes have been made in perfecting methods for culture of normal mammary tissue and other hormone-dependent secondary sexual tissues (38, 39), research on normal prostate has been limited. Observations presented here clearly show that normal adult rat prostatic epithelial cells may be maintained in pure monolayer culture for substantial periods of time. When the techniques of collagenase and trypsin digestion followed by Ficoll purification are employed, essentially pure epithelial preparations can be obtained, and with additives to the medium such as native serum or conditioned medium, these preparations consistently produce viable monolayers. The acinar explant technique, described by Webber (46, 48) and Sanford (40), in our hands failed to produce pure monolayers of epithelial cells with reliability and in sufficient quantities to be of value. Thus, collagenase-Ficoll preparative methods offer several advantages for obtaining pure cultures of not only tumour cells, as shown by Pretlow (31, 32), but normal cells in sufficient quantities for extensive investigation. The techniques relieve the need for tedious cell cloning and can be performed with a minimum of time, expense, and equipment.

The epithelial nature of cells in this study is confirmed both by the presence of numerous desmosomes and acid phosphatase activity. Acid phosphatase activity in prostatic tissue has been shown to be tartaric acid-inhibitable (13, 15, 29). Additionally, the enzyme activity is not significantly reduced after five hours incubation in 10% formalin, whereas acid phosphatase activity in other cell types is totally inhibited by the treatment (48, 50).

### Morphology of Normal Cells in Vivo and in Vitro

Correlative scanning and transmission electron microscopy of tissue blocks demonstrates the presence of an apocrine mode of secretion, as well as the merocrine mode, typically associated with epithelia. Several investigators have suggested that apocrine secretion occurs in areas of dog, rat, rabbit, and human prostate (20, 23), and our observations confirm these reports. The nature of secretory product(s) in both the merocrine granules and secretory bodies is unknown.

Apocrine secretion bodies described here are not believed to be artifacts of fixation, since cells with apocrine activity were observed focally rather than distributed homogeneously throughout the acinus. Furthermore, fixation of the internal contents of the saccules is comparable to that of

the rest of the cytoplasm, which is well preserved.

Apical craters described in recent studies of human prostate (2, 10, 45, 46, 47), were not found in rat specimens. Perhaps the craters represent points of detachment or rupture of the saccules. Since vigorous washing of the cut surface is necessary to remove prostatic secretion from the cell for viewing in the SEM, they may be artifactual. The craters are missing from our specimens, which were prepared by the more gentle method of Epon removal. Epoxide solvent removes plastic as well as secretion from the cell surfaces without physically damaging the membranes, as shown in recent studies (10, 56).

When prostatic acini were disassembled and cells plated on a flat plastic substratum, accompanying morphological alterations are typical of the differentiative changes which most specialized cell types undergo when placed in vitro. Most noticeable was the transformation from a columnar shape with stratified organization of secretory organelles to a flattened, squamous morphology. Regression of organelles associated with synthesis of secretory product accompanied the changes in cell shape. Loss of differentiation in specialised cells may well reflect the lack of a proper substratum, cell-to-cell contacts, and sufficiently high cell densities to permit structural organization into a secreting epithelium. In addition, maintenance of the differentiated state may require special factors or inducing substances present in the extracellular milieu, produced either in the prostatic stroma, the epithelial cells themselves or distant sites missing in tissue culture preparations (11, 28).

Extreme morphological variations and loss of secretory capacity of cells placed in vitro demonstrate a dependence upon factors in the cellular environment for normal cytological architecture and differentiation. Attempts to ameliorate the morphological dissimilarities between in vivo and in vitro grown cells by culturing on different substrate, such as floating collagen membranes (5) and cellulose sponges (37, 38, 39), are currently being investigated.

Besides loss of secretory capacity, the disappearance of microvilli from the cell surface and induction of microfilament bundles in the cytoplasm are other morphological consequences related to the spreading of cells on the culture dish. The presence of surface microvilli can be strongly correlated to cell shape in vitro in many cell types, most notably fibroblasts (5, 34, 54, 55, 57). Rounded, mitotic cells are covered with microvilli, as are cells grown on poorly adhesive substrata which do not allow flattening. Interphase cells grown on adhesive substrata progressively lose their surface microvilli as the cells spread and flatten.

Also associated with the flat morphology of untransformed cells in vitro is the accumulation of microfilaments of 10 nm diameter into dense bundles that traverse the cytoplasm in close proximity with the substratum (reviewed by Willingham and Pastan, 53). Common in other cell types, they have been referred to as "stress fibres" or "cables" (33). These bundles have been visualised in fibroblasts and liver epithelial cells by immunofluorescent labelling of fixed, permeabilized cells with antibodies to actin, myosin, and tropomyosin (17, 18, 52). The development of actomyosin cables is correlated to variations in cell shape as are the surface features above; they are absent from rounded or mitotic cells and reappear as cells spread on the substratum and assume a tenuous morphology. Cables have also been described in mammary epithelial cells in vitro (38, 39). When grown on smooth collagen substrate, cells are flattened and possess numerous cables. Release of the collagen layer from the culture dish causes the collagen disc to shrink dramatically in diameter. As the disc shrinks, cells assume a rounded morphology with surface microvilli and ruffles, and cables disappear. Similarly treated collagen discs without cells do not contract. Such observations, together with immunofluorescent data on the molecular composition of cables, suggests they possess contractile ability.

## Differences in Morphology Between Normal and R3327H Cells

The R3327H prostatic adenocarcinoma cell line was isolated from a spontaneous tumour in the dorso-lateral lobe of a Copenhagen rat by Dunning in 1961 (41, 42). The line is considered to be an appropriate model of human prostatic adenocarcinoma, since it exhibits both androgen dependent and independent phases of growth in host animals, as is often the case in human disease (19). The cells are classified as well-differentiated, since the tumour grows in an adenocarcinomatous pattern subcutaneously.

Although certain malignant mammary cells can form blister-like structures in vitro (30), to our knowledge only R3327H forms spheroidal cysts. These hollow balls of cells are similar in size and morphology to the acinus-like structures formed by the tumour cells in vivo. Similarly, the cell surfaces of R3327H grown both in vivo and in vitro exhibit identical morphologies. These observations suggest that the morphology of adenocarcinoma cells is not as dependent upon environmental conditions as is that of normal cells, although they exhibit anchorage dependence of growth and arrange themselves in an acinar pattern.

The differences seen in ultrastructure between normal and malignant cells maintained in identical environments, while substantial, are not un-

expected. With few exceptions, many kinds of malignant cells have been described as having a more rounded shape, an increase in surface microvilli and ruffles, and lacking a fully organized actomyosin cytoskeleton typical of their normal counterparts (8, 12, 33, 35, 55).

The many common structural variations among normal and transformed cells such as breast, fibroblast, and prostate have stimulated the suggestion that these characteristics might constitute a "morphological phenotype of transformation" (55).

Willingham et al. (55), have convincingly shown in fibroblasts that expression of the malignant phenotype is related to the adhesive properties of the transformed membrane. That is, malignant fibroblasts exhibit a decreased adhesivity to most tissue culture substrata, and as a result the cells cannot spread, are more rounded, covered with microvilli, and lack microfilament bundles. Three possible causes of decreased adhesiveness in these cells have been identified: deficiency in specific glycoproteins of the surface, a reduction in surface carbohydrate, and a decrease in intracellular cAMP levels (55). Remarkably, normal morphology can be restored by correcting for the above deficiencies in experimental systems; however, growth control can only be partially restored, suggesting that defective adhesion and the resulting morphological alterations may be dissociable from growth control in these cells.

The expression of morphological features characteristic of the malignant phenotype by R3327H cells is suggestive of altered adhesive properties of their surfaces. The loss of many malignent cells from culture dishes during processing for electron microscopy supports this contention. Preliminary studies in our laboratory show differences in lectin binding sites between normal and R3327H cell surfaces, which may be indicative of structural differences in the plasma membranes.

The above morphological manifestations of the malignant cells can also be directly related to another property of tumours, metastasis. Release of metastatic cells from tumours may be dependent upon their decreased adhesivity (3). Nicolson has shown that site selection by spreading metastatic cells is dependent upon surface membrane characteristics (24, 25). Since prostatic adenocarcinoma is a disease that causes death almost exclusively by metastasis, further investigation of surface membrane characteristics in R3327H and normal cells may yield information useful both to understanding the metastatic process and preventing its occurrence.

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