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The Epithelial Origin of a Stromal Cell Population in Adenocarcinoma of the Rat Prostate

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Dunning R3327-H rat prostate adenocarcinoma cells, when grown in syngeneic (Copenhagen) rats or nude mice, produce tumors with prominent hypercellular stroma. The authors have previously demonstrated the presence of anomalous steroid-sensitive cells in both the epithelium and stromal compartments of this model system. In order to better understand the histogenesis of these cells, the authors studied samples of the tumor which were radiolabeled overnight with tritiated dihydrotestosterone (^{3}H-DHT). Frozen sections of the tissues were thaw-mounted onto autoradiographic emulsion-coated slides to permit silver grain identification in association with nuclei of androgen-sensitive cells. Surprisingly, numerous silver grains were found to be associated with nuclei of large cells within the stroma. Therefore, these cells were termed "epithelioid" pending confirmation of their origin. To further define these cells and their relationship to the surrounding matrix, autoradiograms have now been examined immunohistochemically with antibodies directed against the basement membrane glycoprotein, laminin, as well as antibodies specific for intermediate cytoskeletal filaments. Following identification of acinar basement membranes, epithelioid cells were identifiable both in the stroma and in the acinar epithelial cell layer. Histochemical staining with acid phosphatase, a marker for prostatic epithelium, was performed and shown to be present in acinar epithelial cells as well as in epithelioid cells. Additionally, fluorescence-activated cell sorting was employed to characterize the DNA content of cell types within the H tumor. Epithelioid cells were found to be in highest concentration in an aneuploid peak with a ploidy of approximately 6N. The autoradiographic, immunohistochemical, cytometric, and ultramicroscopic studies suggest that 1) epithelioid cells are epithelial derived stromal cells; 2) these epithelioid cells arise by pathologic division of aneuploid neoplastic precursor cells of approximately 3N ploidy, which are found within the prostatic epithelium; and 3) the resulting 6N cells degrade the basement membrane locally, invade the stroma, and populate it. Here, they can be distinguished from fibroblasts by their size, acid phosphatase activity, and hormone receptor content. Thus, the term "epithelioid" is inappropriate; and these cells should be regarded simply as large neoplastic epithelial (LNE) cells. The presence of this cell type suggests that this tumor subline represents a useful naturally occurring model for the study of the initial stages of neoplastic transformation. (Am J Pathol 1987, 128:555–565)

THE DUNNING R3327-H (H) rat prostatic adenocarcinoma is a neoplastic cell subline which originated from one of the original rats to which the R3327 tumor was passed.[1] The hormone sensitivity of this tumor was initially described by Voight and Dunning,[2] who observed that the tumor grew more rapidly in intact males than in castrated males. The original tumor was a well-differentiated papillary adenocarcinoma that bore considerable histologic resemblance to the normal dorsal prostate.[1]

These neoplasms occasionally lose their characteristic well-differentiated morphology and begin to proliferate rapidly. Thus, numerous sublines of this

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tumor have arisen.[3-7] Investigators at the Johns Hopkins University School of Medicine who extensively characterized the original R3327 tumor used the suffix "H" to identify it as a hormonally sensitive subline.[6,8] Thus, studies describing the original R3327 adenocarcinoma may refer to it as R3327 or R3327-H.[8,9] The present study uses the latter designation.

The H tumor contains androgen[9-12] and estrogen receptors.[9,10] Following orchiectomy[10] or estrogen priming,[13] progestogen receptors can be demonstrated as well, suggesting the functionality of the estrogen receptor.[13] Orchiectomy of male rat[14] or athymic mouse[15] hosts results in a transient growth suppression lasting approximately 60 days, followed by a return to the former growth rate. The resulting tumor is insensitive to further alterations in circulating androgen concentration and is designated the HI (hormone-insensitive) tumor.[16-17]

The stroma of this tumor model is characterized by enhanced cellularity, compared with the stroma of the rat dorsal prostate from which it was derived.[1] We have previously shown that nuclei of a prominent subpopulation of these cells have a high concentration of receptors for dihydrotestosterone (DHT) and estradiol (E_2).[18] Since the histogenesis of these cells was uncertain, we suggested the name "epithelioid cells" to distinguish them from normal fibroblasts which lack substantial hormone receptors.[18] As we now show, these cells can be identified both within the boundary of the acinar basement membrane and in the stroma. Thus, the term "epithelioid" is no longer appropriate. This cell type represents a population of large neoplastic epithelial (LNE) cells. We have further clarified the localization of these large cells possessing enhanced capacity to bind androgens. Electron-microscopic and immunohistochemical studies have been performed to demonstrate the relationship between these cells and the extracellular matrix and have provided further evidence of their invasive capacity. Flow cytometric analysis was performed, and the uniqueness of the DNA ploidy exploited for separation and study of this population.

Materials and Methods

Tumor Inoculation and Incubation

Ten male nude (bg/bg) mice (NIH) were implanted subcutaneously under acepromazine/ketamine anesthesia with 3 cu mm pieces of Dunning R3327-H tumor in the inguinal region. After 6 months the tumors had grown to approximately 1 cu cm in volume. Mice were castrated 24 hours before surgical excision of the tumors to allow for clearance of endogenous serum androgens. Tissues were placed in 4 C RPMI 1640 culture medium containing 100 U/ml penicillin and 100 μg/ml streptomycin and minced with razor blades into small pieces 1–2 cu mm. Tissues were incubated overnight as described below in closed 50-ml tubes in a shaking water bath (37 C). Tissue viability was maintained in RPMI 1640 culture medium with antibiotics, 2 mM HEPES buffer, and 1 nM ^{3}H-DHT (210 Ci/mM, New England Nuclear, Cambridge, Mass) to permit maximal hormone receptor binding.[18] Competition studies were performed by the addition of 100 nM unlabeled DHT to the incubation medium. After incubation, the tissues were washed for 4 hours with hourly changes of 10 ml RPMI 1640 medium containing 3.5% (wt/vol) bovine serum albumin (Fraction V, Sigma Chemical Co., St. Louis, Mo).[19]

Autoradiography

After incubation, tissues were prepared for autoradiography by the method of Beckman et al.[18] Briefly, tissues were blotted, mounted on brass cryostat stubs, frozen in isopentane at −70 C, and stored in liquid nitrogen until processing for autoradiography. Sections (4μ) were cut and thaw-mounted onto emulsion-coated slides (Kodak NTB-3). Slides were developed weekly and examined for the presence of cells with nuclear androgen receptor complexes, as evidenced by a concentration of silver grains over cell nuclei. When sufficient silver grains had accumulated over nuclei to permit identification of steroid-sensitive ells, the remaining slides in the box were removed in a darkroom, and then developed in Kodak D-19 developer (45 seconds at 15 C) followed by Kodak Fixer (5 minutes at 15 C).[20]

Immunohistochemistry

Autoradiograms which were prepared for combined autoradiography and immunohistochemistry were immunostained following the method of Keefer et al.[21] Prior to photographic development, slides were placed in a 4% paraformaldehyde solution (4 C, pH 7.0) for 60 seconds. Subsequent to photographic development, slides were washed for 5 minutes in running tap water and then placed in 0.05 M Tris buffer (pH 7.6) for 5 minutes. Slides were treated for an additional 5 minutes in 1% normal sheep serum (NSS) to block nonspecific binding sites prior to application of the antibody bridge.

A double peroxidase–antiperoxidase method[22] was used for immunohistochemical demonstration of la-

minin and keratin proteins. Rabbit anti-laminin antiserum (Bethesda Research Laboratories, Bethesda, Md) was used at a concentration of 1:100 in Tris buffer supplemented with 1% NSS. Rabbit anti-keratin antiserum (Lipshaw, Detroit, Mich) was used undiluted as supplied. This latter polyclonal antibody recognizes a large family of both high and intermediate weight keratins. Both antisera were applied overnight at room temperature (RT). Each set of slides immunostained included one control slide for which normal rabbit serum was substituted for the primary antiserum to control for nonspecific deposition of reaction product.

Intracellular vimentin filaments were demonstrated immunohistochemically with a monoclonal antibody kit (Hybritech, Inc., San Diego, Calif) following the published protocol.

Acid Phosphatase Cytochemistry

Cells containing acid phosphatase enzyme were demonstrated utilizing Barka's modification of Burstone's acid phosphatase method as outlined by Brinn and Pickett.[23] This procedure yields a red reaction product wherever the acid phosphatase enzyme is located.

Fluorescence-Activated Cell Sorting

H tumors were dissociated enzymatically in a solution of 0.25% collagenase with constant stirring for 60 minutes at 37 C. Free cells in the supernatant were removed every 10 minutes and diluted in 3.5% BSA to stop the enzymatic reaction. Fresh enzyme solution was added to replace the volume of supernatant removed. Viable tumor cells were maintained in the same culture medium as described above for organ culture of solid tumor pieces. Hoechst dye #33342 was added to achieve a final concentration in the media of 1 μM. This dye concentration was 20% of that recommended by Shapiro.[24] Preliminary studies indicated that the low concentration of Hoechst dye in the solution broadened the peaks of the histograms (C V = 7.5% versus 4% for 5 μM Hoechst), but superior cell viability was obtained over the long period of time (3 hours) required for the sort.

Cells were incubated for 45 minutes at 37 C before sorting. Flow cytometry was performed using a Coulter Epics V system equipped with an argon laser and the MDADS (Coulter Electronics, Hialeah, Fla) data acquisition system. Fluorescence of Hoechst dye bound to DNA was activated by a 363-nm argon laser excitation beam at 20 mW. Emission was recorded through a 418-nm short-pass interference filter. Forward angle light scatter and DNA content parameters were used for sorting cells. The three peaks of fluorescence identified by DNA fluorescence intensity were set at Channels 52, 109, and 166. These peaks were sorted into two populations. Cells with a mean channel position of 52 were sorted together by collecting all cells falling between Channels 30 and 71 into a single collection vial. Cells in the two peaks between Channels 71 and 180 were collected together. Subsequently, the distinct peaks of cells with mean values at Channels 109 and 166 were separated by a second sorting pass. Cells in each of the three populations resulting from this two-step sorting were stained with the Giemsa stain and examined microscopically.

Transmission Electron Microscopy

H tumor pieces 1–2 mm in diameter were fixed in a mixture of 0.1 M phosphate buffer, 2% paraformaldehyde, and 1.25% glutaraldehyde with 0.01% picric acid (Ito-Karnovsky fixative).[25] After overnight fixation, tissues were dehydrated through a graded ethanol series and embedded in Epon resin. Thin (900 nm) sections were cut and mounted on 100-mesh copper grids for ultrastructural study utilizing a Zeiss 109 electron microscope.

Morphometric Analysis

Measurement of nuclear areas was performed on the Zeiss Videoplan Image Analysis System with digitizing tablet. Photomicrographs (1000×) were placed on the tablet, and a stylus was used to circumscribe the nuclear boundaries of epithelial and LNE cells (recognized by the large numbers of silver grains present over the nucleus). Silver grains were recorded by the point counting function. This made it possible to record both the number of silver grains within each nucleus and the nuclear area within which they were contained. The standard measurement programs which are contained within the Zeiss Videoplan statistical program package were used.

Results

Histologic comparison of tissues from the H tumor (Figure 1) and the normal rat dorsal prostate (Figure 2) revealed both to be characterized by variable numbers of glandular acini consisting of cytologically bland epithelial cells and myoepithelial-like basal cells. The stroma from the normal prostate was loose with a supporting fibrovascular network and infiltrating macrophage population. The H tumor acinar epithelium consists of one to two cell layers. The adlu-

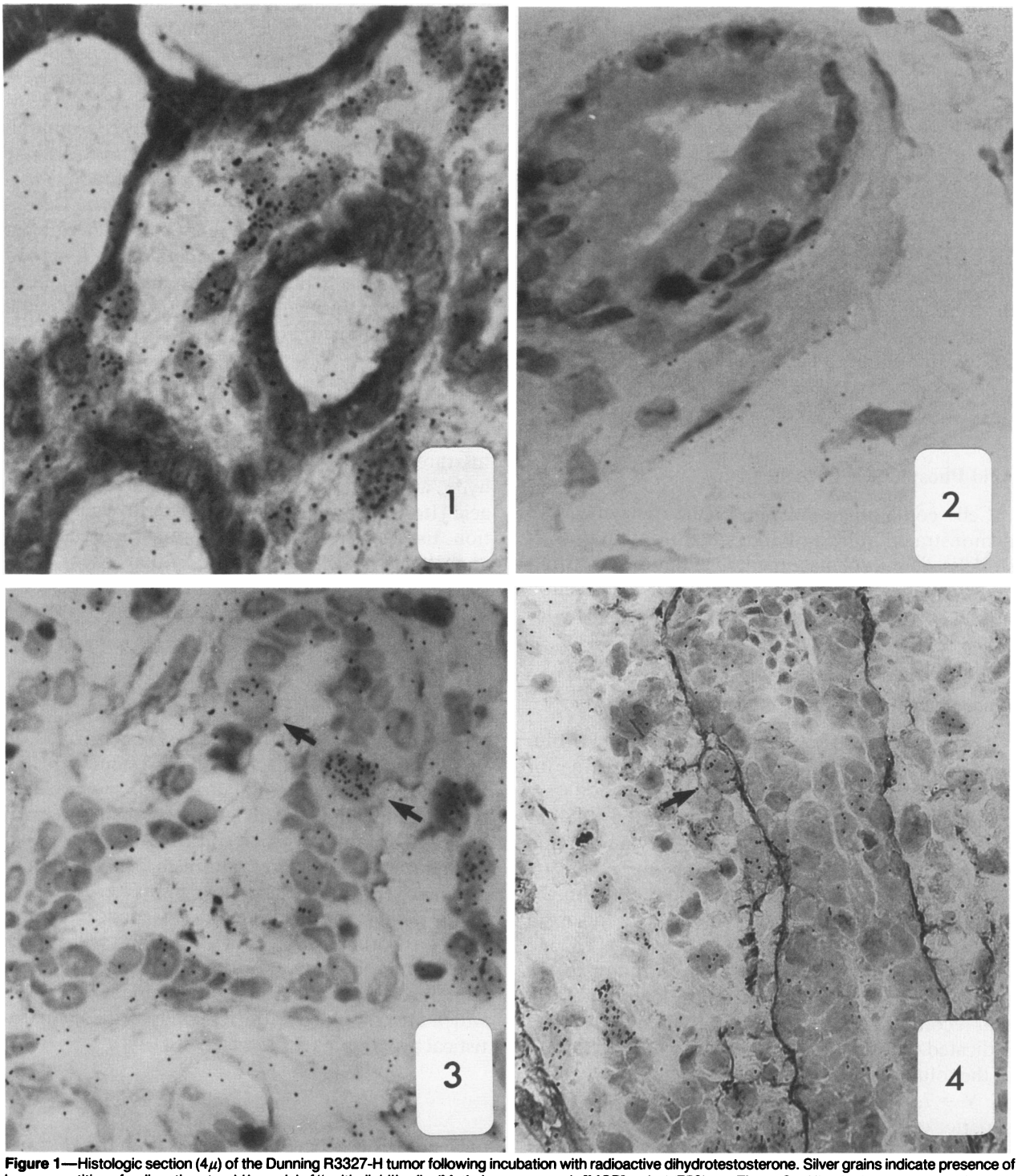
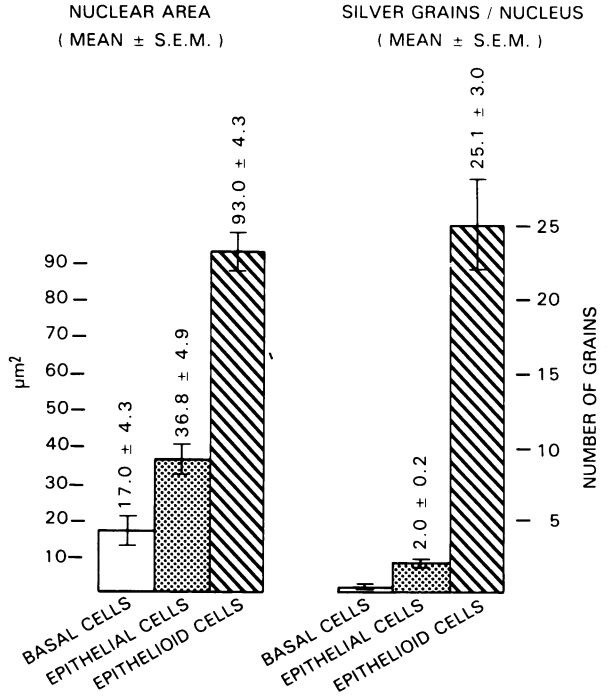


Figure 1—Histologic section (4 μ) of the Dunning R3327-H tumor following incubation with radioactive dihydrotestosterone. Silver grains indicate presence of larger quantities of radioactive steroid in nuclei of "epithelioid" cells. (Methyl green–pyronin [MGP] stain, ×750) **Figure 2**—Histologic section (4 μ) of normal rat dorsal prostate stained histochemically for acid phosphatase (dark areas at apical portion of epithelial cells). Few cells are present in the stroma, which is largely free of acid phosphatase reaction product with the exception of lysosomal activity in occasional macrophages. (×750) **Figure 3**—A well-developed basement membrane surrounds the basal cell layer of acini. A light immunohistochemical reaction reveals the presence of laminin present within the epithelial basement membrane which surrounds the acinus. Intraacinar "epithelioid" cells (*arrows*) were identified by the presence of increased numbers of nuclear-associated silver grains. Extraacinar cells as well as large intraacinar cells contain increased numbers of nuclear silver grains, resulting from receptor bound ^{3}H-DHT. (MGP, ×350) **Figure 4** — A large cell at the margin of the acinus appears to be exiting from the basement membrane, which exists as a filmy "halo" around the cell (*large arrow*). Other cells with increased numbers of nuclear silver grains appear in the stroma. (MGP, ×350)

minal layer is composed of small epithelial cells, which, by morphometric analysis, have a mean nuclear area of 36.8 ± 4.9 sq μ and smaller basal cells having a mean nuclear area of 17 ± 4.3 sq μ (Table 1) which usually form a continuous layer surrounding the acinus. Additionally, the stromal compartment of the H tumor contained many cells with a large nuclear area (93.0 ± 4.3 sq μ; Figure 1) not found in the normal prostate stroma. These cells were identifiable by large numbers of silver grains concentrated within the boundaries of the nuclear membrane. Localization of silver grains in cells of H tumors grown in nude mice and incubated in vitro with ^{3}H-DHT indicated the presence of androgen associated with nuclei of large cells in the stroma which we have previously termed "epithelioid" cells.[18] Autoradiograms of the H tumor were evaluated to determine the numbers of silver grains present over nuclei of basal, epithelial and "epithelioid" cell types. Although "epithelioid" cell nuclei were roughly three times as large as epithelial cell nuclei, their mean silver grain content was greater than ten times that of the epithelial cells (Table 1). Thus, it is unlikely that the increased DHT-associated radioactivity can be explained by the difference in nuclear area.

Further examination of tumor tissue sections which had been immunohistochemically stained with antibodies to laminin revealed similar large cells

Table 1—Mean Cell Sizes and Silver Grain Content of Different Cell Types Within the H Tumor



within the acinar epithelial layer, (Figure 3, arrows) within duplicated layers of periacinar basement membrane (Figure 4, arrow) as well as in the periacinar stroma. In some instances, laminin immunoreactivity was less intense in proximity to these cells.

Ultrastructural analysis of H tumor acinar morphology revealed a stratified cuboidal epithelium composed of a relatively regular basal cell layer underlying one to two acinar epithelial cells with a generally intact basement membrane (Figures 5–7). Cells adjacent to the lumen were joined by tight junctions and contained apical microvilli and abundant rough endoplasmic reticulum (RER). Within the acinar epithelium occasional "light cells" were observed. The cytoplasm of these cells was less electron dense than that of neighboring cells (Figures 5 and 6). Light cells also appeared to be poorly anchored to surrounding epithelial cells, lacking the desmosomes which characterized the epithelium. The ultrastructure of these cells varied, with some being relatively void of cytoplasmic inclusions, while others appeared highly vacuolated with irregularities of the nuclear and cytoplasmic membranes (Figure 8). Occasionally, continuities could be seen between the dilated RER and the nuclear membrane (Figure 8). Irregular nuclear profiles were common (Figure 9). Light cells could be observed both in the adluminal cell layer (Figure 6) and in the basal cell layer (Figure 8). Typically, basal cells were associated with well-formed basal laminae (Figure 7). However, when light cells were in contact with the region of the basal lamina, areas were apparent in which it was absent or exhibited abnormalities such as folding and reduplication (Figure 8). Similar cells with dilated RER, irregular cell membranes, and poor attachments to the collagenous matrix were observable in the stroma. Figure 9 illustrates one such cell, with an enlarged irregular nucleus, in the process of invading the periacinar stroma. Normal epithelial cells with apical microvilli are evident in the upper right hand corner of the figure. The large arrow identifies the probable course of movement of this cell from its original location within the epithelial layer into the stroma. Signs of recent proteolytic activity such as collagen fragments, degraded basal lamina, and cytoplasmic debris are evident in the vicinity of the large arrow. The smaller arrow identifies an area in which continuity exists between this cell and the epithelial basal lamina (see inset for detail). Thus, the ultrastructural evidence suggests that these "light cells" correspond to the "epithelioid cells" illustrated in Figures 1, 3, and 4.

Acid phosphatase, a marker enzyme for prostatic epithelium, but not prostatic stroma, was identified histochemically in both normal murine dorsal pros-

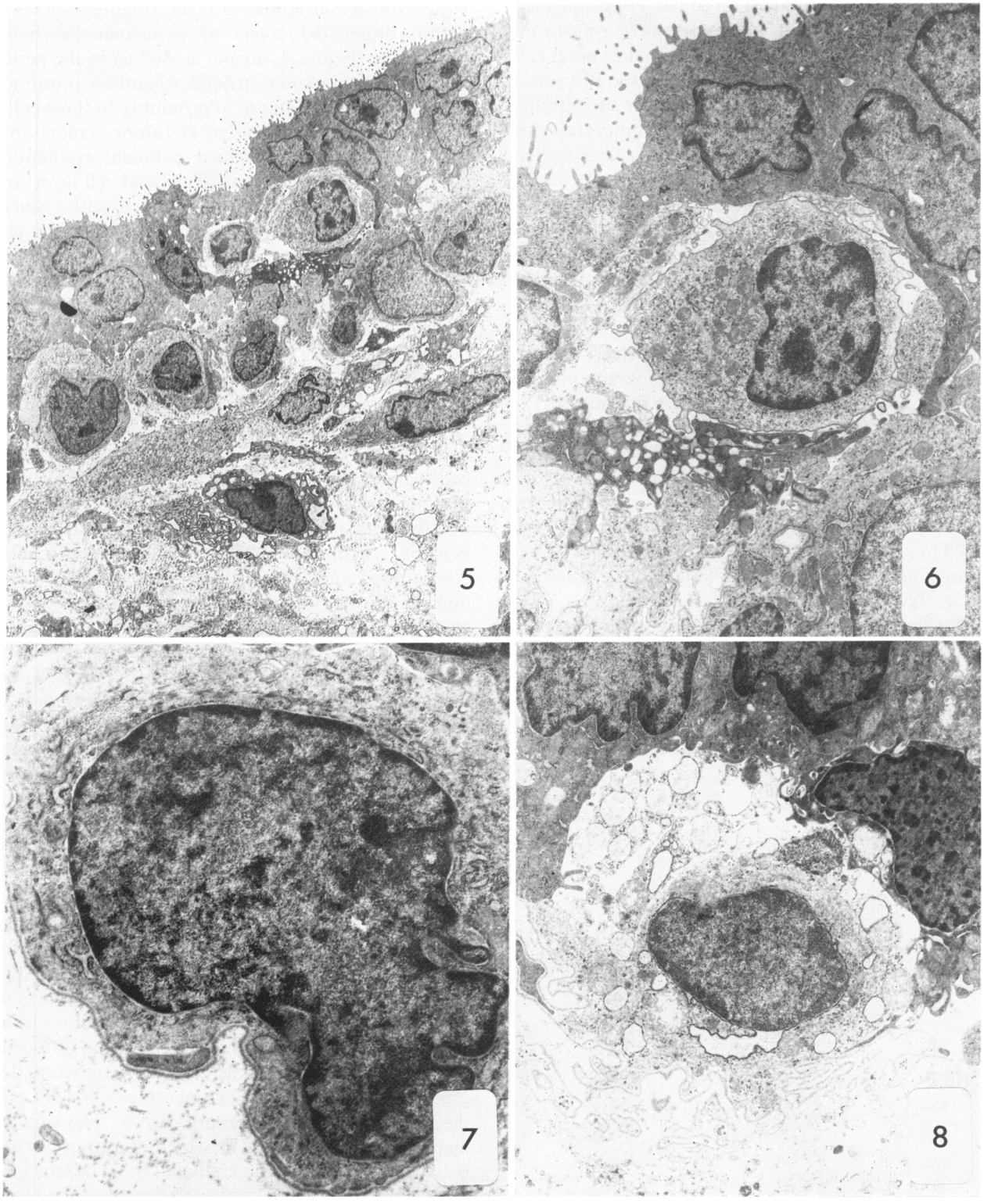


Figure 5—Electron micrograph of normal acinus of the H tumor. Cells appear relatively innocuous. A large "light cell" is present in the center of the field, which is poorly attached to the adjacent epithelial cells. "Light cells" are believed to be precursors of epithelioid cells. (×1500) **Figure 6**—Higher magnification of the "light cell" in Figure 5. Desmosomes are absent and a fluid filled area exists in the vicinity of the cell. (×3400) **Figure 7**—Normal basal cells have well-formed basal laminae and normal ultrastructure. (×7000) **Figure 8**—"Light cells" which have reached the basal lamina reveal increased distortion of the ultrastructure, including vacuolization within the RER and distortion of the nuclear membrane. Continuities frequently exist between the RER and the nucleolemma (×3400)

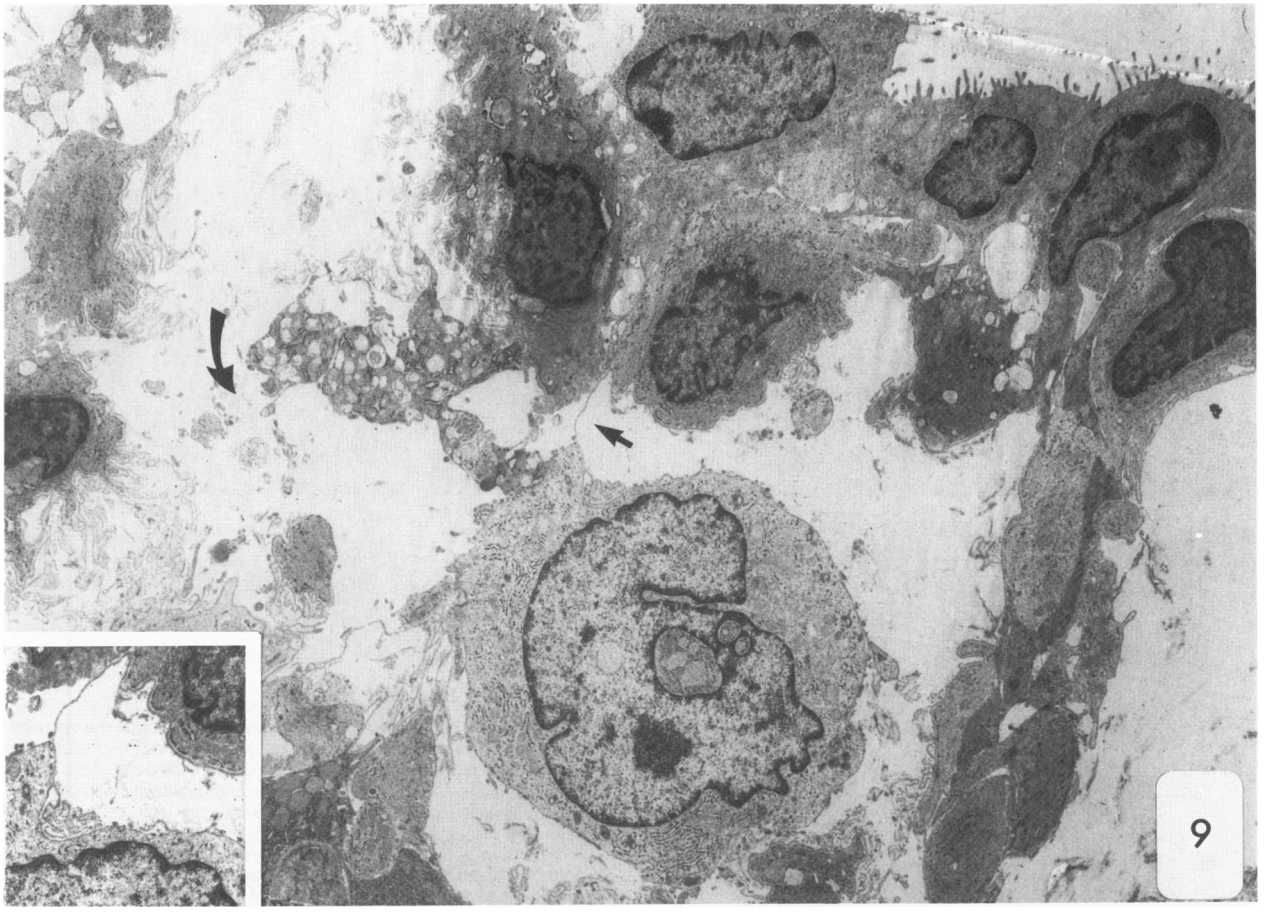


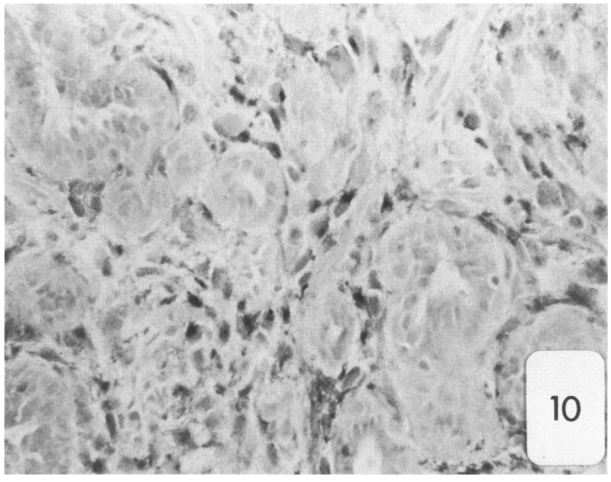
Figure 9—Light cells which migrate into the stroma from the acinar epithelium degrade basement membrane and stroma in their path (*large arrow*) leaving behind collagen fragments, reduplicated basal lamina, and cytoplasmic debris. The prominent, irregularly shaped nucleus is a key morphologic feature of this cell type. Continuity of the epithelial basal lamina with this cell is still evident in the region of the *small arrow*, enlarged in the **inset**. (×2100; **inset**, ×3400)

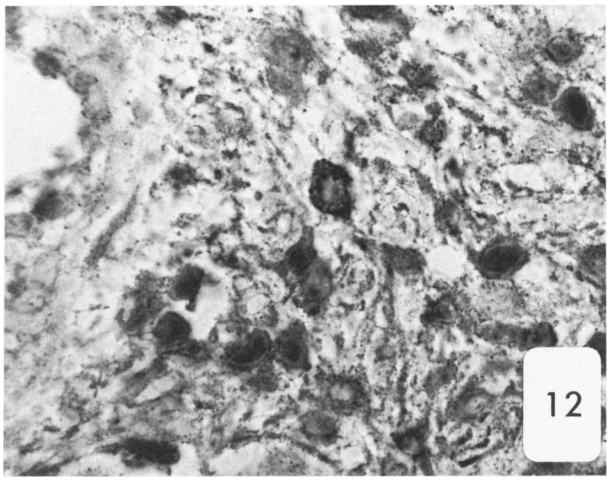
tate cells (Figure 2) and in the H tumor (Figure 10). Within the H tumor, the amount of acid phosphatase reaction product produced in large "epithelioid" cells was so great that, for illustrative purposes, the reaction had to be stopped prior to the appearance of product in the acinar epithelial cells (Figure 10). Long-term incubations revealed product in acinar epithelial cells as well.

A polyclonal antibody to keratin stained periacinar cells intensely (Figure 11). Longer incubation times revealed the presence of weak keratin immunoreactivity in acinar epithelial cells and epithelioid cells as well (not illustrated). A monoclonal antibody to vimentin filaments revealed intense immunoreactivity in occasional "epithelioid" cells and lower levels of immunoreactivity in fibroblasts and other stromal "epithelioid" cells (Figure 12). The acinar epithelium did not stain with vimentin.

Fluorescence activated cell sorting following enzymatic digestion separated the H tumor into three populations of cells on the basis of DNA content (Figure 13). Microscopic examination of cells within each of these peaks following separation revealed that the first peak consisted primarily of fibroblasts and normal diploid epithelial cells. Cells in the second peak consisted of a mixture of dividing cells and other cells which did not appear to be undergoing mitosis. Occasional cells in this peak were characterized by larger nuclei, which appeared to be morphologically irregular. Cells in the third population consisted mainly of very large cells with highly enlarged nuclei, although some smaller cells (<5%) were present as doublets and triplets.

From the mean channel position of the three peaks at 52, 109, and 166, and the microscopic examination of the contents of each peak, it was concluded that four populations of cells exist in this tumor: 1) a diploid/pseudodiploid population (2N), consisting of normal cells and adenocarcinoma cells in G_0/G_1; 2) a small number of 3N cells, consisting of abnormal adenocarcinoma stem cells, and cells in S phase; 3) a 4N population, consisting of G_2/M phase cells from the





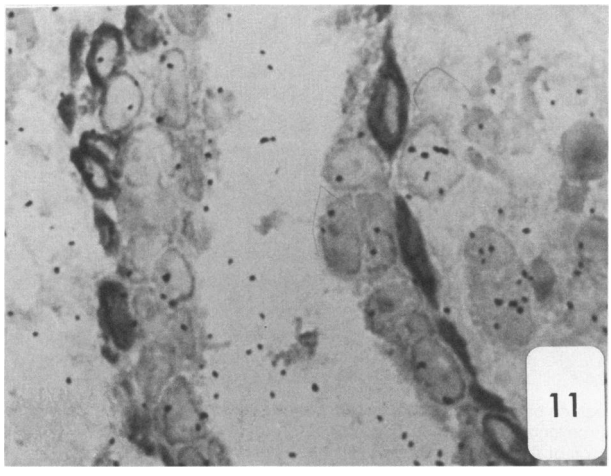


Figure 10—Acid phosphatase activity is present primarily in the epithelioid cells of the H tumor. In order to visualize epithelial acid phosphatase, prolonged reaction times are necessary, which obscure the detail of the stromal localization. (×350) **Figure 11**—Keratin immunoreactivity following immunostaining with a polyclonal antiserum revealed cytokeratin filaments primarily in the myoepithelial-like cells which surround prostatic acini. Longer staining times reveal weak immunoreactivity in epithelial and epithelioid cells. (×100) **Figure 12**—Vimentin immunoreactivity is present in fibroblasts as well as epithelioid cells, but not epithelial cells. (×750)

first population; and 4) a 6N population, consisting of some triplets of the 2N population and aneuploid cells resulting from mitosis of the 3N stem cell population.

Discussion

We have previously reported that the H tumor is characterized by the presence of anomalous large steroid sensitive cells in the epithelial and stromal compartments, and suggested that they be called "epithelioid cells" pending confirmation of their epithelial origin.[18] In pursuit of that goal, histochemical and immunohistochemical staining of the tumor was performed in conjunction with electron-microscopic and flow-cytometric analysis of the cells of interest.

The keratin family of intermediate filaments have been widely used as markers for epithelial cells.[26] We therefore used an immunohistochemical approach to characterize the distribution of this filament type within the H tumor. We were surprised to observe that there was immunolocalization within the basal cells and that immunoreactivity in the epithelial cells was much weaker under these experimental conditions. It was felt that this approach was supportive, but not definitive, of the epithelial origin of "epithelioid" cells, since the epithelial cells themselves stained weakly for this class of filaments.

Because vimentin commonly is present in the cytoplasm of mesenchymal cells, we postulated that vimentin immunoreactivity would be observed in stromal fibroblasts, but not in epithelial or "epithelioid" cells. As anticipated, nearly all cell types within the stroma contained vimentin. Interestingly, occasional large stromal cells were intensely stained as well. Although at first confusing, others have reported that neoplasms can acquire the ability to co-express intermediate cytoskeletal proteins.[27,28] Thus, it was concluded that immunoreactivity of the cytoskeletal protein vimentin would not be a useful indicator of the histogenesis of the "epithelioid" cell type.

Acid phosphatase, an enzyme known to be present in prostatic epithelium, but not in mesenchymally derived prostatic elements, was demonstrated histo-

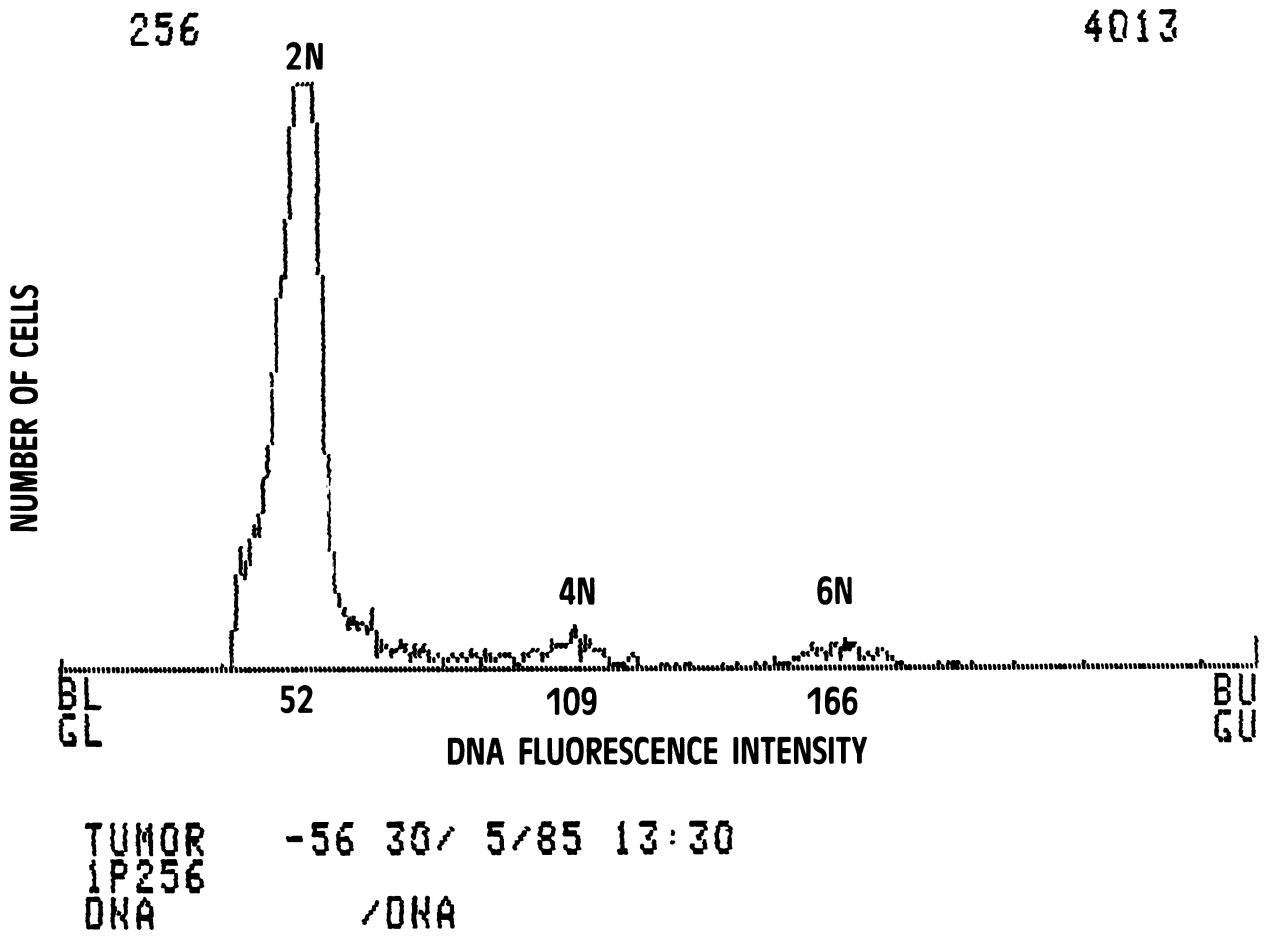


Figure 13—MDADS computer graphic representation of cell ploidy analysis prior to fluorescence-activated cell sorting of the Dunning R3327-H tumor. Mean channel numbers and approximate ploidy are indicated on the abscissa. The fluorescent DNA stain Hoechst #33342 was employed for ploidy analysis. Smaller numbers of cells were present in the two peaks with DNA contents greater than 2N because of smaller numbers of dividing cells and apparent increased susceptibility of these cells to degradation during enzymatic dispersion.

chemically in sections of H tumor and normal mouse prostate.[29] "Epithelioid" cells were observed to contain large quantities of this enzyme, providing support for the epithelial origin of these cells. In an attempt to further confirm the cells' epithelial origin, autoradiograms were stained with antibodies directed against specific components of the basement membrane.[30] The results of this approach revealed that the basement membrane, which was ordinarily present as an intact sheath around the entire acinus, was occasionally absent or fragmented in the vicinity of "epithelioid" cells. This observation was confirmed electron microscopically.

Electron microscopy revealed numerous instances of reduplication of the basement membrane in the vicinity of the "light cells," which we believe represent precursors of the "epithelioid" cell. Anomalies of basement membrane, cell surface and cell–cell attachments were typical of these cells. These irregularities suggest a defect in the synthesis or secretion of basement membrane proteins or an increased production of proteolytic enzymes by this cell type. It is likely that these cells have acquired a phenotype which permits synthesis of enzymes which degrade the extracellular matrix prior to invading the stroma.

Autoradiographically identifiable "epithelioid" cells were also observed in close juxtaposition to the basement membrane on the stromal side (Figures 1 and 4). The identification of a light cell population of precursor or *in situ* cells which can be seen invading the basement membrane and extending into the stroma again supports, but does not irrefutably confirm, the origin and direction of tumor cell migration away from the acinus. However, the weight of the evidence suggests that these large, steroid-sensitive cells in the H tumor epithelium should be classified as large neoplastic epithelial (LNE) cells, rather than "epithelioid" cells.

Digestion of the H tumor presented considerable difficulties. The digestion process seemed to be more detrimental to the viability of the LNE cells than to acinar epithelial cells. Light and electron micrographs of tumor pieces taken at various intervals during the digestion process revealed selective degradation of the larger cells, which might be expected in view of the apparent fragility of the cell membrane and RER of this cell type. Thus, a much lower yield of this cell type in proportion to acinar epithelial cells was obtained by the enzymatic approach than by conventional mechanical disruption of the tumor (mincing).

Flow cytometry revealed that the H tumor consists of three cell populations: 1) an apparent 2N population, 2) a population having 1.5 to two times the DNA content of the first group (3N to 4N): and 3) a population which contained primarily the LNE cell type. The second population was heterogeneous by Giemsa-stained light microscopy and contained mitotically active cells as well as a few larger cells. The segregation of most LNE cells to the third peak population suggests that these cells are aneuploid and have an abnormal DNA content, which is approximately 6N. No mitotic figures were present in this population. Likewise, we have never observed mitotic figures in the stroma of this tumor in conventional histologic sections. Thus, we speculate that these large cells result from an incomplete division of 3N stem cells present in the epithelial layer of the H tumor and have little or no further capacity to undergo mitoses.

Benson et al[31] and Claflin et al[32] have described the flow cytometric analysis of the H tumor. Claflin et al reported peaks at 2N , 2.8N, and 4N but did not see a 6N peak. The poorer resolution obtained with the vital dye Hoechst #33342 obscured the distinction between 2.8N and 4N, which were both included in our second peak. The peak which we observed at 6N contained some triplets of 2N epithelial cells, but consisted primarily of the larger LNE cells. In light of the fragility of these large cells, it is possible that Claflin et al my have disrupted the majority during the dissociation process.

In light of the morphologic evidence that LNE cells are present within the H tumor acinar epithelium, the pathologic evidence of their ability to degrade the extracellular matrix, and the histochemical evidence that they have the capacity to secrete acid phosphatase, we conclude that these cells are epithelial in origin. Immunohistochemical evidence that both epithelial and LNE cells contain low quantities of cytokeratin in contrast to periacinar cells provides a further confirmation of their epithelial identity. We surmise that their presence in the stroma is the result of their migration out of the epithelial cell layer due either to a preexisting invasive phenotype or to the acquisition of this phenotype during transformation.

Thus, we believe that these cells can be identified as a subset of epithelial cells which have acquired the capacity to invade the stroma locally. In fact, it is possible that LNE cells are responsible for the rare reports of metastatic ability of the H tumor.[4] Thus, the H tumor represents a naturally occurring model system for the study of the early events of tumor progression.

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