Induction of Hyperplasia and Its Suppression by Hydrocortisone in Organ-cultured Rat Urinary Bladder

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SUMMARY

Urinary bladders from Fischer rats organ cultured in a chemically defined medium, Ham's F12, underwent transitional cell hyperplasia which persisted for the duration of the culture period (10 days). The hyperplastic response was initiated at 2 days of culture in basal epithelial cells as evidenced by [3H]thymidine autoradiography. After 2 days, cells classified as intermediate cells were observed replicating DNA in increasing numbers, whereas the frequency of basal cells replicating DNA decreased. The peak periods of basal and intermediate cell DNA replication were at 2 and 5 days, respectively. The total increase in the number of cells in the epithelium during a 10-day culture period was approximately 2.6-fold. The appearance of DNA-replicating cells before the appearance of mitotic figures indicated that the cells of the transitional epithelium are primarily G1 cells.

The hyperplastic response in the transitional epithelium was significantly inhibited by hydrocortisone. Epithelia cultured in the presence of hydrocortisone also displayed less atypia than those epithelia cultured in its absence. Hydrocortisone concentrations of 2.1 and 21 μM inhibited hyperplasia by 75 and 84%, respectively. Cells replicating DNA at 2 days of culture were considerably less sensitive to the hydrocortisone inhibition of [3H]thymidine incorporation into DNA than cells replicating DNA at 4 days of culture. The possibility is discussed that basal and intermediate cells may have different sensitivities to hydrocortisone.

INTRODUCTION

There have been numerous reports in the literature which demonstrate the existence of multiple noninvasive microscopic lesions, ranging from hyperplasia through increasing degrees of epithelial atypia to carcinoma in situ, in areas of cystoscopically normal-appearing human bladder epithelium which lie peripheral to invasive tumors (1, 4, 9, 13, 18–21). The implication of these observations is that some of these lesions may be precursors to invasive cancer and that they exist for some period of time before its appearance. Recent evidence (5) demonstrates more definitively that human bladder cancer develops over a considerable period of time and is preceded by a preneoplastic phase characterized by progressive degrees of epithelial hyperplasia, dysplasia, and nuclear atypia. A knowledge of the development of preneoplastic lesions in the transitional epithelium would add considerably to our understanding of the preneoplastic state. Unfortunately, the analysis of this problem in man is difficult because of the apparently long duration and gradual development of preneoplasia. The problem in man has therefore remained poorly understood; this is also the case for experimental carcinogen-induced preneoplasia of the bladder. An experimental system, in which the preneoplastic phase of cancer development is accelerated under carefully controlled conditions, would be of considerable help in studying this clinical problem.

In an earlier paper (16), procedures were described for the successful organ culture of normal and carcinogen-treated rat urinary bladder in various chemically defined culture media. One medium, Waymouth's MB752/1 (25), allowed the long-term culture of transitional epithelium. A small degree of basal cell hyperplasia was observed; however, the epithelium remained transitional. This was not the case in Ham's Medium F12 (8). Although long-term cultures were maintained, the epithelium displayed moderate to marked hyperplasia with dysplasia and produced extensive nodular downgrowths into the submucosa.

Although the etiology of hyperplasia and dysplasia in the preneoplastic condition is obviously quite different from that observed in Medium F12, the developmental mechanisms by which hyperplastic and dysplastic states are established in the 2 conditions may be quite similar. A study of the response of bladder epithelium to growth in Medium F12 would thus provide useful information for understanding the development of preneoplastic lesions in the transitional epithelium. More importantly, the system provides a means of testing agents which may be potentially useful in the control or reversal of preneoplastic bladder lesions. A study of the response of bladder epithelium in Medium F12 would thus provide useful information for understanding the development of preneoplastic lesions in the transitional epithelium. Further, the system provides a means of testing agents which may be potentially useful in the control or reversal of preneoplastic bladder lesions. A study of the response of bladder epithelium in Medium F12 was therefore undertaken to determine how hyperplasia and dysplasia develop and how they can be controlled. In this paper, an analysis of hyperplasia and its control by hydrocortisone is presented.

MATERIALS AND METHODS

Animals. Male Fischer 344 rats were obtained from the Charles River Breeding Laboratory, Wilmington, Mass., and used at 7 weeks of age.

Organ Culture Procedure. Animals were anesthetized with ether. Urinary bladders were removed aseptically and
cut into equal halves, longitudinally. These hemibladder explants were cultured, with the epithelium positioned dorsally, on stainless steel grids in 60-mm plastic culture dishes containing 3 ml of Ham's Medium F12 (8). The medium contained 100 units of penicillin and 100 μg of streptomycin per ml. Cultures were maintained at 34.5° in a gas phase of 4.5% CO₂ in air. Medium was changed every 2 or 3 days.

In experiments investigating the effects of hydrocortisone on hyperplasia, sterile aqueous hydrocortisone-21-sodium succinate (The Upjohn Co., Kalamazoo, Mich.) was used. At each change of medium, the hormone was added as a freshly prepared solution. Insulin (crystalline, bovine; Eli Lilly and Co., Indianapolis, Ind.), prepared as a sterile stock solution in 4 mM HCl, was included at a concentration of 1 μg/ml in all media used in hydrocortisone experiments, including control media.

[^3H]Tdr Labeling of DNA. Tissues were labeled with 15 μCi of [^3H]Tdr (specific activity, 20 Ci/m mole; New England Nuclear, Boston, Mass.) in 3 ml of medium. Tissues to be used for autoradiography were labeled for 1 hr, and tissues for specific activity measurements were labeled for 3 hr. During the labeling periods, dishes were tilted to ensure that the tissues were completely submerged in the labeling medium.

Histology and Autoradiography. Tissues were fixed in buffered neutral formalin, embedded in glycol methacrylate, and sectioned at 2 μm. For autoradiography, sections were coated with Kodak NTB2 liquid emulsion and stored for 14 days at 4°C. Autoradiographs were developed for 5 min in D19 Kodak developer, rinsed in water, and fixed for 10 min in Kodak fixer. Sections were stained with hematoxylin and phloxine B.

Scoring of Data. To quantify the hyperplastic response, the numbers of nuclei, labeled nuclei, and mitotic figures per 150-μm length (unit length) of epithelium were scored on 1 section from each of 4 or 5 explants/time point. Only flat unit lengths of epithelia were counted. Folded and invaginated areas of epithelia were not counted. In the case of nodular downgrowths which were frequently encountered at 7 and 10 days of culture, if part of a unit length was flat but also contained a nodular area, it was counted. If, however, the unit length encompassed a length of epithelium which was entirely nodular, it was not counted.

In the area of the cut surface, a small portion of the bladder epithelium becomes attenuated and displays an abnormally high labeling and mitotic index. These wound areas were not scored. A nucleus was considered labeled if 7 or more exposed grains were observed in the emulsion above it. In cases where the grain density above a nucleus was such that the grains coalesced but with obviously numerous grains present, the nucleus was scored as labeled. Labeled nuclei and mitotic figures were expressed as percentage of total cells, while nuclear number was expressed as number per unit length.

The distinction between basal and intermediate cells was made according to their position in the epithelium. Basal cells were those cells most proximal to the epithelial-submucosal border. All other cells, which were separated from the border by another cell (basal cell), were classified as intermediate cells.

Specific Activity of[^3H]Tdr Incorporation into DNA. After labeling, tissues were held on ice in 3 ml of Dulbecco’s phosphate-buffered saline (3). Each tissue was transferred to a plastic dish containing 0.5 ml of cold calcium- and magnesium-free phosphate-buffered saline containing 20 mM EDTA, and the epithelium was scraped from its collage nous supporting layer with a scalpel. Essentially all of the epithelium was removed by this method without damaging or including submucosal tissues (Fig. 1). The surface of the dish was washed twice with fresh phosphate-buffered saline containing 20 mM EDTA to ensure complete recovery. A total of 1.5 ml of cell suspension was obtained. This was made 0.2 n HClO₄ and held on ice for 15 min. The precipitated cell suspension was centrifuged and washed twice with cold 0.2 n HClO₄.

To the final pellet, 0.6 ml of 1.6 n HClO₄ was added, and the DNA was hydrolyzed for 20 min at 70°C. After centrifugation, the deoxyribose in the hydrolysate was measured by a modification of the procedure of Giles and Myers (7). To 0.5 ml of the DNA hydrolysate, 0.5 ml of 4% diphenylamine in glacial acetic acid and 0.025 ml of 0.2% aqueous acetaldehyde were added. The samples were incubated at 35°C for 18 hr and then read in the spectrophotometer as previously described (7).

RESULTS

Development of Hyperplasia. The uncultured bladder epithelium showed a low frequency of [^3H]Tdr-labeled nuclei (<0.1%). When bladder was cultured in Ham’s medium, this condition persisted for the 1st 24 hr. After this period, a large increase was observed in the frequency of labeled cells (Chart 1). This trend continued until 4 days of culture and then declined. At 10 days of culture, however, the frequency of cells undergoing DNA synthesis was still considerably above that of normal bladder.

Cells undergoing DNA replication were also scored ac-

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1. The abbreviation used is: [^3H]Tdr, [^3H]thymidine.

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Chart 1. Time course of [^3H]Tdr incorporation into DNA of bladder epithelium cultured in Ham’s Medium F12. Each point represents the mean percentage of labeled epithelial cells from 4 or 5 cultures ± S.E. All tissues were labeled for 1 hr.
Induction and Control of Bladder Hyperplasia

According to whether they were basal or intermediate cells. At 2 days of culture, greater than 80% of the labeled cells were classified as basal cells. At 3 days, label was nearly equally distributed between intermediate and basal cells (Chart 2). By 4 and 5 days, however, intermediate cells were the predominant cells engaged in DNA synthesis. This pattern subsequently reversed, and by 10 days of culture basal cells were again the predominant DNA-synthesizing cells. Only 2 labeled superficial cells were encountered in the entire experiment.

That the DNA synthesis which was observed during the culture period was followed by cell division is shown in Chart 3 by the pattern of increase in the frequency of mitosis. Mitotic figures were not seen until the 2nd day of culture at which time 2 were observed out of a total of 2527 counted cells. The increase in frequency of mitotic figures followed, by a slight lag, the increase in labeled cells. The highest frequency of mitotic figures was observed at 3 and 4 days of culture, after which the frequency declined. As was the case for cell labeling, the frequency of mitotic figures at 10 days of culture remained considerably above that of the uncultured bladder.

It is evident from the histology that the increases in DNA synthesis and mitosis resulted in more epithelial cells (Figs. 2 to 4). It was also possible to obtain a quantitative estimation of increased cell number during the culture period by counting the number of nuclei per flat unit length of epithelium (Chart 4). Cell number was essentially constant for the 1st 2 days. Between 2 and 7 days of culture, the number of cells increased linearly. The magnitude of increase in nuclei per unit length of epithelium between 2 and 10 days was approximately 2.6-fold.

Inhibition of Hyperplasia with Hydrocortisone. Hydrocortisone has been shown to inhibit the DNA synthesis and growth of some cells (2, 6, 11, 22). Having established the features of the hyperplastic response in F12 medium, the ability of hydrocortisone to control this hyperplasia was examined. In bladders cultured continuously for 3 days in the presence of various concentrations of hydrocortisone, the incorporation of [3H]TdR into DNA was strongly inhibited at concentrations of 2.1 and 21 μM (Chart 5). A small degree of inhibition was observed at 0.21 μM.

It was a consistent observation that the sensitivity of [3H]TdR incorporation to hydrocortisone depended upon the day of culture on which the tissues were labeled. Cells synthesizing DNA at 2 days of culture were considerably less sensitive to hydrocortisone than those synthesizing DNA at 4 days (Table 1). DNA-replicating cells at 2 days of culture showed essentially no inhibition of [3H]TdR incorporation into DNA by 0.21 μM hydrocortisone, whereas those cells replicating at 4 days were inhibited by approximately 60% with the same concentration. At a 10-fold higher concentration, cells replicating DNA at 2 days showed a greater
degree of inhibition than at the lower concentration, but the inhibition was still less than that observed in 4-day replicating cells. That the inhibition of [3H]TdR incorporation into DNA was due primarily to the inhibition of DNA synthesis is shown by the suppression of epithelial hyperplasia (Chart 6). Hydrocortisone present continuously in the medium for 7 days at a concentration of 2.1 mM caused a 75% inhibition of epithelial hyperplasia compared to controls. At a 10-fold higher concentration of hydrocortisone, the inhibition was 84%. In addition to this striking suppression of hyperplasia, epithelia cultured in the presence of hydrocortisone (Fig. 6) did not display the atypia characteristic of epithelia cultured in the absence of hydrocortisone (Fig. 5). Epithelial cells also displayed an apparent increase in cytoplasmic area as a result of hydrocortisone treatment. No evidence of epithelial degeneration or other manifestations of toxicity to hydrocortisone was observed.

**DISCUSSION**

The transitional epithelium is not a rapidly renewing tissue (10). However, as the result of some conditions, such as exposure to carcinogens or infection, it displays a striking capacity for proliferation and growth (14). It has been demonstrated in this study that culture in Ham’s Medium F12 is another condition that results in marked proliferative activity and growth in the transitional epithelium. Although the period of intense proliferation is of relatively brief duration in Medium F12, it should be emphasized that cell proliferation continues throughout the duration of the culture period. This is in contrast to some chemically defined culture systems in which only a limited period of proliferation is supported (17).

The absence of mitotic activity before the appearance of [3H]TdR incorporation into DNA indicates that the transitional epithelium of the rat bladder is composed primarily of cells that are in the G₁ phase of the cell cycle. It is possible, however, that division by a small population of G₂ cells could have occurred during the 1st 12 hr of culture and was not detected.

The time sequence of the appearance of [3H]TdR-labeled cells in the basal and intermediate layers of the epithelium suggests that hyperplasia begins in basal cells. Labeling is subsequently seen in intermediate cells, but it is not possible to determine from this study whether this labeling is in “original” intermediate cells or in “daughter” basal cells that have migrated into the intermediate zone after division.

**Table 1**

<table>
<thead>
<tr>
<th>Time in culture (days)</th>
<th>Experiment</th>
<th>Hydrocortisone, 0.21 μM</th>
<th>Hydrocortisone, 2.1 μM</th>
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<td>0.5</td>
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</tr>
<tr>
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<td>2</td>
<td>53</td>
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**Chart 6.** Inhibition of hyperplasia with hydrocortisone. Tissues were cultured in Ham’s F12 medium continuously for 7 days in the absence of hydrocortisone (Control) or at hydrocortisone concentrations of 2.1 or 21 μM. Some tissues were not cultured (0 Day). These were fixed immediately after removal from the animal and indicate initial values. Data are expressed as the mean number of nuclei per unit length (150 μm) of epithelium from 5 cultures ± S.E.
It is possible that some labeled cells were originally intermediate cells, since it has been shown in the mouse bladder that all 3 types of transitional epithelial cells (basal, intermediate, and superficial) retain the capacity for proliferation (10, 24).

Since the highest frequency of [3H]TdR-labeled cells occurred at 4 days of culture, it would be expected that the peak in mitotic activity would occur subsequent to 4 days. This was not the case; mitotic activity peaked at 3 to 4 days. To resolve this discrepancy, a more detailed analysis of the cell-cycle kinetics in the various cell populations of organ-cultured transitional epithelium will be required.

It has been suggested recently that some tissues, which normally do not undergo rapid renewal but have retained the capacity for rapid growth, are particularly sensitive to the hydrocortisone suppression of [3H]Tdr incorporation into DNA (12). We have demonstrated in the present study that the transitional epithelium of the urinary bladder can be added to this class of tissues and that this suppression is probably due exclusively to the suppression of DNA synthesis.

The temporal change in hydrocortisone sensitivity in culture is of particular interest. Epithelial cells synthesizing DNA at 2 days are considerably less sensitive to hydrocortisone than those synthesizing DNA at 4 days. Since the predominant DNA-synthesizing cells at 2 and 4 days are basal and intermediate cells, respectively, the data suggest that intermediate cells may be considerably more sensitive to hydrocortisone. Whether this difference in sensitivity reflects some general change in the epithelium such as increased permeability to hydrocortisone or whether it indicates an actual difference in responsiveness of the 2 cell types will require more study. In connection with this latter possibility, it has been shown in squamous epithelial cells of the mouse forestomach that a period exists (called “R phase”) during the last 6 to 9 hr of G1 when cells become insensitive to hydrocortisone and thus can no longer be stopped from eventually entering S phase (6). Since the sequence of events described in this paper indicates that the basal cells of the transitional epithelium are also in G1 when the peak in mitotic activity would occur subsequent to 4 days, it is possible that these cells may accumulate at, or just before, a point in G1 which is similar to “R phase.”

Insulin was routinely included as a culture medium component in the hydrocortisone experiments because it has been shown that insulin and hydrocortisone display synergistic action in some culture systems (15, 23). Recent evidence (D. H. Reese and R. D. Friedman, unpublished observation) shows that insulin alone has no apparent effect on the hyperplastic response of bladder epithelium in Medium F12 and that insulin is not necessary for the hydrocortisone inhibition of this hyperplasia.

The concentration of hydrocortisone necessary to inhibit DNA synthesis in the present study is at least 10 times higher than that required to inhibit DNA synthesis in some other epithelial cells (11). Whether this greater requirement for hydrocortisone is a characteristic of the transitional epithelium, organ culture, serum-free medium, or some unknown entity will require further study.

Acknowledgments

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References

Fig. 1. A bladder explant cultured for 7 days in Ham's Medium F12. One-half of the epithelium was scraped away from the explant (see "Materials and Methods"). The explant was then fixed for histology. The plane of the histological section was made at right angles to the plane of scraping so that the photo shows adjacent scraped and unscraped areas of the epithelium. Note the complete removal of epithelium from the scraped portion without apparent damage to submucosal tissues. H & E, × 145.

Fig. 2. Autoradiograph of a bladder explant labeled for 1 hr with [3H]TdR immediately after surgical removal from the animal. Hematoxylin-phloxine B, × 360.

Fig. 3. Autoradiograph of a bladder explant cultured in Ham's Medium F12 for 4 days before [3H]TdR labeling for 1 hr. Hematoxylin-phloxine B, × 360.

Fig. 4. Autoradiograph of a bladder explant cultured in Ham's Medium F12 for 10 days before [3H]TdR labeling for 1 hr. Hematoxylin-phloxine B, × 360.
Fig. 5. Area of an epithelial downgrowth in a bladder explant cultured for 7 days in Ham’s Medium F12. This explant is from the same experiment as tissue shown in Fig. 6. Note the extensive dysplasia. H & E, × 360.

Fig. 6. Bladder explant cultured for 7 days in Ham’s Medium F12 in the presence of 21 μM hydrocortisone. Note the normal transitional morphology of the epithelium. H & E, × 360.
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