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Histomorphometry and Cell Kinetics of Normal Human Bladder Mucosa in vitro*

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Summary. In this study, we have quantified the morphologic and kinetic parameters of explant cultured normal adult human urinary bladder mucosa. Quantitative parameters studied were urothelial height, cell density, labelling index and mitotic index. For these studies, urinary bladders from seven adults with no previous history of urologic disease were obtained at autopsy. Mucosal explants were maintained in rocking culture on Gelfoam rafts for up to 33 days using supplemented CMRL 1066 medium. Prior to sampling, cultures were treated with tritiated thymidine and colchicine to investigate tissue kinetics. Data was based on histologic autoradiograms. During culture, urothelial cells retained normal polarity. During the first week of culture, urothelial height increased and cell density decreased. DNA synthesis and mitotic activity occurred primarily among basal cells. DNA synthesis was first noted on day 2 of culture; mitotic activity began after 3 days of culture. Morphologically, human urothelium was well maintained; DNA synthesis and mitotic activity was variable but continued throughout culture.

Key words: Human urinary bladder, Urothelium, Explant culture, Kinetics.

Introduction

Cancer of the urinary bladder in humans has been linked to occupational exposure to certain aromatic amines such as 4-aminobiphenyl, benzidine, and 2-napthylamine used in the dye, rubber, paper and leather industries [14]. Some of these compounds have subsequently been shown to be potent bladder carcinogens in animals in vivo [1, 14]. However, comparable in vivo experiments cannot be undertaken in humans and it may not be appropriate to extrapolate from animal data to man. Thus, development of an in vitro system using human urinary bladder tissue is essential to study the etiology and pathogenesis of bladder cancer and would facilitate interspecies comparisons [4, 23]. Many authors [3, 5, 7, 9, 10, 16] feel that in vitro studies of bladder carcinogenesis should be undertaken in an organ explant culture system rather than in a cell culture system because of the strong modulating effects connective tissue stroma exerts on the morphology of the urothelium [7]. In a cell culture system, the stroma is absent, while in an explant culture system, the epithelial-stromal interactions are preserved, perhaps allowing a more in vivo-like tissue response following carcinogen treatment in vitro.

In order to conduct adequately controlled experiments, significant amounts of tissue are required and tissue utilized should be from individuals without evidence of urologic disease. Previously, normal human urothelial cells have been recovered from urine and bladder washings of adults [12] and neonates [22]. Urothelial cells have also been obtained in vitro as primary outgrowths from ureteral explants [18]. Knowles et al. [9, 10] established and maintained explants of bladder tissue obtained at cystoscopy and Peterson et al. [15] isolated cells from tissue obtained in this manner. However, neither urothelium obtained from washings of the urinary tract, ureters, nor surgical tissue obtained by cytoscopy from older individuals with clinical disease can be considered representative of normal urothelium of the urinary bladder.

Our laboratory has a unique resource for the collection of normal adult human urinary bladder urothelium which we have used in previous experimental studies [3, 5]. These tissues are obtained from patients at Immediate Autopsy (IA) within 1–2 h of somatic death [24]. Patients included in this series succumbed to brain death following vehicular accidents or suicide attempts, and usually are 16–30 years of age with no underlying urological disease.

Stoner and coworkers [2, 19–21] have studied the metabolism and binding of several known carcinogens to

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the DNA of cultured human bladder tissue obtained at autopsy. These studies utilized a serum-free explant culture system different than the one described below, and were short-term studies. Although the authors state that outgrowth cell viability was maintained up to 28 days in vitro, the issue of explant morphology for this period of time in culture was not addressed. Recently, Kirk et al. [8] described a serum-free system for the selective growth of urothelial cells in vitro. Again, these studies concentrated on cell outgrowth and viability, not explant morphology and kinetics in vitro. Studies in our laboratory with a serum-free culture system were unsuccessful in adequately maintaining urothelial explants for kinetic and morphologic studies (unpublished results).

Before experiments of urinary bladder carcinogenesis can be undertaken in vitro, the system to be used must be reproducible and well-characterized. In this paper, we have examined the effects of culture conditions on several quantitative parameters relevant to a subsequent detailed investigation of the acute and chronic effects of known carcinogens and promoters on human urinary bladder mucosa morphology. The results which follow represent a synthesis of data from seven individual IA cases obtained over a period of 1 year.

Materials and Methods

The entire urinary bladder was obtained from 5 males, aged 16 to 61 and 2 females, ages 19 and 40 at Immediate Autopsy [18], and placed into cold L-15 medium [11] (Gibco, Grand Island, NY).

Using aseptic technique in a laminar flow hood (Bellco Glass Co., Vineland, NJ), the entire mucosa was trimmed from the underlying muscle and connective tissue. The mucosa was then placed in L-15 medium supplemented with antibiotics: 600 U penicillin per ml, (Biofluids, Inc., Rockville, MD) and 600 μg streptomycin per ml (Biofluids), and 1.25 μg Amphotericin B per ml (Gibco) at 37 °C for 2 h to reduce the possibility of subsequent contamination of cultures. Explants 0.5 cm^2 were prepared and placed on Gelfoam (Upjohn CO, Kalamzoo, MI) rafts in 60 mm petri dishes (Costar, Cambridge, MA), 3–4 explants per dish with the urothelial surface uppermost. Mucosa to be cultured was selected based on gross appearance; areas of hemorrhage and obvious physical trauma were avoided. Five ml of a synthetic medium, CMRL-1066 (Gibco) was added to each dish. This medium was supplemented with 0.45 μg/ml ferrous sulfate (Sigma Chemical Co., St. Louis, MO) [9, 10], 1 μg/ml hydrocortisone hemisuccinate (Schwartz/Mann, Rockville, MD), 10% (v/v) fetal bovine serum (Gibco), 300 U penicillin/300 μg/ml streptomycin (Biofluids), and 0.625 μg/ml Fungizone (Gibco). A single lot of fetal bovine serum was used in each series of experiments.

Culture dishes were maintained in a controlled atmosphere chamber (Bellco) in an atmosphere of 50% N_2, 45% O_2, and a 5% CO_2 (Air Products, Inc., Baltimore, MD) [3, 5]. The chamber was rocked at 3 cycles per minute on a rocker platform (Bellco) to facilitate nutrient and gas exchange.

At intervals during culture, explants were dosed with tritiated thymidine (^{3}HTdR) (6.7 Ci/mmol) (New England Nuclear, Boston, MA) in fresh culture medium for 6 h at an activity of 5 or 15 μCi/ml to label cells during DNA synthesis. After ^{3}HTdR exposure, the medium was changed, and fresh medium containing 1 μg/ml colchicine (Sigma) was added to cultures for another 6 h to arrest mitosis of cells at metaphase, followed by fixation in 4% formaldehyde-1% glutaraldehyde [11] and paraffin embedding. Treatment of cultures with ^{3}HTdR and colchicine, the sequence of medium changes, and samplings were initiated at approximately the same time of day ± 1 h to minimize diurnal variation.

After an adequate period of fixation, culture explants were bisected, processed, and embedded flat in the paraffin block. The explants were then vertically sectioned (5 μm).

Histologic sections for autoradiography were deparaffinized and coated with diluted NTB-2 liquid emulsion (Eastman-Kodak, Rochester, NY); 2 parts emulsion: 1 part distilled water. Coated sections were exposed for 2 weeks in the dark at 4 °C, developed for 2 min at room temperature in Dektol (Kodak) diluted 1 : 1 with distilled water, rinsed in tap water, then fixed 4 min in NH-5 Hypo Concentrate (Geico Division, Whittaker Corp., Delaware Water Gap, PA) diluted 1 part: 3 parts with tap water, and finally washed for 10 min in running tap water. Autoradiograms were then stained with hematoxylin and eosin (H & E), dehydrated, cleared and mounted.

Quantitative parameters measured in autoradiograms were: Urothelial height, cell (nuclear) density, labelling index, and mitotic index. Data was collected from approximately five adjacent 225 μm segments of urothelium per explant. Urothelium less than 3 cell layers in thickness, or at or near the cut margins was excluded. Urothelial height and cell (nuclear) denisty were determined using a calibrated ocular micrometer. Height and length were measured in μm; cell density was expressed as the number of nuclei per mm^2 after determining the area in mm^2. Labelling index (% of urothelial cells showing ^{3}HTdR uptake) and mitotic index (% of metaphase-arrested urothelial cells) were also determined.

Means and standard errors of each parameter were calculated for each explant. Regression analysis was performed using the method of least squares. The correlation coefficient was tested for significance using Student's t-test.

Results

Prior to culture, human urinary bladder mucosa appeared normal by histopathologic criteria (Fig. 1). Histologically, the urothelium was composed of basal cells, intermediate and superficial cells arranged in an ascending pattern of differentiation. During culture, superficial cells were generally lost from the urothelium (Figs. 1, 2). However, the remaining urothelial cells (basal and intermediate cells) retained their normal polarity. During culture, DNA synthesis and mitotic activity occurred predominantly in the basal layer (Fig. 2). Cut surfaces of the explants were covered by migrating urothelial cells by two days culture. Urothelial cells also began to migrate into the underlying Gelfoam raft by 3 days of culture. Widened intercellular spaces appeared in the urothelium as early as 2 days culture (Fig. 2); intraepithelial lumina, sometimes containing cell debris, appeared as early as 12 days culture. At later intervals, mild necrosis of the deep connective tissue was observed, but not of the epithelial cells.

Although variation was evident among the different patients with respect to quantitative parameters, data obtained from the same patient was consistent during culture for each parameter examined, and similar trends in data were noted for the tissue from each of the different patients.

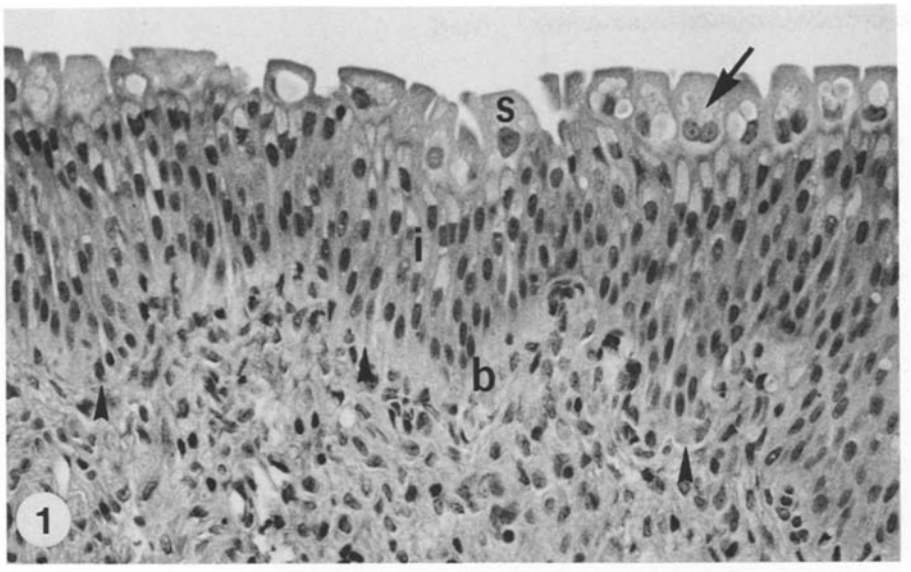

Fig. 1. 0-time sample of human urinary bladder mucosa obtained at autopsy. Urothelial tissue appears normal by histopathologic criteria, with basal (*b*), intermediate (*i*) and superficial cells (*s*) arranged in the usual ascending pattern of differentiation from the basement membrane (*arrowheads*) to the luminal surface. Some of the superficial cells are binucleated (*arrow*). This appearance is typical of tissue used in experiments prior to explant culture. Hematoxylin and eosin, x315

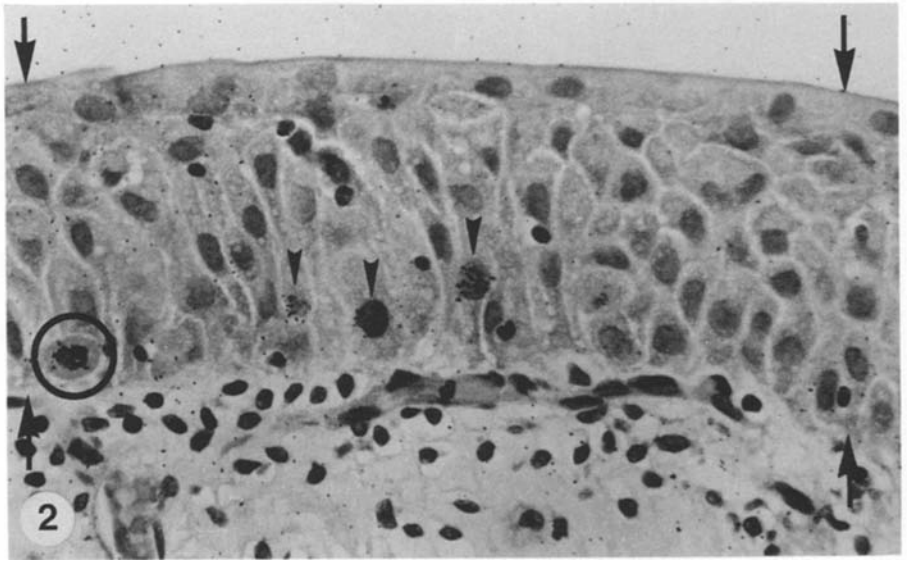

Fig. 2. Four day culture of normal human urothelium in explant culture. An area of urothelium from which data is collected is indicated by arrows. Cells within the 225 μm linear distance and the urothelial height are scored as unlabeled, labeled or mitotic. The cells retain normal polarity, and the urothelial cells near the luminal surface appear somewhat flattened or squamous-like. Most of the mitotic and DNA synthetic activity, as indicated by labeled mitotic figure (*circle*) and labeled cells (*arrowheads*) occurs in the basal layer. Widened intracellular spaces are present, predominantly in the lower cell layers. Autoradiogram, hematoxylin and eosin, x504

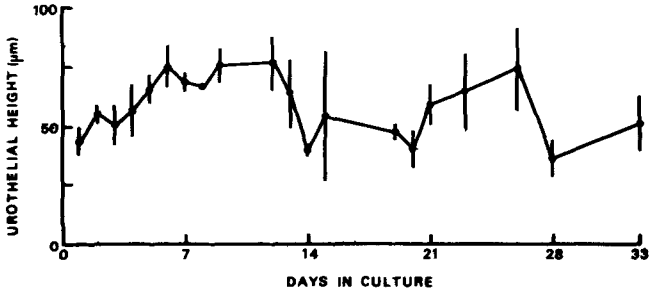

Fig. 3. Changes in urothelial height of normal human during explant culture up to 33 days. Each data point represents the mean and SE of 4–9 explants from 1–7 bladders sampled at each time interval. An increase in urothelial height occurs during the first week of culture, becoming variable thereafter; though overall, height is maintained near 0-time values

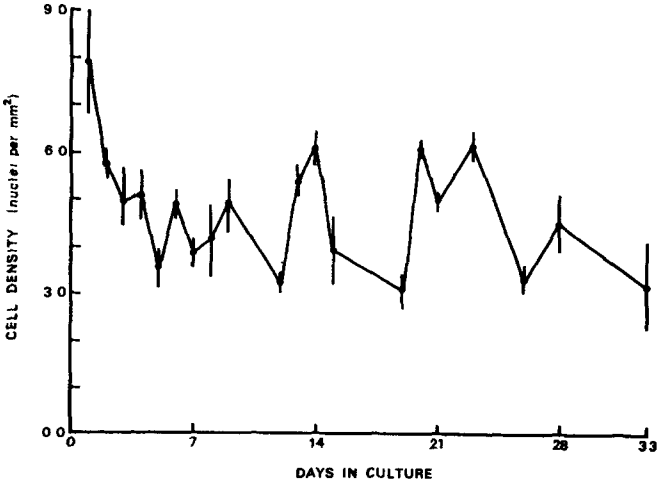

Fig. 4. Changes in cell (nuclear) density of normal human urothelium during explant culture up to 33 days. Each data point represents the mean and SE of 4–9 explants from 1–3 bladders sampled at each time interval. A decline in cell density compared to 0-time values occurs during the first week of culture, followed by a plateau or slight decline of values thereafter

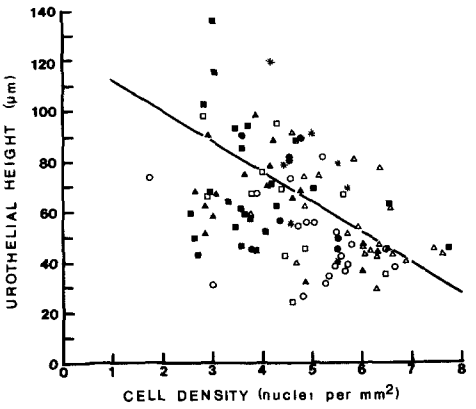

Fig. 5. Urothelial height plotted against cell (nuclear) density of normal human urothelium in explant culture up to 33 days. Each data point are the means from each explant counted from seven different bladders, represented by the different symbols. The apparent inverse correlation is significant ($p < 0.005$)

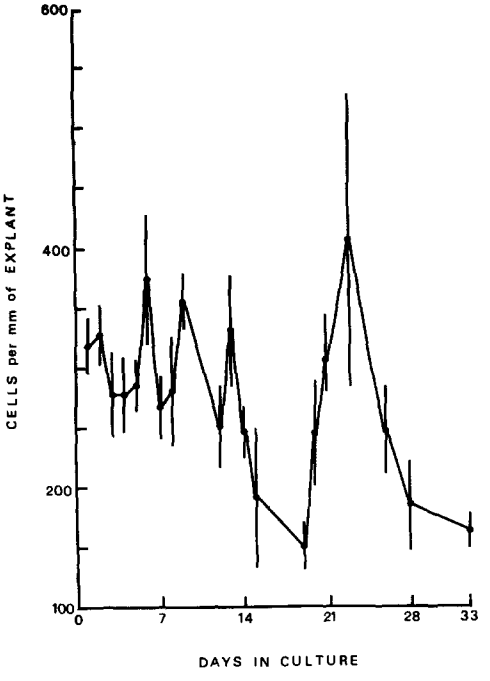

Fig. 6. Changes in the number of urothelial cells per mm of normal human urothelium during explant culture up to 33 days. Each data point represents the mean and SE of 4–9 explants from 1–3 bladders sampled at each time interval. Data are highly variable over the period, but there appears to be a trend toward a general decline in cell numbers, which is consistent with the decline in cell density (Fig. 4). A somewhat cyclical pattern of changes in cell numbers is observed over time in culture

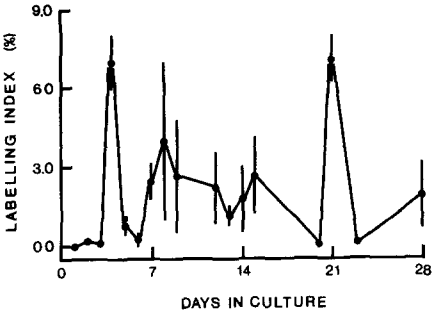

Fig. 7. Changes in the labelling index in autoradiograms of normal human urothelium during explant culture up to 28 days. Each data point represents the mean and SE of 4–9 explants from 1–3 bladders sampled at each time interval. Prior to sampling, cultures were treated with 15 μCi/ml ^{3}HTdR for 6 hours. Significant DNA synthesis is initiated after 3–4 days of culture, but remains highly variable thereafter

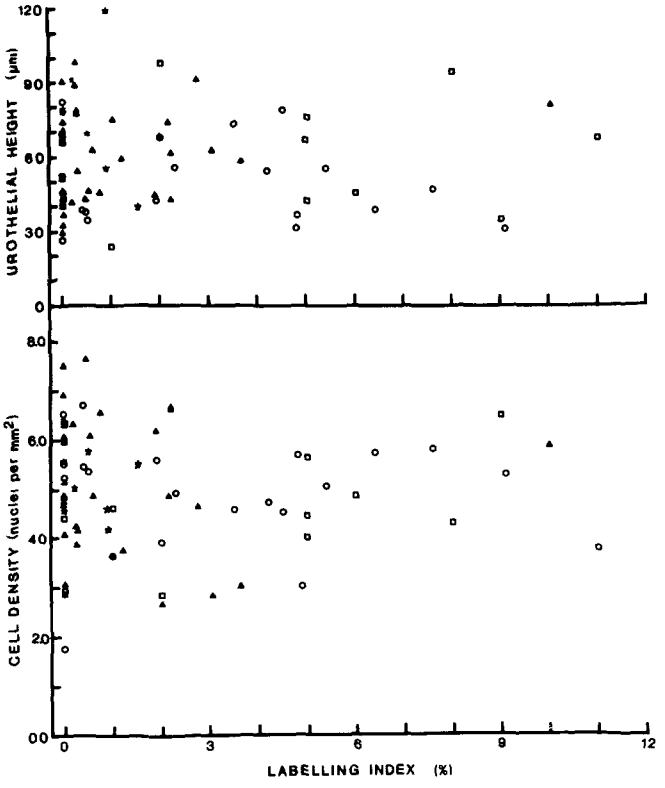

Fig. 8. Urothelial height and cell (nuclear) density of normal human urothelium during explant culture up to 28 days plotted against the labelling index. Data shown are the means from each explant from seven different bladders, represented by the different symbols. No clear relationship exists between the labelling index and either urothelial height or cell (nuclear) density

Prior to culture, the thickness of the urothelium ranged from 40 μm to 100 μm, with a mean value of 65 μm. During the first 1–2 weeks of culture, however, there was a general increase in urothelial thickness, with a maximum of up to 135 μm (Fig. 3) despite the loss of the superficial cell layer. Thereafter, the urothelium varied in thickness within the range observed during the first 1–2 weeks culture.

Despite the increase in urothelial thickness up to 1–2 weeks culture, urothelial cell (nuclear) density sharply decreased over this interval, and thereafter plateaued or

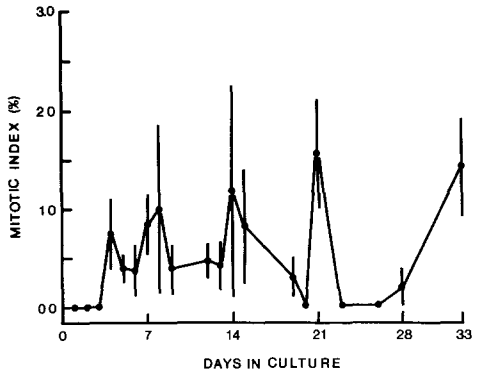

Fig. 9. Changes in the mitotic index in autoradiograms of normal human urothelium in explant culture up to 33 days. Each data point represents the mean and SE of 4–9 explants from 1–3 bladders sampled at each time interval. Prior to sampling, cultures were treated with 1 μg/ml colchicine for 6 h. Significant mitotic activity is initiated after about 4 days culture, but remains highly variable thereafter

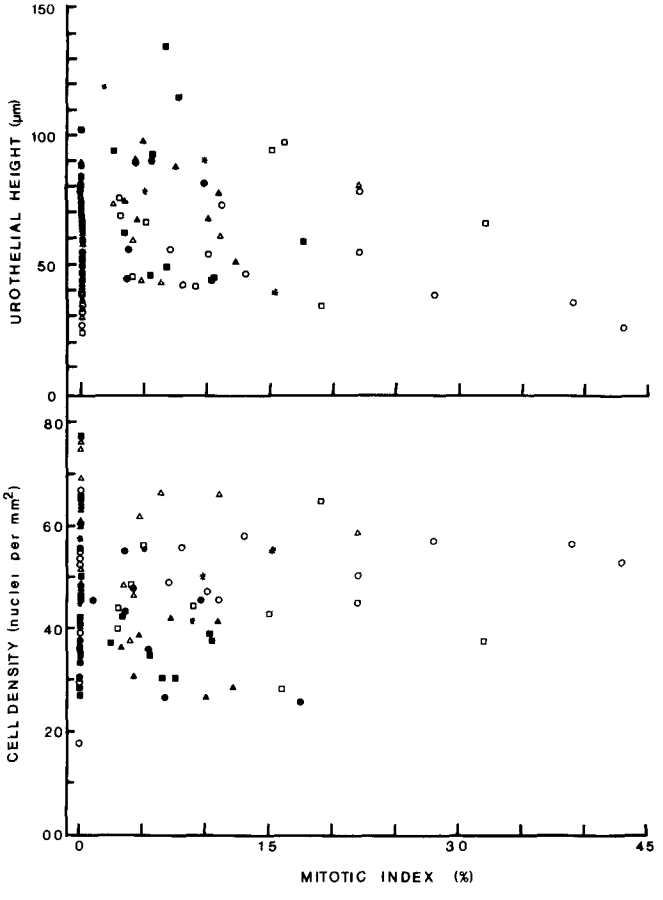

Fig. 10. Urothelial height and cell (nuclear) density of normal human urothelium during explant culture up to 33 days plotted against the mitotic index. Data shown are the means from each explant from seven different bladders, represented by the different symbols. No clear relationship exists between the mitotic index and either urothelial height or cell (nuclear) density, although the mitotic index may be somewhat increased at lower urothelial heights

declined at a slower rate (Fig. 4). A somewhat cyclical variation in cell density was noted, particularly during days 12–33 of culture. The mean cell density per explant over time in culture was 4.7 nuclei/mm^2 and the 99% confidence interval was ± 0.33.

A plot of values of urothelial thickness against cell (nuclear) density revealed a significant ($p < 0.005$) inverse correlation. The equation of the line resulting from a least squares analysis of the data was $y = 124.5–12.13(x)$ ($n = 112$) (Fig. 5). With the decline in cell density with time in culture, there also was a general decrease in the total number of urothelial cells per mm of urothelial length (Fig. 6). The cyclical variation of cell density over time in culture (Fig. 4) is even more apparent when examined in this manner.

A lag of approximately 2 days culture was noted prior to the initiation of DNA synthesis in the explants as determined by ^{3}HTdR uptake. This lag was followed by a peak of DNA synthesis activity around 4 days in culture, which fell to near zero by 6 days of culture. From 7 to 15 days culture, a relatively consistent level of DNA synthetic activity was maintained, and on day 21 of culture a peak of DNA synthetic activity was seen similar to that on day 4 of culture (Fig. 7). DNA synthesis was not related to the height of the urothelium, nor to urothelial cell (nuclear) density (Fig. 8).

There was a lag of about 4 days prior to the initiation of cell division, based on the appearance of mitotic figures. The initial lag period was followed by consistent mitotic activity which lasted until day 15 of culture after which time mitotic activity was highly variable (Fig. 9). Values over this interval were variable, but significantly ($p < 0.005$) correlated with DNA synthetic activity ($r = 0.788$). Mitotic activity seemed to increase somewhat as urothelial height decreased, but remained consistent over the range of cell densities per explant (Fig. 10).

Discussion

In the present study, marked alterations in morphological quantitative parameters were observed in human urinary bladder mucosa during explant culture. Morphologic changes include the loss of the superficial cell layer, an initial increase in urothelial thickness, and reductions in cell (nuclear) density and number of urothelial cells per unit length of the explant urothelium. We have previously reported the loss of superficial cells in explant culture based on the lack of expression of asymetric unit membrane and the presence multiple nuclei [5]. The apparent discrepancy between the increase in urothelial height and reduction in cell density could be explained by an increase in intercellular space, which became more prominent with increasing periods of cultures. This morphologic finding has been reported previously with explant cultures of human tissue [3, 5, 9, 10, 16]. Urothelial height and cell (nuclear) density had a significant inverse correlation, but this correlation

accounts for only 33% of the relationship; the remaining 67% may be related to the decline in cells per unit length of urothelium and individual patient variability. Contributing to this increase in urothelial height and decrease in cell density is the appearance of occasional intraepithelial lumina, which sometimes contain cell debris. The presence of intraepithelial lumina have been noted in previous studies [3, 5, 9, 10, 16]. There may also be a reduction in individual cell size, though no confirmatory quantitative data are available. The decline in cells per unit length of explant urothelium may be due to the migration of cells from the explant into the gelfoam support.

Despite these various modulations, the urothelium remained viable from a morphologic standpoint and also that DNA synthesis continued throughout culture. The major difference compared to 0-time culture was the loss of well-differentiated superficial cells, as noted previously [3, 5, 9, 10, 16]. Knowles et al. [9, 10] report the reappearance of differentiated superficial cells by 14–21 days of culture in supplemented Ham's F-12 medium. CMRL 1066 has higher levels of thymidine, contains other nucleotides, and possesses cofactors necessary for cell proliferation compared to Ham's F-12. The purpose of this study was not to establish a differentiated state, but to maintain human urothelium in a viable state in culture so that future experiments of urinary bladder carcinogenesis can be conducted in vitro.

As further evidence of the viability of explant cultures, DNA synthesis and mitotic activity occurred predominantly in the basal layer of the urothelium as has been previously observed in experimental animals in vivo [6], and in cultured human urothelium in vitro [9, 10]. The absence of mitotic activity before the appearance of labelled cells indicates that human urothelium is composed primarily of cells that are in the G_1 phase of the cell cycle [17]. Increased levels of DNA synthesis and mitotic activity coincide with, or shortly follow, the epithelialization of the cut surfaces of the explant and the subsequent migration of epithelial cells into the supporting Gelfoam sponge. Knowles et al. [9, 10] report that 24 h labelling indices may reach as high as 50% on the cut surfaces of the explant around 7 days of culture. The peaks of the labelling and mitotic indices seen at 4 days culture on the original urothelial surface of the explant probably reflect the time at which migration of cells over the cut surfaces of the explant has been completed, and the original number of cells present has been restored. The lower but variable levels of DNA synthesis and mitotic activity which follow may reflect only maintenance of the urothelium necessary to replace those cells continually lost from the explant by sloughing or continued migration.

Although a low O_2 concentration is usually used for the cultivation of mammalian epithelium in vitro, we have found previously [3, 5, 16] that human urothelium in organ culture grows and is maintained well in an O_2 concentration of 45%. This may be due to increased metabolic requirements and the ability of human urothelium in vitro to detoxify oxygen free radicals (L. Marzella, personal communication). The increased rate of cell division and migration observed in the explants supports this hypothesis.

Most previous reports of explant cultured urothelium have utilized scored culture dishes [2, 3, 5, 8, 18–24] or stainless steel grids [7, 9, 10, 17] as a support medium for the tissue in culture. However, we have utilized Gelfoam rafts in this report and previously [16] because of the avidity with which the explants attach to this material and the ability to identify the luminal surface of the explant reliably following paraffin sectioning. Additionally, this support medium may induce continued cell migration during culture and subsequent DNA synthesis and cell division in the original urothelium.

The cyclical nature of the cell density (Figs. 4, 6) in cultured urothelium is not due solely to interindividual genetic variability because tissue from two or more individuals were sampled at all time points. Because of this fact, and that culture were treated and sampled at approximately the same time at each interval, this cyclical variability is probably due to reasons inherent to the tissue, or culture conditions. It is also noteworthy that the largest increase in nuclear density seen at day 21 of culture in Figs. 4 and 6, coincide with peaks of DNA synthetic activity (Fig. 7) and mitotic activity (Fig. 9). The individuals from whom the data was collected at day 21 did not demonstrate high LI or MI at other times in culture inconsistant with the general trend.

Initially, kinetic data was expressed as a percent of all cells in a given length of urothelium. However, as is well known, mitosis in bladder urothelium is restricted for the most part to basal cells adjacent to the basal lamina [6]. Consequently, the majority of urothelial cells in the urinary bladder are post-mitotic. Therefore, we also expressed kinetic data per unit length of urothelium in an attempt to reduce the variability in the data (not shown). However, the resultant plots were essentially identical to plots of the labeling index or mitotic index over time. Plots of kinetic data as a function of urothelial height or cell (nuclear) density similarly failed to show any clear relationship (Figs. 8, 10). This leads to the conclusion that cell division among basal cells in urothelium of the urinary bladder is random. This conclusion, coupled with the relatively low rates of mitosis and interindividual genetic variation probably account for the wide variability in the data. Thus, in subsequent experimental protocols, given a finite number of explants available for experimental purpose, the number of parameters should be restricted and the frequency of sampling reduced so that additional explants are available for quantitative analyses at each time point for each parameter studied.

The results of previously published preliminary studies on the acute effects of the direct-acting carcinogen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) or the tumor promoter saccharin, failed to show a clear dose response with respect to any of the parameters in the present study at sublethal concentrations [16]. The apparent lack of a

dose-related response to sublethal concentrations of these agents could be due to the variability alluded to above.

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