

In Vitro Culture of Cells Exfoliated in the Urine by Patients with Diabetes Mellitus

CAROL J. DETRISAC, RONALD K. MAYFIELD, JOHN A. COLWELL, A. JULIAN GARVIN,
and DONALD A. SENS, *Departments of Medicine and Pathology, Medical
University of South Carolina, Charleston, South Carolina 29425*

ABSTRACT As an approach to facilitate the understanding of the progression of diabetic renal disease, we assessed the urine of diabetic patients and normal volunteers for the presence of cells that could be cultured in vitro. The results suggest that both normal control subjects and diabetic patients, without clinically detectable microangiopathy, exfoliate few culturable cells into the urine. In contrast, diabetics with documented retinopathy but without nephropathy exfoliate substantially higher numbers of culturable cells (5.2 cells/100 ml urine), whereas diabetics with both retinopathy and advanced nephropathy exfoliate even greater numbers of culturable cells (50.8 cells/100 ml urine). The cells that are exfoliated and culturable can be divided into five distinct cell types based on morphology at the light microscope level. The exfoliated cells proliferate at clonal density after isolation from urine and are epithelial in appearance. These data suggest that the culture of cells from urine might have diagnostic value as an early indicator of diabetic renal disease and provide a convenient, noninvasive new source of human kidney epithelial cells.

INTRODUCTION

The study at a cellular level of renal dysfunction in diabetes mellitus and other disease states is currently hindered by the unavailability of human epithelial cells of defined kidney origin that can be maintained

in vitro. This effectively prevents detailed biochemical analysis of events occurring at the cellular level in both normally functioning and diseased kidneys. We sought a simple, noninvasive method for obtaining epithelial cells, which would have a likely probability of being of kidney origin and would be uncontaminated with fibroblasts. The urine from diabetics with advanced nephropathy was chosen as a source of these cells.

The use of urine as a possible source of epithelial cells for in vitro culture was first described by Sutherland and Bain (1) who demonstrated that viable epithelial cells could be cultured from urine of newborn infants. This and subsequent studies (1-4) demonstrated that the exfoliation of cells was limited to newborn infants during the first few weeks of life and, thereafter, only rarely did viable cell exfoliation occur. These cells were thought to originate from the mucosa of the urinary bladder, although definitive proof was not obtained (4). The epithelial nature of these cells was confirmed by their reaction with antibody against human keratin (4). This study demonstrates that urine from diabetics with advanced nephropathy provides a convenient source of epithelial cells for culture. In addition, the presence of increased numbers of epithelial cells from the urine of patients with early microvascular disease may provide information regarding the progression of the disease state.

METHODS

Study subjects. 19 diabetic patients and 8 nondiabetic volunteer controls were studied. The protocol was approved by the Institutional Review Board for Human Research of the Medical University of South Carolina, Charleston, SC. Of the diabetics, 14 were insulin-dependent and five were non-insulin-dependent. All but one patient were being treated with insulin. Within the diabetic group, eight were

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free of retinopathy (negative direct ophthalmoscopy) and nephropathy (negative urine protein by Albustix (Ames Division, Miles Laboratories, Inc., Elkhart, IN); <300 mg/liter). Six patients had retinopathy (five with background and one with proliferated), but no nephropathy, as determined by repeated negative screening for urinary protein with Albustix. Five had proliferative retinopathy and advanced nephropathy (with a mean excretion of 3.21 ± 1.24 g urinary protein/24 h and a creatinine clearance of <50% of normal). Mean age in the three groups was not significantly different (41 ± 5 , 36 ± 2 , and 38 ± 6 yr, respectively). Duration of diabetes was >10 yr in the latter two groups (13 ± 3 and 19 ± 2 yr, respectively) but was <10 yr in those patients without complications (6 ± 1). Hypertension was present in all patients with nephropathy, in two of those with retinopathy alone, and in four patients with neither retinal nor renal disease.

Collection of urine and cell culture. First voided morning urines were obtained by clean-catch technique into a sterile 1-liter plastic container and processed immediately. The cells from each sample were isolated from the urine by repeated centrifugation with 2–50-ml sterile centrifuge tubes. The urines were centrifuged for 10 min at 800 g, the supernatant removed, and depending on sample volume, new urine was added. The resulting cell pellets were each resuspended into 5 ml of Dulbecco's Modified Eagles' Medium containing 15% vol/vol fetal calf serum and transferred to a 15-ml sterile centrifuge tube and centrifuged. The resulting cell pellet was then resuspended into 10 ml of identical growth media and equally distributed among 10–24-well Costar culture dishes (1 cm² per well; Costar, Data Packaging, Cambridge, MA) with each well containing 1 ml of growth media. The plates were incubated at 37°C in a 5% CO₂:95% air, humidified incubator and fed fresh media every 3 d. The cultures were allowed to grow for 3 wk, at which time they were photographed, fixed, and stained to assess colony formation. Briefly, the growth medium was removed and the cells fixed in calcium acetate-buffered formalin for 20 min at 4°C. The fixed cells were then rinsed with 70% ethanol followed by three rinses with distilled water. The cells were then stained with toluidine blue (0.5 g/100 ml 1% sodium borate) for 2 min and washed with distilled water until residual stain was removed. The wells in which growth had occurred could be readily ascertained visually.

RESULTS

Urine of diabetic patients as a potential source of epithelial cells. To determine whether the urine from patients with diabetic nephropathy would serve as a potential source of epithelial cells, we initially tested the urine of five patients with advanced nephropathy. These patients yielded large numbers (51 cells/100 ml of urine) of viable cells that proliferated from clonal density in vitro. The cells grown from the urine of one of these patients has been extensively characterized. These cells were photographed in situ after 3 wk in culture with an inverted phase-contrast microscope and developed as contact prints. Of 211 photographs examined, five morphologically distinct cell types could be distinguished. Cultures photographed at 3-d intervals throughout the 3-wk growth period revealed that each cell type was unique and did not represent

a change from one cell type to another during growth. A representative photograph of each cell type is depicted in Fig. 1 as well as a photograph of a skin-derived human diploid fibroblast grown under similar conditions. Inspection of the photographs demonstrates that the cells cultured from urine possess a morphology which is quite distinct from that of a fibroblast. The epithelial nature of each of these cell types has been confirmed by ultrastructural examination with electron microscopy, which demonstrates numerous sites of cell-to-cell interaction (i.e., desmosomes, tight junctions, and intermediary junctions).

Several investigations were carried out with these cells. It was observed that optimum plating efficiency occurred when the cell pellets obtained were washed with cell culture medium to remove the last traces of urine. This finding was extended by the demonstration that both normal and diabetic urine were inhibitory to cell growth at concentrations >1%. In no instance was added urine stimulatory to cell growth or noted to increase plating efficiency (tested between 0.1 and 15%). In addition, as the cells proliferated from clonal density (the ability of one cell to initiate proliferation in a single well), it was determined that an equal number of cells proliferated when placed together in a single culture vessel (area equal to 10 cm²) as when plated alone in a single well of a 24-well plate. We also tested a number of different cell culture media for their ability to support cell growth (Ham's F10, Ham's F12, Medium 199, and Minimal Essential Medium) and none were demonstrated to be superior or inferior to Dulbecco's Modified Eagles' Medium. The concentration of fetal calf serum was also tested and 15% was found to be optimal for growth. We tested several urine samples from one of the patients with nephropathy and found that the yield of cells remained within 20% on four first voided urines. In addition, while morning urines were used to obtain larger volumes, cell yields per unit volume of urine remained similar during the first three daily voids. Divided urines, one-half processed immediately and the remainder stored at 4°C, revealed that exfoliated cells were stable in urine for at least 4 h.

Relationship of urinary cell exfoliation to diabetic complications. After the finding of a large number of culturable epithelial cells exfoliated into the urine of patients with nephropathy, urines from 14 diabetics with retinopathy but without clinically apparent renal disease were screened as were patients without microvascular disease. Eight nondiabetic volunteers were also studied. The results depicted in Table I indicate that normal volunteers and patients with diabetes mellitus free of complications exfoliate few culturable cells into their urine. However, uncomplicated diabetics do yield a small but significantly greater number

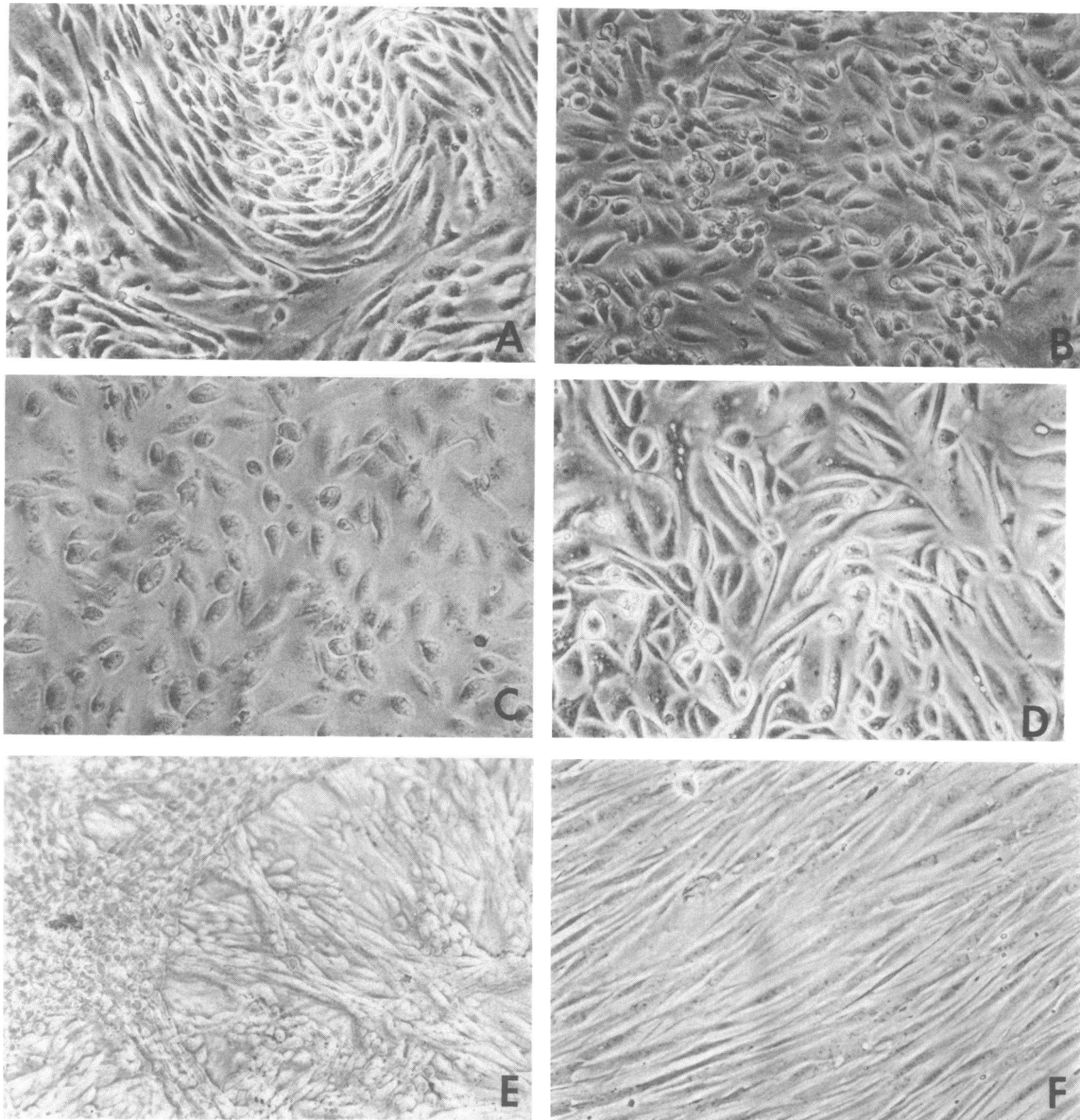


FIGURE 1. Cell types exfoliated by a diabetic with nephropathy. A morning void urine was obtained and cells prepared as described in Table I except that before fixation and staining the cells were photographed (X100) with an Olympus 1M inverted microscope (Olympus Corporation of America, New Hyde Park, NY). The cells obtained from urine were of five morphological distinct types (A-E) and the approximate frequency of appearance in order was 45, 20, 15, 15, and 5 percent. A human diploid fibroblast grown in identical growth media is shown to demonstrate typical fibroblast morphology (F).

of viable cells than do control subjects. Of interest is the finding that patients with retinopathy alone exfoliate an intermediate, but significant, number of culturable cells compared to uncomplicated and nephropathy patients. In the limited number of patients studied, neither the degree of proteinuria nor glycosuria could be correlated with the number of cells exfoliated

within any individual group of diabetics. Urinary cytologic examination was performed on the urines of eight normal volunteers and four diabetics with nephropathy to determine if there was a large difference in total cell content of the urine samples. Although urine from diabetics with nephropathy demonstrated a minority population of morphologically distinct cell

TABLE I
Urinary Cell Exfoliation as a Function
of Diabetic Complications

Patient	n	Urine volume	Cells/100 ml urine
		ml	
Normal volunteer	8	126.3±7.3	0.13±0.12
Diabetic—no complications	8	341.3±16.2	1.3±0.35
Diabetic—retinopathy	6	370.0±21.0	5.2±2.1
Diabetic—nephropathy	5	438.1±35.0	50.8±5.8

The results presented are the mean±SEM. The number of cells exfoliated from normal volunteers compared with diabetics without complications was significant at $P < 0.01$. All other groups compared to any single group were significant at $P < 0.001$. The urine volumes of the volunteer group were significantly different when compared to all diabetic groups ($P < 0.001$). Within the diabetic group, the only significant difference in urine volume occurred between those without complications and those with nephropathy ($P < 0.02$).

types not found in normal urine, there was not a significant difference in total cell content.

DISCUSSION

This study demonstrates for the first time that the urine of diabetic patients with nephropathy contains viable cells that can be cultured *in vitro*. Preliminary data suggests that cell exfoliation into the urine may also serve as an indicator of the progression of diabetic complications. This is suggested by the data demonstrating an increasing gradient of viable cells exfoliated from patients without complications to patients with retinopathy and nephropathy. The origin of the exfoliated cells, although not conclusively shown, can be inferred from indirect evidence to be the kidney. Diabetic patients without clinical nephropathy fail to exfoliate large numbers of cells into the urine, whereas in those with nephropathy, exfoliation is markedly increased. However, the other potential sites of exfoliation within the genitourinary tract, i.e. ureter, bladder, prostate, and urethra, were not excluded, even though these tissues were not obviously diseased in the patients studied. Increased urine flow rate can be excluded as the major factor because diabetics without complications had similar urine outputs as those affected with nephropathy.

The identification of the origin within the kidney of each cell type is currently under investigation. The five cell types possess neither a fibroblastic nor smooth muscle morphology, suggesting that the types exfoliated are all of epithelial origin. This is further confirmed by ultrastructural examination with electron

microscopy, which demonstrates that each cell type has numerous points of cell-to-cell interaction consistent with an epithelial origin. The evidence for the existence of five cell types rests solely on morphological observation. Further proof for the existence of five cell types was obtained by photography at 3-d intervals to insure that growth state and degree of confluency did not influence morphology. In all instances morphology remained unique to each cell type. Final proof of both uniqueness and site of origin will rest on histochemical and immunological (hybridoma) techniques. Continuing studies are somewhat hindered by the finding that although each cell type proliferated from clonal density in serum-containing media, attempted subculture by using trypsin followed by growth in media containing serum results in cell death. We have found (data not presented) that hormone-supplemented serum-free media is necessary to affect subculture and we are currently defining the growth media for each individual cell type.

Studies are in progress to determine if the current finding in patients with diabetes mellitus is unique to this disease state or is more generally applicable to renal disease. Preliminary studies on 20 patients with hypertension have demonstrated that those without proteinuria exfoliate no culturable cells into the urine (16 patients), whereas those with proteinuria, and creatinine clearances similar to diabetic patients with nephropathy, exfoliate an average of five or less cells per 100 ml of urine. No other diseases with associated proteinuria have yet been assessed. Of central importance is the possibility that the time during disease progression that an individual cell type is exfoliated, as well as the number exfoliated, may prove of clinical significance.

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