Assessing the differentiation state of cultured bovine urothelial cells: elevated synthesis of stratification-related K5 and K6 keratins and persistent expression of uroplakin I

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Summary

Although significant progress has recently been made in culturing mammalian urothelial cells, relatively little is known about their biochemical differentiation. In this paper, we assessed the differentiation state of cultured bovine urothelial cells by analyzing their keratins and a cell surface marker, uroplakin I. Urothelial cells were serially cultured either in a serum-free medium, or in a serum-containing medium in the presence of 3T3 feeder cells, with similar results. Despite their stratified appearance, both normal urothelium and cultured urothelial cells synthesize mainly K8, K18 and K19, keratins that are typically seen in simple epithelia. However, cultured urothelial cells synthesize a greatly increased amount of K5 and K6 keratins, which are usually expressed by stratified epithelia but present only in trace amounts in normal urothelium. These data indicate that, as far as keratin synthesis is concerned, cultured urothelial cells undergo an altered pattern of differentiation towards a more 'stratified phenotype'; this unusual finding has interesting implications for urothelial evolution. In the meantime, many superficial cells in cultured urothelial colonies make uroplakin I, a 27×10^3 Mr protein subunit of the asymmetrical unit membrane (AUM) characteristic of urothelial (superficial) umbrella cells. These results indicate that cultured urothelial cells undergo, at least in part, AUM biogenesis. Cultured urothelial cells thus provide a useful experimental model system for studying certain early steps of AUM formation.

Key words: urothelial differentiation, keratins, uroplakin I.

Introduction

Urothelium is a multi-layered epithelium covering the mucosal surface of a large part of the urinary track including the ureter, the bladder and the urethra. This epithelium exhibits several unique biological properties, perhaps the most striking of which is its developmental plasticity. In fact, urothelium provides one of the rare examples in which a postnatal mammalian epithelium can 'transdifferentiate' into a different cell type, in this case, a bona fide prostatic epithelium. This change occurs under the directive influence of embryonic urogenital sinus mesenchyme (Cunha et al. 1983; Neubauer et al. 1983). Such a plasticity is also reflected in its being able to give rise to a bewildering spectrum of tumors, ranging from the more common transitional cell carcinomas, to squamous cell carcinomas, adenocarcinomas and small cell carcinomas (Mostofi, 1954; Melicow, 1974; Koss, 1975; Bryan, 1983). When transplanted ectopically, urothelium exhibits the peculiar property of being able to induce bone formation (Huggins, 1931; Roberts et al. 1974). Finally, urothelium undergoes a unique form of membrane specialization. The apical surface of its superficial umbrella cells is covered with numerous rigid-looking plaques, which, in cross-section, can be seen as an asymmetrical unit membrane (AUM), with its luminal leaflet twice as thick as its cytoplasmic one (Hicks, 1965; Koss, 1969). These plaques also form fusiform vesicles in the cytoplasm of superficial cells, and it has been said that during bladder distention these specialized vesicles can insert into apical membrane and contribute to an increased surface area (Porter and Bonneville, 1964; Hicks, 1966; Minsky and Chlapowski, 1978; Lewis and deMoura, 1984). The molecular bases of these fascinating phenomena are largely unknown, but their analyses could be facilitated if one can grow, under well-defined in vitro conditions, urothelial cells that are capable of reproducing at least in part some of these phenomena.

For this reason, plus the potential usefulness of cultured urothelial cells as a model for studying bladder carcinogenesis, many papers have been published describing the in vitro growth of urothelium. However, despite the significant progress made in the past decade in expanding under in vitro conditions, serially or even clonally, rat and human urothelial cells (Chlapowski and Haynes, 1979; Pauli et al. 1980; Reznikoff et al. 1983, 1987; Wu et al. 1982; Chlapowski et al. 1983; Kirk et al. 1985), very little is known about the differentiation state of such cultured cells. This is largely due to a lack of well-defined biochemical markers of urothelial differentiation. Conse-
quenty, analysis of cultured urothelium has so far been limited to the morphological level, and many uncertainties remain. For example, in general, cultured urothelial cells seem to lose their ability to undergo membrane specialization, since they do not form a recognizable asymmetrical unit membrane (Pauli et al. 1980; Reznikoff et al. 1987). The only known exception was reported by Howlett et al. (1986), who placed a rat urothelial sheet on a collagen gel containing live 3T3 feeder cells. This tissue 'recombinant' was placed at the air/liquid interface. However, even in this case the expression of AUM in the superficial cells was limited. Moreover, since urothelial replication was somewhat limited in this 'organ culture' system it was unclear how much of these mature AUMs were formed de novo.

Recent data suggest that two classes of well-defined cellular proteins can serve as biochemical markers of urothelial differentiation. The first is keratin, which is a group of about 30 water-insoluble cytoskeletal proteins that form intermediate filaments in almost all epithelial cells (Moll et al. 1982; Heid et al. 1986; Lynch et al. 1986). These proteins can be divided into a relatively acidic (Type I) subfamily and a relatively basic (Type II) subfamily. Moreover, each acidic keratin tends to coexpress with a specific basic keratin (K5/K14) are synthesized by the basal cells of all stratified epithelia and may therefore be regarded as markers for basal keratinocytes (Woodcock-Mitchell et al. 1982; Nelson and Sun, 1983; Skerrow and Skerrow, 1983; Stoler et al. 1988). In contrast, K1/K10 keratins are mainly found in the suprabasal layers of keratinized epidermis and can therefore be regarded as markers for skin-type differentiation (Fuchs and Green, 1980; Woodcock-Mitchell et al. 1982; Stoler et al. 1988). Interestingly, under hyperproliferative or other conditions when epidermal cells somehow 'fail' to differentiate normally, the suprabasal cells cease to synthesize K1/K10 and start to make K6/K16 keratins (markers for 'hyperproliferation' or an 'alternative pathway of differentiation'; Weiss et al. 1984; Stoler et al. 1986; Schermer et al. 1989). In the cases of simple epithelia, one tends to find smaller keratins such as K7 and acidic K19 (Moll et al. 1982). These 'rules' thus provide a useful framework for analyzing keratin expression in cultured urothelium.

Another class of newly discovered markers of urothelial differentiation is uroplakin, a group of integral membrane proteins that form the asymmetrical unit membrane (Yu et al. 1990). Using a monoclonal antibody, AE31, which we generated against bovine AUM, we have recently identified a urothelium-specific 27×10^3 M_r protein (uroplakin I; Yu et al. 1990). Since this protein is expressed only in superficial umbrella cells and is associated with the apical (thickened) leaflet of AUM, it provides a marker for an advanced stage of urothelial differentiation (Yu et al. 1990).

In this paper, we describe the in vitro cultivation of bovine urothelial cells and an analysis of their differentiation state. A comparison of the keratins expressed by bovine urothelium in vivo and in vitro revealed an elevated synthesis of K5 and K6 keratins by cultured cells. Meanwhile, most of the superficial cells in such a culture retain their ability to synthesize the 27×10^3 M_r uroplakin I and therefore can apparently achieve a significant degree of membrane specialization. The implications of these results on urothelial biology and the usefulness of cultured urothelial cells as an experimental system for analyzing urothelial differentiation will be discussed.

Materials and methods

Cultivation of bovine urothelial cells in a serum-containing medium

Bovine urinary bladders were obtained from local abattoir and were transported to the laboratory on ice. They were cut open, washed with phosphate-buffered saline (PBS), and the mucosa (containing the epithelium and surface stroma) was dissected from underlying muscles. The mucosal sheets were incubated with trypsin (2.5 mg/ml in 0.5× Dulbecco's modified Eagle's Medium (DMEM) and Ham's F12 medium containing 20% fetal calf serum, hydrocortisone (0.5 μg ml^-1), EGF (15 ng ml^-1), insulin (5 μg ml^-1) and cholera toxin (5 ng ml^-1), in the presence of mitomycin-treated 3T3 feeder cells (cf. Wu et al. 1982; Rheinwald and O'Connell, 1984).

Urothelial cells were subcultured by first spraying the culture with 0.01% EDTA (in PBS) to remove the 3T3 feeder cells and any contaminating fibroblasts, then trypsinizing the remaining urothelial cells with a PBS solution containing 0.1% trypsin and 0.01% EDTA for 30 min at 37°C. The released single cells were replated as above.

Cultivation of bovine urothelial cells in a serum-free medium

Bovine urothelial cells were isolated as above, and were plated in a 1:1 mixture of DMEM and Ham's F12 media supplemented with the following components: hydrocortisone (0.5 μg ml^-1), EGF (15 ng ml^-1), insulin (5 μg ml^-1), cholera toxin (5 ng ml^-1), aldosterone (10 ng ml^-1), Bel-1-estradiol (0.3 ng ml^-1), dihydrotestosterone (15 ng ml^-1), selenium (0.172 μg ml^-1), somatostatin (1 ng ml^-1), T3 (20 μg ml^-1) and testosterone (2.5 ng ml^-1), and transferin (5 μg ml; modified from Detrisac and Moon, 1986). Cells were subcultured as described above.

Keratin analysis

Keratins were extracted from bovine urothelium and cultured urothelial cells as described previously (Cooper and Sun, 1986). Proteins were separated either by SDS–PAGE, or by two-dimensional PAGE in which the first-dimensional separation was nonequilibrium pH gradient electrophoresis and the second dimension was SDS–PAGE. For immunoblotting, protein bands were electroelutically transferred to a sheet of nitrocellulose paper, which was then stained with Past Green to visualize the proteins. After the unoccupied binding sites were blocked by 3% bovine serum albumin in PBS, the paper was stained with various antibodies using the Fast Green/peroxidase–anti-peroxidase technique (Woodcock-Mitchell et al. 1982; Eichner et al. 1984).

Monoclonal antibodies used in this study include: AE1 and AE3 (Woodcock-Mitchell et al. 1982; Tseng et al. 1982; Sun et al. 1984b; Lynch et al. 1985; AE14 (Lynch et al. 1986); AE22 (Locamis et al. unpublished data); 10.11 (Brabon et al. 1984; Cooper et al. 1985); and AE8 (Cooper et al. 1985; Pang et al. unpublished data).

Indirect immunofluorescence staining

Fresh bovine bladder tissues were frozen in O.C.T. embedding medium (Miles Inc.; Elkhart, IN) and cryo-cut into 6 μm sections. Cultured cells were grown on 12 mm plastic coverslips, fixed with cold methanol/acetic acid (1:1) for 10 min, and stored in PBS with 0.1% sodium azide at 4°C. Indirect immunofluorescence staining was carried out as described earlier (Sun and Green, 1978).
Electron microscopy

For conventional electron microscopy, cultured urothelial cells were fixed for 2 h with 2.5% glutaraldehyde and with 1% OsO4, and then processed routinely. For immuno-localization of cell surface epitopes, cultured cells were fixed with Zamboni’s fixative (15% picric acid, 2% paraformaldehyde, PBS) for 2 h, incubated with primary antibodies at 37°C for 1 h, and incubated with secondary antibody conjugated with 5 nm colloidal gold particles. The specimens were further fixed with 2.5% glutaraldehyde and with 1% OsO4 each for 2 h, and then processed routinely.

Results

Serial cultivation of bovine urothelial cells

Although most previous work on cultured urothelial cells has been conducted using human or rat tissues, we chose to study bovine urothelium because: (1) we can routinely obtain large quantities of fresh bovine urothelium, a crucial consideration for the biochemical characterization of relatively minor membrane proteins like uroplakins (Yu et al. 1990); (2) we have previously performed a detailed analysis of bovine keratins and uroplakin I, and have a panel of monoclonal antibodies reactive to these bovine molecules (Cooper and Sun, 1986; Yu et al. 1990). This latter consideration excludes the use of rat or mouse urothelia, since many of our mouse antibodies do not react with rodent antigens.

It has been well established that all stratified squamous epithelial cells (keratinocytes) can be serially cultivated in the presence of lethally irradiated 3T3 fibroblast (feeder) cells in DMEM containing 20% fetal calf serum and a few additives including hydrocortisone and epidermal growth factor (Rheinwald and Green, 1975; Doran et al. 1950; Rheinwald, 1980; Sun et al. 1984a). Under these conditions, bovine urothelial cells also grow well. Even better growth can be achieved, however, in a 1:1 mixture of DMEM and Ham’s F12 medium (Fig. 1). In this medium, dispersed bovine urothelial cells behave similarly to human urothelial cells (Wu et al. 1982) and undergo clonal growth that is totally feeder-dependent (Fig. 1). The morphology of these urothelial colonies (Fig. 2) is similar to that of cultured epidermal cells (Sun and Green, 1976). Young colonies consist primarily of small basal cells, while older colonies occasionally show large, binucleated, squamous cells that appear to have attained a more advanced degree of differentiation (Fig. 2). After an initial period of exponential growth with a doubling time of approximately 24 h, the cells reach a confluent density of roughly 5×10^6 cells/60 mm dish (Fig. 3). In comparison with the number of cells initially plated per dish (~10^4), this represents a population expansion of at least 50 folds, or three to four cell doublings. The confluent cultures can be maintained with a steady-state density of ~5×10^4 cells/60 mm dish for about 2 weeks during which cell proliferation is apparently balanced by the shedding of superficial cells.

Subculturing can be achieved, usually with a higher plating efficiency if carried out shortly before confluency, by removing the 3T3 feeder cells and any contaminating fibroblasts with EDTA followed by trypsinization of the remaining urothelial cells. The released urothelial cells can then be plated under the same conditions, and clonal growth resumes. Typically, with a split ratio of 1:10, we can subculture the cells four to six times; this amounts to a total expansion ratio of 10^3–10^6 folds.

In another series of experiments, we tested various serum-free media and found that the condition previously described by Detrisac and Moon (1986) for growing mouse urothelial cells worked well also for growing bovine urothelial cells. Under these conditions, bovine urothelial cells undergo clonal expansion (Fig. 4). The morphology of these urothelial colonies resembles that of cells grown with serum, except that cells at the advancing edge of the colony can be seen more clearly (since there are no feeder cells obstructing the view) to form ruffles and pseudopods (Fig. 5A). In many colonies, cells at the edge of the colony pile up forming a wall (Fig. 5B and C), and superficial cells in the center of the colony tend to form ‘cornified’ cells (Fig. 5D–G), which are morphologically extremely similar to those seen in cultured epidermal colonies (Sun and Green, 1976).

Under these conditions urothelial cells have a lower plating efficiency and grow more slowly than in serum-containing medium, with a doubling time of about 36 h instead of 24 h (Fig. 3, lower curve). However, cells can reach about the same saturating density (5×10^6 cells/60 mm dish) as in the serum-containing medium. Subculturing can be carried out (at a split ratio of 1:10) for three to four times amounting to a total expansion factor of 10^3–10^6 folds.

Keratin expression

To assess the differentiation state of these cultured urothelial cells, we analyzed their keratins and compared them with those of in vivo bovine urothelium by one- and two-dimensional PAGE coupled with immunoblotting...
Fig. 2. Morphology of bovine urothelial cells cultured in a serum-containing medium in the presence of mitomycin-treated 3T3 feeder cells. Colonies that have grown for 3, 7, 12 and 18 days, as described in Fig. 1, are shown in A–D, respectively. Note that most urothelial cells are relatively small and uniform in size, and are similar in appearance to cultured skin and corneal keratinocytes. Also note mitotic figures and, in older cultures, some large, squamous, superficial cells, some of which are binucleated. Bar, 100 μm.

using a panel of monoclonal antibodies. Despite its stratified morphology, normal bovine urothelium expresses primarily K8, K18 and K19 (Figs 6 and 7A), keratins of the 'simple-epithelial type'. A small but significant amount of K1 (a marker for skin-type differentiation or keratinization) was also detectable by Coomassie blue staining (Fig. 6A, lane 1, and Fig. 7A). In addition, a minute quantity of K5 can be detected by immunoblotting of a grossly overloaded gel using AE3 antibody (Fig. 6C, lane 1). Overall, this keratin pattern is quite similar to that of normal human urothelium, except that human urothelium possesses K13 (an 'esophageal-type' differentiation marker) instead of K1 (Moll et al. 1988).

Cultured bovine urothelial cells continue to make K8, K18 and K19 (Fig. 6A, lane 2; Fig. 7B). In addition, these cells synthesize large amounts of K5 and K6, to the extent that these two keratins are now as abundant as any of the major simple-epithelial keratins (Figs 6 and 7B). Although Coomassie Blue staining showed that K1 keratin is diminished in cultured cells, autoradiographic analysis of keratins synthesized by [35S]methionine-labeled urothelial cells revealed clearly a continued, albeit low level, of K1 (and possibly K2) synthesis (Fig. 7B). These biosynthetic data plus the modulated amounts of K1 in vivo versus in vitro rule out the possibility that the K1 keratin present in our urothelial cytoskeletal preparations is due to stratum corneum contamination.

Immunofluorescent staining of in vivo bovine urothelium and cultured urothelial cells was performed to assess the histological distribution of keratins (Fig. 8). AE1 is a monoclonal antibody known to recognize multiple acidic keratins (Tseng et al. 1982; Eichner et al. 1984; Sun et al. 1984). However, of all the acidic keratins expressed by bovine urothelium both in vivo and in culture, AE1 recognizes only K19 (Fig. 6B). The histological distribution of K19 can therefore be assessed by AE1 staining. In normal urothelium, AE1 stains the superficial umbrella cells strongly and all other cell layers moderately (Fig. 8A). Consistent with this result, cultured cells are AE1-positive, indicating that all urothelial cells contain K19 keratin (Fig. 8B). Staining of normal urothelium with AE3, another antibody that reacts with all known basic keratins (Tseng et al. 1982; Sun et al. 1984b; Lynch et al. 1986), showed strong reactivity in basal cells, and weaker reactivity in all upper cells (Fig. 8C). Cultured urothelial cells, however, are uniformly AE3-positive (Fig. 8D). The staining of urothelium with 10.11, a monoclonal antibody
cell density (~5x10^5 to 7x10^5 cells/60 mm dish) was achieved. Cells grow faster in serum-containing medium, a similar final density of 10^5 cells per dish was obtained in serum-free medium. Bovine urothelial cells (5x10^3 cells per 60 mm dish) were plated either in a 1:1 (v/v) mixture of DMEM and F12 media containing 20% fetal calf serum (with 3T3 feeder; • •), or in a serum-free medium (without 3T3 feeder; see Materials and methods; ○—○). At different time points, urothelial cells were trypsinized and counted after the 3T3 feeder cells and any contaminating bladder fibroblasts were selectively removed by spraying the dishes with a solution of 0.125% EDTA in PBS. Cultures grown in serum-free medium are always free from fibroblast contamination. Note that, although urothelial cells were trypsinized and counted after the 3T3 feeder cells and any contaminating bladder fibroblasts were selectively removed by spraying the dishes with a solution of 0.125% EDTA in PBS. Cultures grown in serum-free medium are always free from fibroblast contamination. Note that, although urothelial cells grow faster in serum-containing medium, a similar final cell density (~5x10^6 to 7x10^6 cells/60 mm dish) was achieved in both growth conditions.

Membrane specialization

Ultrastructural examination of cultured urothelial colonies clearly showed multiple cell layers (Fig. 9B; cf. in vivo urothelium shown in Fig. 9A), indicating that urothelial stratification can occur even when the nutrients come from the above in agreement with Pauli et al. (1988; for a different view, see Chlapowski and Haynes, 1979). The cells are characterized by abundant desmosomes and tonofilaments (Figs 9C and D). Some of the superficial cells appear necrotic, and possess an electron-dense submembranous domain similar to cornified envelopes (Figs 9B and E) seen in the superficial cells of other more typical stratified epithelia (Sun and Green, 1976; Green, 1977). Most other superficial cells, however, appear healthy (Fig. 9C). The apical surfaces of these cells are populated with numerous microvilli covered with glycocalyx-like material (Fig. 9C). No apical plaques or asymmetrical unit membranes were discernible. These morphological features are therefore quite similar to those observed in vivo in carcinogen-treated as well as in hyperplastic urothelium (Hicks and Wakefield, 1976; Norman et al. 1987).

Although no mature AUM plaques are detectable, many superficial cells are stained strongly by AE31 (Fig. 10) indicating the presence of the 27 x 10^6 M_r subunit of AUM (uroplakin I; Yu et al. 1990). Since in vivo this protein is limited to the superficial umbrella cells (Figs 10A and B; Yu et al. 1990), its presence in many of the superficial cells in cultured urothelial colonies suggests that such cells must have achieved an advanced stage of membrane specialization (Figs 10C and D). This conclusion is supported by the observation that AE32, another monoclonal antibody that we raised recently against a 85 x 10^6 M_r urothelial glycoprotein characteristic of umbrella cells, also stains many superficial cells of cultured urothelium (Yu et al. unpublished data). Ultrastructural localization data showed that both AE31 and AE32 antigens are associated with the apical surface of superficial cells (Fig. 11 and Yu et al. unpublished data).

Discussion

A 'reversed' mode of urothelial differentiation?

It is well known that the program of keratin expression can be perturbed when stratified epithelial cells are placed in a different growth environment (Fuchs and Green, 1978; Doran et al. 1980; Eichner et al. 1984). Therefore, the finding that cultured urothelial cells synthesize keratins different from in vivo urothelium is not surprising. What is unexpected, however, is that cultured urothelial cells turn on large amounts of keratins that are larger in size than their normal major keratins (Fig. 6). This is in sharp contrast to the general pattern that cultured stratified epithelia over-produce mainly smaller, presumably more...
primitive keratins (Wu et al. 1982; Sun et al. 1984b; Schermer et al. 1986, 1989).

Related data were obtained by Moll et al. (1988), who recently demonstrated that K13 keratin is present mainly in the 'undifferentiated' basal cells and a few intermediate cell layers of human urothelium. This is quite different

Fig. 5. Morphology of bovine urothelial cells cultured in a serum-free medium. Approximately $5 \times 10^4$ cells were plated per 60 mm dish and grown for 2 weeks. (A) Edge of an expanding colony showing membrane ruffling, a feature also commonly seen in colonies growing in serum-containing medium (cf. Fig. 2A and B). Note the absence of feeder cells. (B) and (C) Edges of two large colonies exhibiting 'thickened' or heavily stratified borders. The formation of the thickened borders by cultured normal urothelial cells suggests that this feature is unrelated to malignancy (cf. Chlapowski et al. 1983). (D) Formation of some superficial cells with peculiar linear cytoplasmic patterns. Similar cells have been seen in cultured skin, corneal and thymic keratinocytes (Sun et al. 1984b, and unpublished observations). (E)–(G) Formation of some superficial squamous cells with necrotic nuclei; these cells are morphologically similar to the cornified cells frequently seen in cultured skin keratinocytes (Sun and Green, 1976; Green, 1977). Bar, 100 $\mu$m.
hydrates are present in the basal cells; these carbo-
thelium. Note the presence of K8, K16/K17, K18 and K19 in both in vivo urothelium and cultured urothelial cells. Also note the presence of K1 keratins in urothelium, and its diminished presence in cultured urothelial cells. In contrast, K5 and K6, which are barely detectable in vivo, are greatly increased in culture. (B) AE1 (known to react with many acidic keratins). Note the strong staining of K19. (C) AE3 (known to react with all basic keratins). Note the strong staining of K1, K5, K6, K8 and some lower Mr bands (presumably degradation products). (D) AE14 (known to react with K5 and some high sulfur, hair-related proteins). Note the selective staining of K5 in cultured cells, with very little staining of the same region in lane 1. (E) AE23 (known to react predominantly with K1). Note the detection of K1 mainly in vivo. (F) 10.11 (reacts with K18). Note the weak staining of K18 in vivo. (G) AE8 (K18). Note the detection of K18 in bovine urothelium, both in vivo and in culture. Lanes 3 and 4 are controls showing the Fast-green-staining and AE8-staining patterns, respectively, of cow esophageal keratins.

from other K13-expressing stratified epithelia in which K13 is mainly associated with suprabasal cells and is barely detectable in the basal layer (Van Muijen et al. 1986; Franke et al. 1986). On the basis of this unexpected K13 expression pattern, Moll et al. (1988) suggested that urothelial differentiation proceeds in a 'reversed' direction as compared with other K13-expressing stratified epithelia.

In addition, Orntoft et al. (1987, 1988) observed an unusual distribution of blood group antigens in bladder epithelium, with complex antigens like LeY and ALey in all cell layers, and a unique expression of most of the less complex antigens in the luminal cell layer. This distribution is distinctly different from those of epidermis and oral epithelia, in which the simple blood group carbohydrates are present in the basal cells; these carbohydrates are then extended by the addition of monosaccharides as the cells mature, leading to the formation of more complex structures in the upper cell layers (Orntoft et al. 1988).

Together, these results establish a unique pattern of urothelial differentiation in which a 'more complex' basal cell phenotype is expressed as early as K13 expression, and this phenotype is maintained through all layers of the urothelium. Such differentiation is apparently hindered in cultured urothelial cells, which actually undergo elevated proliferation and ultimately exhibit a 'reversed' differentiation pattern, presumably for functional gains such as improved stretchability. This may explain why the K5 and K6 keratins are no longer expressed in normal urothelium but can be turned on in cultured urothelium.

Our hypothesis that urothelium evolved in a sequence of 'a simple epithelium—a relatively typical stratified epithelium (K13+, K5+, K6+, complex sugar)—an unusual stratified epithelium with superficial cells possessing some simple epithelial features (K13+, K5+, K6+, simple sugar),' although highly speculative, provides a conceptual framework that can explain at least some of the enigmatic properties of urothelium, e.g. the behavior of cultured urothelial cells and the 'reversed' differentiation as defined by some markers. This hypothesis can be tested in part by comparing the keratin patterns of urothelia in various mammalian species to see whether stratified keratins may be more predominant in lower species. Alternatively, one can analyze keratin changes during urothelial development to see whether K5 and K6 keratins are expressed transiently during a particular period of embryonic development.

Urothelial differentiation: a hypothesis

A possible explanation for these paradoxical observations is that urothelial cells may revert to a more 'primitive' phenotype when they are placed in a cell culture environment designed to encourage hyperproliferation (for a related discussion on keratinocytes, see Schermer et al. 1988). Normal urothelium is one of the slowest self-renewing tissues in the body, with a labeling index of 0.2% to 1% (Messier and Leblond, 1960; Locher and Cooper, 1970; Martin, 1972). In comparison, cultured urothelial cells, with a doubling time of 24-48 h (Fig. 3), are clearly hyperproliferative. According to this hypothesis, the keratin pattern of cultured urothelial cells may reflect those of primitive urothelium, which may at one time have synthesized a large quantity of K5 and K6 keratins but later evolved to shut off these two genes during normal differentiation, presumably for functional gains such as improved stretchability. This may explain why the K5 and K6 keratins are no longer expressed in normal urothelium but can be turned on in cultured urothelium.

Urothelial differentiation
Fig. 7. Analyses of urothelial keratins by two-dimensional immunoblotting. The water-insoluble, cytoskeletal proteins of: (A) in vivo bovine urothelium, and (B) cultured urothelial cells were separated by two-dimensional PAGE, transferred electrophoretically to nitrocellulose paper, and stained (A and B) with Fast Green to reveal total protein patterns. (A') The in vivo proteins were additionally stained immunochemically with a mixture of AE1, AE3 and alF antibodies (MaK for mouse anti-keratin antibodies) to identify intermediate filament proteins. (B') Cultured cell proteins (prepared from [35S]methionine-labeled cells) were visualized by autoradiography to determine whether K1 keratin is biosynthetically labeled. Arrows marked 1 and 2 denote the directions of the first-dimensional charge separation (NEpHGH) and 2nd-dimensional SDS–PAGE separation, respectively (see Materials and methods). The downward, thick arrow marks a side lane containing an identical sample resolved only during the second-dimensional separation; this is included to facilitate the correlation between the one- and two-dimensional gel patterns of the keratins. B and P in A and B are protein standards bovine serum albumin and 3-phosphoglycerate kinase, respectively.

Possible significance of K1 expression in urothelium
Normal human urothelium expresses K13 in its basal and intermediate cell layers (Moll et al. 1988). Bovine urothelium is different in that it lacks K13 but contains a small but significant amount of K1 (Figs 6 and 7). This high molecular weight keratin has previously been shown to be expressed in suprabasal cells of the keratinized epidermis, and has sometimes been referred to as a marker for keratinization (Fuchs and Green, 1980; Woodcock-Mitchell et al. 1982; Skerrow and Skerrow, 1983; Tseng et al. 1984; Cooper et al. 1985). The detection of this keratin in a non-keratinized urothelium is therefore unexpected. A trivial explanation is that this keratin may come from human stratum corneum, which frequently contaminates solutions and glassware (Ochs, 1983). We deem this unlikely, however, because the amount of this keratin is greatly reduced in samples of cultured urothelial keratins prepared in parallel (Fig. 6). Moreover, cultured urothelial cells can incorporate [35S]methionine into a small, but still significant, amount of K1 (Fig. 7B'). These results strongly suggest that bovine urothelial cells can make K1 both in vivo and in culture. The finding that even under culture conditions that usually discourage keratinization (Fuchs and Green, 1978; Eichner et al. 1984) urothelial cells can still make a significant amount of this keratin is surprising, and may be related to the known propensity of urothelium to undergo frank orthokeratinization during vitamin A deficiency (Hicks, 1969; Molloy and Laskin, 1988).

Uncoupled expression of K5 and K14
Although under normal circumstances K5 and K6 tend to coexpress with K14/K15 and K16, respectively, cultured urothelial cells provide a clear example that this coexpression can be uncoupled. Other examples of uncoupled expression of normally 'paired' keratins include K3 and K12. When cultured rabbit corneal epithelial cells reach confluence K3 keratin expression precedes K12 expression (Schmer et al. 1986). Similarly, Roop et al. (1987) observed that expression of K1 precedes that of K10 in cultured mouse epidermal keratinocytes that were induced to differentiate by high levels of calcium. Uncoupled expression of K4 and K13 keratins has also been noted (Moll et al. 1982; Van Muijen et al. 1986). Finally, Dhonauilly and coworkers have recently found that under certain conditions K12 expression can precede K3 (D. Dhonauilly et al. unpublished data). These results indicate that the paired expression of specific acid and
Fig. 8. Keratin localization in normal bovine urothelium (A, C, E, G) and cultured urothelial cells (B, D, F, H). Monoclonal antibodies used include: (A) and (B) AE1 recognizing K19 (see Fig. 6B); (C) and (D) AE3 specific for all basic keratins; (E) and (F) 10.11 antibody specific for K18 of simple epithelia; (G) and (H) AE14 specific for K5. For the staining of in vivo tissue, frozen sections (6 μm thick) of bovine bladder were used. Cultured urothelial cells were grown on glass coverslips, fixed and permeabilized with cold methanol/acetone, followed by immunofluorescent staining. Bar, 50 μm.

basic keratins is not rigidly coupled and that there is no obligatory order of expression.

It is unclear which acidic keratins copolymerize with the overproduced K5 and K6 keratins in cultured urothelial cells, but it is possible that K18 and/or K19 can fulfill such a role (Schermer et al. 1986).

AUM maturation
As mentioned earlier, the apical surface of urothelium is covered with numerous plaques composed of asymmetrical unit membrane (AUM). Detailed ultrastructural analyses showed that these AUMs are composed of semicrystalline, hexagonal arrays of 12 nm protein particles (Hicks and
Ketterer, 1969, 1970; Vergara et al. 1969; Warren and Hicks, 1970; Brisson and Wade, 1983; Taylor and Robertson, 1984). The protein composition of AUM has been studied extensively and a large number of putative protein subunits have been identified (Ketterer et al. 1973; Vergara et al. 1974; Caruthers and Bonneville, 1977; Stubbs et al. 1979; Trejosiewcz et al. 1984). However, whether any of these proteins are truly AUM-associated in situ has not yet been conclusively demonstrated. Using a monoclonal antibody (AE31) against bovine AUM, we have recently identified a $27\times10^3 M_r$ protein that can be localized on the luminal (thicker) leaflet of apical AUMs.
Fig. 10. Cultured urothelial cells express AE31 antigen, a marker for an advanced stage of urothelial differentiation. (A) A frozen section (6 µm) of bovine bladder mucosa; phase-contrast image. (B) The same field as in A demonstrating the immunofluorescent staining of superficial umbrella cells by a mouse monoclonal antibody, AE31. This antibody recognizes a 27×10^3 Mr protein subunit of the asymmetrical unit membrane, a urothelium-specific membrane specialization (Yu et al. 1990). Arrows and broken line denote the epithelial-mesenchymal junction. (C) and (D) A confluent, primary culture of bovine urothelial cells double-stained using: C, a rabbit antiserum to keratin, and D, mouse antibody AE31. Note that a subpopulation of the keratin-positive cells, usually superficially located, are AE31-positive. Bar, 50 µm.

(Yu et al. 1990). This protein is urothelium-specific; it is differentiation-dependent, as it is restricted to the superficial umbrella cells; it is relatively acidic (pl ~5.8); it partitions into the detergent phase during Triton X-114 phase separation; and it forms a stable complex with a 15×10^3 M_r and a 47×10^3 M_r protein (Yu et al. 1990). On the basis of its association with urothelial plaques, we have named this 27×10^3 M_r protein uroplakin I (Yu et al. 1990).

Many superficial cells in our subcultured urothelial colonies stain positively with AE31. Since these cells have undergone a significant degree of in vitro replication (>10^3 folds), the uroplakin I in these cells is almost certainly de novo synthesized. This conclusion is supported by our recent observation that in these cells uroplakin I can be biosynthetically labeled with [35S]methionine (Yu et al. 1990). Interestingly, these uroplakin-positive cells do not form morphologically recognizable AUMs (Fig. 9). This could mean that cultured urothelial cells do not make enough uroplakin I to form such a structure. Alternatively, these cells may lack additional protein subunits (e.g. the 15×10^3 M_r and/or the 47×10^3 M_r protein), or are deficient in other aspects of membrane maturation (e.g. lipid changes) that are necessary for later steps of AUM assembly. Experiments are in progress to distinguish between these possibilities.

The ultrastructure and anti-keratin staining properties of superficial umbrella cells are strikingly different from those of the underlying cell layers (Ramaekers et al. 1985; Moll et al. 1988; Schaafsma et al. 1989). In addition, these umbrella cells apparently can divide in response to wounding (Walker, 1960; Levi et al. 1969). On the basis of these observations, one may question whether these umbrella cells belong to a compartment separate and independent from the underlying cell layers (see, e.g. Schaafsma et al. 1989). This is an important issue because, if proved to be correct, this will fundamentally alter our concepts on urothelial homeostasis and differentiation. In our culture system, we found that small urothelial clones containing no AE31- or AE32-positive cells can later give rise to many superficial cells that express both AE31 and AE32 antigens that are characteristic of in vivo umbrella cells. Thus, at least in our culture conditions basal cells can readily generate umbrella-like cells. This finding, plus the extremely low labeling index of the umbrella cells, makes it highly unlikely that under normal circumstances...
Cultured urothelial cells. Primary cultures of bovine urothelial cells were grown for 12 days, washed with PBS, and incubated with the AE31 antibody. The cells were then washed, fixed, and further processed for the immunolocalization of the bound antibodies. Note the linear but sparse labeling of the apical cell surface by AE31 antibody. Bars, 0.25 μm in A; and 0.08 μm in B.

umbrella and lower-layered cells belong to two separate cellular compartments.

Cultured urothelial cells as a differentiating system

In this paper, we have shown that cultured bovine urothelial cells can undergo a significant degree of population expansion (10^2-10^7 folds; Figs 1-5). These cultured cells thus provide an ample source of fresh cell populations expansion (10^3-10^7 folds; Figs 1-5). These cultured cells express greatly elevated levels of K5 and K6 keratins, suggesting that they undergo an altered pattern of differentiation towards a more 'stratified phenotype' (Figs 6-8). We have also shown that these cultured urothelial cells provide a useful model system for studying the biosynthesis of uroplakin I and possibly some initial steps of AUM assembly (Figs 9-11; also see Yu et al. 1990). Further improvement of the culture system by, e.g. lifting cultured urothelium to the air/liquid interface (Howlett et al., 1986), may lead to a more complete maturation of AUM, thereby allowing studies on some later steps of AUM formation. Finally, these cultured urothelial cells should be useful as a transformation host for cDNAs and genes coding for AUM proteins. Such transformation experiments may unravel the mechanisms of targeting and transport of AUM proteins to the apical surface. These experiments should also yield useful information regarding the cis- and trans-controlling elements that are responsible for the urothelium specificity and differentiation-dependent expression of uroplakins.

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