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Membrane specialization and synchronized cell death in developing rat transitional epithelium

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INTRODUCTION

The transitional epithelium lining the mammalian urinary bladder has a much lower permeability to water and small charged ions than is found in other mucous membranes (Johnson, Cavert, Lifson & Visscher, 1951; Marucci, Shoemaker, Wase, Strauss & Geyer, 1954; Englund, 1956; Blandy, 1964; Hicks, 1965, 1966*a*; McIntyre & Williams, 1969). Bladder impermeability is a passive phenomenon, not dependent on tissue respiration, as is reflected by the sparseness of mitochondria in the epithelium (Walker, 1960; Hicks, 1965). The luminal surface of the superficial cells is limited by an unusually thick, angular plasma membrane (Hicks, 1965; Porter, Kenyon & Badenhausen, 1967; Koss, 1969) and damage to, or modification of, this membrane results in a greatly increased flux of water and ions across the bladder wall. The evidence suggests that this specialized membrane is the barrier to exchange of water and ions between urine and intracellular fluid (Hicks, 1966*a*; 1969). In the adult rodent there is a turnover of thick membrane in the long-lived superficial cells, new membrane being assembled in the Golgi complex and migrating to the luminal surface in the form of fusiform vacuoles (Hicks, 1966*b*; Koss, 1969; Lavin & Koss, 1971).

The formation of the luminal barrier membrane in the fetus and neonate has not been described. An earlier histological investigation of the development of polyploidy in the superficial cells of young mouse transitional epithelium did not extend to ultrastructural observations (Walker, 1958). In the present paper the development of the urothelium in the fetal and neonatal rat is described, with particular reference to the differentiation of the thick luminal membrane. A more detailed account is also given of a previously reported example of cell death in the perinatal period (Firth & Hicks, 1970).

MATERIALS AND METHODS

Between the fifteenth and final days of gestation, pregnant albino rats were killed by dislocation of the neck. The fetuses were removed and their bladders fixed. Suckling rats were killed by decapitation.

Electron microscopy

Bladders were fixed for 6 hours in cacodylate-buffered glutaraldehyde-formaldehyde (Karnovsky, 1965), washed overnight, postfixed in phosphate-buffered osmium

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Fig. 1. Bladder epithelium in a two day old rat. Superficial cells show a variable loss of cytoplasmic density and some ghost cells (*G*) are present. Toluidine blue. × 600.

Fig. 2. Bladder epithelium in a three day old rat. The undamaged basal cells (*B*) are covered by dense, necrotic superficial cells. Toluidine blue. × 770.

tetroxide (Millonig, 1961), dehydrated in ethanol and embedded in Epon. Thin sections were cut, stained with uranyl acetate and lead citrate, and examined in a Philips EM200 electron microscope.

Enzyme cytochemistry

Bladders were fixed in cold cacodylate-buffered 4 % glutaraldehyde for thirty minutes and then rinsed overnight in cold cacodylate-buffered sucrose. 40 μm frozen sections were cut with a freezing microtome, transferred to staining media, and incubated at 37 °C.

Acid phosphatase activity was demonstrated by the modified Gomori method (Gomori, 1952; Holt & Hicks, 1961). Incubations for thiamine pyrophosphatase and inosine diphosphatase activities were carried out in tris-maleate-buffered media at pH 7·2 (Novikoff & Goldfischer, 1961). Substrate blanks were prepared for all incubations.

The tissue slices were rinsed in cold buffer, postfixed in osmium tetroxide, dehydrated in ethanol, and embedded in Epon. Thin sections were cut and stained with uranyl acetate.

Light microscopy

1 μm Epon sections were stained with toluidine blue. Lead phosphate deposits from cytochemical reactions were demonstrated by pre-treatment with warm, dilute ammonium sulphide solution.

All aldehydes and embedding materials were obtained from TAAB Laboratories, Reading, and substrates for cytochemical techniques were supplied by Sigma Chemical Co. Ltd., London.

RESULTS

Histology

On the fifteenth day of gestation the fetal bladder was lined by a single layer of cuboidal cells, many of which were in mitosis. During the next three days these proliferated to form a double layered epithelium with many binucleate superficial cells. Between the nineteenth day of gestation and the second day after birth, scattered superficial cells lost cytoplasmic density and their nuclei swelled. These changes rapidly extended to involve the entire superficial layer (Fig. 1). Many cells were reduced to 'ghosts', which then desquamated into the bladder lumen. In some animals the superficial cells, after first appearing less dense, later shrank down to densely staining, flattened remnants (Fig. 2).

Within a few days the necrotic superficial layer of cells desquamated in adherent sheets to leave a single layer of basal cells. These appeared to remain unaffected, and proliferated to regenerate a new superficial layer by the middle of the second week after birth. A third cell layer was established in the third week, and differentiation into the adult type of transitional epithelium appeared complete. Characteristically, the superficial cells were larger than the rest, more frequently binucleate, and contained densely staining cytoplasmic granules 1·0–3·0 μm in diameter.

Fig. 3. Superficial epithelial cell in a sixteen day fetus. The luminal plasma membrane is not thickened and the cell does not have the angular profile typical of adult superficial cells. $\times 55000$.

Fig. 4. Epithelial cells in a twenty day fetus. The luminal profile is more angular, and a few cytoplasmic fusiform vacuoles (*f*) are present. $\times 25000$.

Ultrastructure

Cell proliferation; 15th to 19th days of gestation

The single layer of cuboidal cells found on the fifteenth day of gestation was morphologically rather undifferentiated. The luminal plasma membrane was unthickened and its unit structure was symmetrical. The luminal surface was covered with blunt, irregular microvilli (Fig. 3). The Golgi complex showed no thickening of cisternal membranes and there were no cytoplasmic thick-membraned vacuoles. Near their luminal margins, the lateral surfaces of the cells were joined by junctional complexes. The epithelium was separated from the lamina propria by a thin, uniform basal lamina.

As a second layer of cells developed between the sixteenth and nineteenth days of gestation, the superficial cells showed some differentiation. The Golgi cisternae occasionally had thickened plaques of membrane, and a few fusiform vacuoles were observed in the apical cytoplasm (Figs. 4, 5). Where the luminal membrane appeared thicker, the cell surface showed an angular profile similar to that of the adult superficial cell.

Necrosis and desquamation; perinatal period

Nuclear and mitochondrial changes were the first indication of superficial cell necrosis. The nuclei became progressively more swollen, the chromatin was coarsely granular, and nucleoli showed segregation into dense granular and less dense fibrillar components.

Mitochondria were also swollen, with reduced matrix density and disrupted cristae (Fig. 6). Little other cytoplasmic damage was detectable at this stage, and the basal cells remained structurally normal in all respects.

As necrosis progressed the cytoplasmic matrix became very electron-lucent. Some of these cells with a rarified matrix desquamated, while others collapsed to become thin and very electron-dense. The Golgi complex retained apparent structural integrity, and cytochemical staining for thiamine pyrophosphatase (Fig. 7) and inosine diphosphatase (Fig. 8) showed both these enzymes to be active and still confined to the Golgi complex.

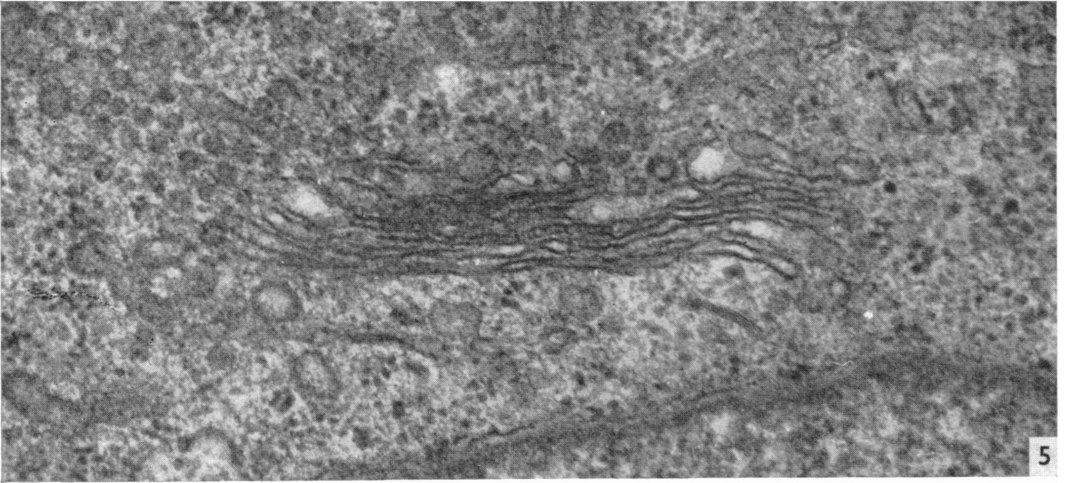
The necrotic cells desquamated in adherent sheets, with some rupturing of plasma membranes in both cell layers. Damage to basal cell membranes appeared to be repaired rapidly, and no other abnormal features were found in this layer.

Cytochemical staining for acid phosphatase confirmed that few lysosomes were present; no increase in activity of this enzyme appeared in necrotic cells.

Proliferation and establishment of an adult-type transitional epithelium; postnatal period

Following desquamation of the dead superficial cells, a new superficial layer was formed by mitosis of the basal cells during the second week after birth. This layer had a few characteristics of differentiated superficial cells, including an angular surface profile and a few cytoplasmic fusiform vacuoles (Fig. 9).

A triple layer of cells, in which the superficial ones showed features characteristic of maturity, was established by the end of the third week after birth (Fig. 10). The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large lysosomes found in adult superficial cells were among the last features to appear; the cytochemically demonstrable lysosomes seen during the third week were relatively small and inconspicuous (Fig. 11).

DISCUSSION

In the adult rat, the assembly of specialized luminal membrane in the Golgi complex of the superficial cells of transitional epithelium has been described both in the intact bladder (Hicks, 1966*b*; Koss, 1969), and in cultured cells (Lavin & Koss, 1971). Formation of fusiform vacuoles of thick membrane occurs both in the intermediate and superficial cell layers, although insertion of thick membrane into the cell surface is only seen in the superficial layer (Hicks, 1965, 1966*b*; Koss, 1969).

In the fetus, fusiform vacuoles appear concurrently with thickening of the luminal surface membrane. At this stage the number of vacuoles is small, and those that are present lie close to the luminal surface; they may be derived from either invaginations of the plasma membrane or from the Golgi complex. The presence of thick membrane in the Golgi complex is a late event in the development of the first generation of superficial cells, and reorganization and differentiation of the molecular structure of the membrane may occur on its way to, or even at, the surface of the cell. A similar situation probably prevails early in the life of the second superficial cell generation, and may relate to the rate at which the membrane is being synthesized and incorporated into the cell surface. Substantial accumulations of fusiform vacuoles are not apparent until several weeks after birth, by which time the rate of cell division has decreased and a steady turnover rate for the cell membrane has probably been established. In the adult bladder, breakdown of thick membrane occurs in the large lysosomes; if the rate of synthesis of membrane exceeds the rate of breakdown, maturation of the membrane will have time to be completed in the Golgi cisternae and the fusiform vacuoles, and the latter will accumulate in the cytoplasm.

During the perinatal period, cell death occurs throughout a population of well differentiated superficial cells. This effect is highly specific, and the less differentiated basal cells remain undamaged. This contrasts with another pattern of cell death during development, in which sporadic necrosis of scattered cells is observed (Manasek, 1969). The possibility that synchronous death of all the superficial cells could be caused by a change in urine composition at birth is not considered likely, as the basal cells are not injured on contact with the urine. Moreover, necrosis sometimes occurs before birth. This may therefore be an example of developmentally determined cell death (Lockshin & Williams, 1965*a*, *b*). Since Glücksmann's comprehensive survey of cell death during development (1951), many studies of the process have been carried out (Zwilling, 1961; Saunders, Gasseling & Saunders, 1962; Weber, 1962; Menkes, Deleanu & Ilies, 1965; Hinchliffe & Ede, 1967; Ballard & Holt, 1968; Hourdry,

Fig. 5. Golgi cisternae with plaques of thickened membrane in a superficial cell of a twenty day fetus. × 52000.

Fig. 6. Epithelial cells in a three day old rat. A necrotic, binucleate superficial cell is partially separated from underlying basal cells (*B*). The nucleus (*n*) is swollen, the nucleolus (*no*) has segregated and mitochondria (*m*) are swollen and vacuolated with damaged cristae. × 12000.

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Fig. 7. Thiamine pyrophosphatase activity in the Golgi complex of a necrotic superficial cell from a three day old rat. × 32000.

Fig. 8. Inosine diphosphatase activity in the Golgi complex of a necrotic superficial cell from the animal illustrated in Fig. 7. × 34000.

Figs 9 and 10. For legends see next page.

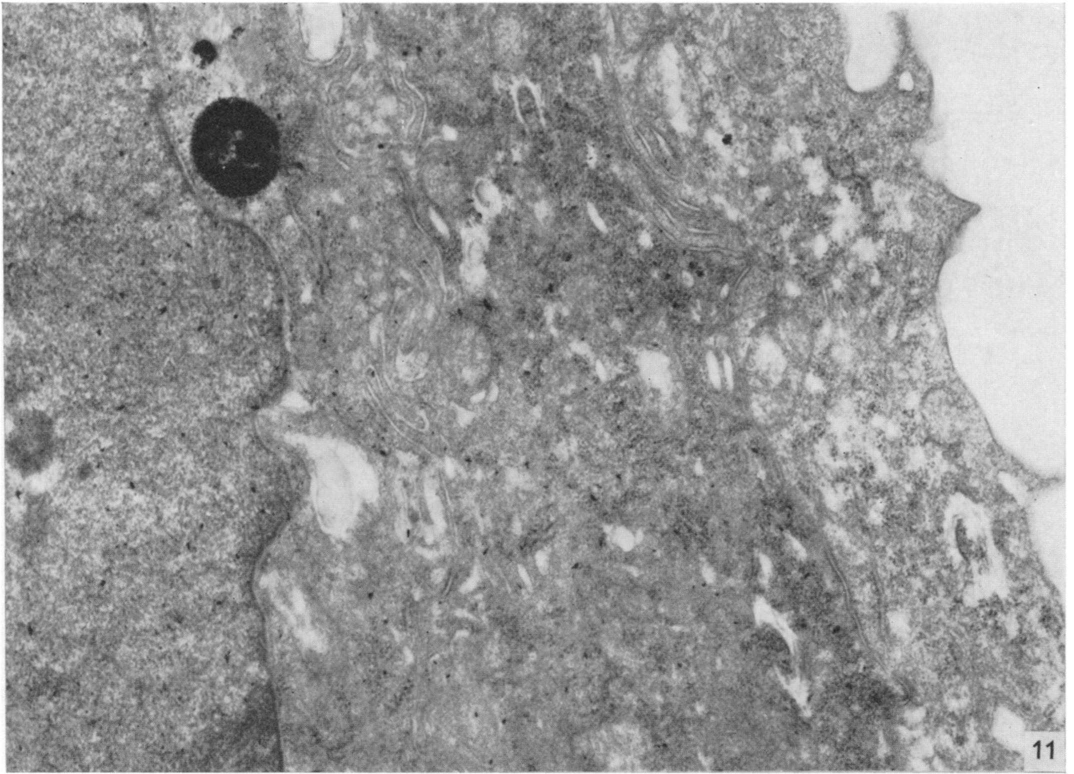

Fig. 11. Eighteen day old rat. A medium sized lysosome in a superficial cell, stained foi acid phosphatase activity. Large lysosomes of the mature type are not apparent until after the third week. × 28000.

1969; Fox, 1970). Lysosome-mediated autolysis has sometimes been suggested as the immediate cause of necrosis, and has been clearly demonstrated in the metamorphosis of insects (Lockshin & Williams, 1965*a*, *b*). However, the evidence in vertebrate systems has not generally supported this theory (Salzmann & Weber, 1963; Ballard & Holt, 1968). Where lysosomal changes are seen, they are probably an autophagic response to damage already initiated (Novikoff & Holzman, 1970).

Mitochondrial damage is a prominent early feature of cell death in a number of vertebrate systems (Bonneville, 1963; Jurand, 1965; Hourdry, 1969, 1971; Fox, 1970). Furthermore, succinic dehydrogenase activity falls sharply in interdigital mesenchyme before morphological damage can be observed (Hammar & Mottet, 1971); this too indicates that mitochondria are a primary site of damage in cell

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Fig. 9. The epithelium in a ten day old rat. Part of a second generation superficial cell is illustrated, showing angularity of the luminal profile and the presence of a few fusiform vacuoles (*f*). × 15000.

Fig. 10. Three week old rat. Part of a superficial cell, showing an angular, thickened luminal membrane and numerous fusiform vacuoles (*f*). × 36000.

death. In the system reported here, severe mitochondrial damage was observed in the early stages of necrosis, but no lysosomal changes were seen. If, indeed, failure of tissue respiration is the initiating event in superficial cell necrosis in the developing bladder, the other changes observed, namely loss of cytoplasmic density and swelling of nuclei, may all follow from failure of energy-dependent enzyme systems concerned with transmembrane transport. Microsegregation of the nucleolus into granular and fibrillar components is commonly seen to precede cell death in chemically injured cells, but its significance in terms of disturbance of physiological function is not yet understood (Svoboda & Higginson, 1968).

The significance of superficial cell death in the developing rat bladder remains obscure. Sometimes cell death has an obvious function, as in the sculpturing of the fetal digits (Ballard & Holt, 1968). However, most of the examples reviewed by Glücksmann (1951) are not yet understood functionally. The results described here suggest that cell death in the developing rat bladder is a programmed event rather than a non-specific toxic effect. Further investigation in organ culture may reveal whether it is mediated by a circulating or local chemical agent, or whether it is a behaviour pattern inherent in the first generation of superficial cells, independent of their tissue association.

SUMMARY

Differentiation of bladder epithelium in the rat is characterized by the production of a short-lived generation of differentiated superficial cells during the last week of gestation. These cells undergo necrosis during the perinatal period and desquamate into the bladder lumen. Proliferation of the undamaged basal cells generates the definitive superficial layer, which matures during the third week after birth.

During maturation of both sets of superficial cells, the thick membrane characteristic of the epithelium appears later in the membrane flow pathway than is normal in the mature tissue. This may be due to a difference in the balance between synthesis and destruction of membrane, leading to a faster flow rate and thus to differentiation further along the pathway.

Necrosis of the first generation of superficial cells is likely to be a determined step in development, and is not caused by lysosomal autolysis. The structural changes seen suggest that a failure of mitochondrial phosphorylation may be the immediate cause of necrosis.

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