Membrane Changes during Urothelial Hyperplasia and Neoplasia

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Summary

Normal mammalian urothelium has a complex pattern of morphogenesis and cell differentiation. The luminal face of the polyploid, superficial cells is limited by a morphologically unique plasma membrane with a highly ordered substructure. This membrane is a convenient marker for normal differentiation in the urothelium. In reversible, benign hyperplasia, following cytotoxic damage, the luminal face of the most superficial layer of cells is limited by an undifferentiated membrane which is comparable to the plasma membrane of normal basal cells. When the hyperplasia regresses, normal differentiation of the urothelium is reestablished, and the normal, specialized surface membrane is again produced.

In both preneoplastic hyperplasia and early neoplasia induced by various carcinogenic regimens, a novel form of differentiation affecting the surface membrane is seen in some superficial cells. Microvilli form on their free luminal face and are limited by a membrane with an unusual, structured glycocalyx, which is not found in normal urothelium or benign hyperplasia. This glycocalyx also develops on some cells in transitional cell carcinoma of the bladder in man. This novel glycocalyx may be a morphological marker for neoplastic transformation in the urothelium.

Introduction

The normal mammalian urinary bladder is lined by a highly differentiated, 3-cell-thick epithelium, the urothelium. Its development, histology, substructure, function, and turnover have been recently reviewed (9) and will not be described here. The membrane limiting the luminal face of the superficial cells is morphologically unique. It is composed of rigid plaques, with a highly ordered substructure of stellate subunits arranged in a hexagonal lattice, separated by thinner, unstructured hinge regions. This membrane is assembled in the Golgi complex of the differentiated superficial cells (6), and its structure, function, and chemistry have been described in some detail (9, 10).

If the urothelium is subjected either to mechanical damage (16) or to cytotoxic damage from a single noncarcinogenic dose of MNU (11, 12), ESNS (8), or cyclophosphamide (12, 14, 15), the basal stem cells divide to form a hyperplastic epithelium composed of many layers of undifferentiated, predominantly diploid cells. This compensatory proliferation is reversible after cessation of the damaging stimulus, and the hyperplasia regresses to form a 3- to 4-cell-thick polyploid urothelium with the normal pattern of differentiation.

By contrast, preneoplastic hyperplasia of the urothelium resulting from treatment of the animals with carcinogens is essentially irreversible, and the proliferation continues until frank tumors are formed. In the early stages of growth, preneoplastic hyperplasia is histologically very similar to benign hyperplasia. This study of the ultrastructural features of cells in benign hyperplasia and preneoplastic hyperplasia was made in an attempt to find a morphological marker for early neoplastic transformation. A change in morphology of the luminal surface membrane of some cells in preneoplastic and early neoplastic urothelia is described; this has also been observed in human transitional cell tumors of the bladder. It appears to be linked with the neoplastic process, for it has not yet been observed in the normal urothelium or in reversible hyperplasia.

Materials and Methods

Specific-pathogen-free Wistar rats were used throughout; they were fed 41B laboratory diet and water ad libitum unless otherwise stated. To induce reversible hyperplasia of the urothelium, a single i.p. injection of cyclophosphamide, 200 mg/kg body weight, was given, or a single dose of 2.0 mg MNU was instilled into the bladder via a urethral catheter (11, 12). Hyperplastic urothelium from rats given a single dose of ESNS (8) were also examined. Bladders from rats maintained on a vitamin A-deficient diet (7) were used for investigating the subcellular changes associated with benign squamous metaplasia. Preneplastic hyperplasia, followed by bladder tumors, was induced in all animals treated either with 4 intravesicular doses of 1.5 mg MNU at biweekly intervals (11) or with weekly s.c. injections of DBN, 200 mg/kg body weight (2). In addition, bladder epithelium was examined from animals fed NFTF as 0.18% of the diet (3) and from rats that had received a single dose of 2.0 mg MNU and that were then maintained on a diet containing either 2.0 or 4.0 g saccharin per kg body weight per day or 1.0 or 2.0 g cyclamate per kg body weight per day (13). Samples of human bladder tumors were obtained after total cystectomy or open surgical biopsy.

For transmission electron microscopy, rat bladders were distended and fixed in osmium tetroxide, embedded in Epon, and thin sections, contrast stained with lead and uranyl salts, were then examined. Two-μm sections of Epon-embedded tissue were stained with toluidine blue for histol-
ogy. For scanning electron microscopy, the bladders were distended and fixed in osmium tetroxide and shadowed lightly with carbon before preparing a gold replica. MNU was a gift from Professor P. N. Magee; DBN was obtained from Eastman Organic Chemicals, Kirkby, Liverpool, England; and NFTF was from Saber Laboratories, Morton Grove, Ill.

Results

**Benign Hyperplasia.** The histological appearance and ultrastructure of benign hyperplasia of the urothelium following a single dose of MNU has been described (11, 12). The epithelium reached maximum thickness after about 2 weeks and at that time the epithelial cells were immature and relatively undifferentiated. During epithelial proliferation, the cells contained increased numbers of filaments that were sometimes aggregated into tonofibrils, structurally abnormal mitochondria, and accumulations of lipid, and there were occasional breaks in the basal lamina through which processes of the basal cells extended into the submucosa (12). The surface of the superficial cells was frequently folded, and isolated, short, stubby microvilli were sometimes observed projecting into the lumen. The free face of these cells, including the microvilli where present, was limited by an undifferentiated, flexible plasma membrane (Fig. 1). In cross-section, this membrane was not asymmetrically thickened, and it had no rigid plaques of hexagonally arranged subunits. Morphologically, it was similar in appearance to the plasma membrane of normal basal cells. After 3 weeks, the normal pattern of differentiation began to be reasserted in the epithelium. The regenerative process was not uniform and proceeded more rapidly in some areas of the bladder than in others, although it was usually complete after 10 weeks. However, from 3 weeks after dosing, some cells could always be found in which the luminal face was again limited by normal, asymmetrically thickened, luminal membrane (Fig. 2).

A similar cycle of proliferation and reversion to normal after cyclophosphamide treatment has been described (12, 14, 15). The subcellular structure of the hyperplastic epithelium a few days after cyclophosphamide treatment was closely comparable to that after a single dose of MNU (12). Again, while the urothelium was hyperplastic the membrane limiting the surface of most superficial cells lacked the normal differentiation (Fig. 3). As the pattern of morphogenesis was reestablished, the normal specialized membrane with plaques of subunits was again assembled and inserted into the free cell surface, as has already been reported (14, 15).

Loss of the normal surface membrane and its replacement by an undifferentiated, flexible membrane have also been illustrated in benign, reversible hyperplasia following ESNS treatment (8).

**Preneoplastic Hyperplasia.** All rats receiving 4 biweekly doses of 1.5 mg MNU into the bladder developed multifocal urothelial tumors that were readily detectable after 20 weeks and, in some animals, as early as 12 weeks after the start of treatment (11). In the few weeks after the last dose of MNU and before the development of histologically diagnos-

able tumors, the epithelium was in a state of preneoplastic hyperplasia. At that time, the cellular substructure of the transitional cells was very similar to that in the proliferative growth phase following a single dose of MNU. Similarly, in rats receiving weekly injections of DBN, the urothelium became hyperplastic after about 12 weeks and increased in thickness and complexity until, after about 30 weeks, histologically definable tumors were formed in all treated animals. There was less cytotoxic damage after DBN than after MNU, but the subcellular changes in the transitional cells of the proliferating epithelium were essentially the same. In both MNU- and DBN-transformed urothelia, some foci underwent squamous metaplasia, and in these areas the subcellular changes were the same as those observed in benign squamous metaplasia induced by a vitamin A deficiency (5, 7).

In these preneoplastic epithelia, some transitional cells at the surface of the epithelium, like those in benign hyperplasia, developed microvilli. However, as the hyperplasia progressed, the number of microvilli per cell increased and an increasing number of cells developed microvilli, until in early transitional cell tumors the free surfaces of many cells were profusely covered with them (Fig. 4).

These microvilli were limited by a flexible membrane that was thinner than the normal luminal membrane and that lacked its characteristic substructure. However, unlike that seen in benign hyperplasia, this membrane was covered with a prominent glycocalyx that appeared either as fine, branching filaments or, more frequently, as a beaded structure. It was present in focal areas of preneoplastic hyperplasia induced either by 4 doses of MNU (Fig. 5) or by weekly injections of DBN (Fig. 6). It was also present on the free luminal face of some, but not all, cells in gross rat bladder tumours produced by multiple doses of MNU, by DBN, by feeding NFTF, or by a single dose of MNU followed by a cyclamate- or saccharin-containing diet (13) (Fig. 7). It was also observed on the surface membrane of some cells in the 3 human transitional cell tumors of the bladder examined (Fig. 8).

This structured glycocalyx was restricted to the free luminal face of transitional cell tumors and preneoplastic transitional epithelium. It was not present on the luminal surface of squamous cell carcinomas or on any other plasma membrane of cells deeper within the epithelium. It was never observed in normal, untreated rat urothelium or in fetal rat bladders (4); thus it appeared to be a qualitative change associated with neoplasia. It was the only morphological change observed which was unique to malignantly transformed urothelium. Quantitative changes occurred in some other organelles, such as tonofilaments, ribosomes, and lysosomes, all of which increased in number, but this was true both for benign and preneoplastic cell proliferation. Cilia were occasionally seen in early tumors, but they were also observed in control, untreated rats, particularly in older animals.

Discussion

The distinguishing characteristic of the cancer cell is not its structure, but its failure to respond to growth-regulating...
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factors, largely unidentified, that control its differentiation in a manner appropriate to the tissue. In consequence, instead of following the path of normal differentiation and programmed cell death, the cancer cell remains locked in the cycle of growth and division. In the urothelium, the active tumor cells frequently appear to be physiologically competent but relatively undifferentiated, but this is true not only for the cells forming a preneoplastic or neoplastic urothelium, but also for normally growing and dividing cells in the proliferative repair phase following noncarcinogenic damage. On theoretical grounds, the chances of detecting a gross morphological marker that would distinguish preneoplastic hyperplasia from normal growth are small. However, reports that experimentally induced murine bladder tumors produce a cross-reactive, tissue-specific tumor antigen (17) suggested that a common arrangement of surface glyco-proteins might be demonstrated morphologically.

If new, specific, antigenic groups were to be inserted into the normal luminal membrane, a change in its hexagonally ordered substructure would be highly probable but, unfortunately, this membrane is produced only by the large, polyploid cells of the normal urothelium, and these take a long time to mature. When the epithelium is proliferating more rapidly than usual, its normal organization is lost and the immature, diploid cells that form the replacement superficial layer do not assemble the thick, structured membrane. Instead, they are limited by a thinner, more flexible plasma membrane, which is comparable in appearance to that limiting the basal cells or stem cells of the normal urothelium. However, as shown here, a novel, structured glycocalyx can be found on the luminal membrane in focal areas of bladders treated with various carcinogenic regimens. Both during the hyperplasia that precedes tumor growth, and also in gross transitional cell tumors. The same type of glycocalyx was found in 3 human transitional cell tumors of the bladder of unknown etiology. Whether this glycocalyx is in any way associated with a tissue-specific tumor antigen or is a totally unrelated phenomenon is not known at the present time.

So far, we have never observed a glycocalyx of this type in the normal bladder or in hyperplastic urothelia that were capable of returning to the normal pattern of differentiation, i.e., in cells still capable of responding to growth control factors. This suggests that it may represent a new pattern of gene expression for the bladder, linked in some way to the neoplastic state. It is interesting that a very similar glycocalyx is seen on the luminal plasma membrane of cystic Walthard cell nests in the human female genital tract. The close histological and ultrastructural similarity between Walthard cell nests and urothelium is frequently emphasized and there is debate as to whether they are derived by transitional metaplasia directly from the coelomic epithelium or by mesonephric metaplasia or urothelial ectopia (1). The presence of the glycocalyx in Walthard cell nests suggests that in bladder tumors it is not produced by genetic mutation, but that it is coded for by a portion of the genome which is normally suppressed but which is readily or preferentially derepressed after neoplastic transformation.

If further investigations with other chemical carcinogens and toxins show this glycocalyx to be a regular or obligatory concomitant of early neoplastic transformation in the urothelium, but to occur only rarely in benign conditions, this glycocalyx could be a useful diagnostic tool for predicting the subsequent behavior of early proliferative lesions of the human bladder. At the moment the results are encouraging, but the correlation observed thus far of new glycocalyx with neoplasia could still prove to be fortuitous, and many more examples are required.

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References

Membrane Changes during Carcinogenesis

All illustrations, except for Fig. 4, are transmission electron micrographs of thin sections of osmium-fixed tissues which have been contrast stained with lead and uranyl salts. Fig. 4 is a scanning electron micrograph of a gold replica of osmium fixed tissue.

Fig. 1. Luminal edge of parts of 2 surface cells from hyperplastic bladder epithelium of a rat treated 2 weeks previously with 1 intravesicular dose of 2.0 mg MNU. The plasma membrane on the urinary face of the cells is approximately 8 nm thick, flexible, and undifferentiated. × 90,000.

Fig. 2. Luminal edge of superficial cell from rat urothelium 12 weeks after a single dose of 2.0 mg MNU. The normal pattern of differentiation has been reestablished and angular, thickened membrane limits the free face of the superficial cell and forms fusiform vacuoles in the cytoplasm. × 39,000. Inset, cross-section of the 12-nm-thick membrane to show its asymmetrical unit structure. The dense leaflet adjacent to the urine is characteristically thicker than that adjacent to the cytoplasm. × 120,000.

Fig. 3. Undifferentiated, approximately 8-nm-thick, membrane limiting urinary face of cell from hyperplastic bladder epithelium of rat treated 4 days previously with single i.p. dose of cyclophosphamide. × 60,000.

Fig. 4. The luminal surface of an early transitional cell tumor. The cells are smaller and more irregular in outline than normal, and their free face is densely covered with microvilli. × 2,100.

Fig. 5. Edge of cell from the bladder epithelium of a rat 12 weeks after commencing treatment with 4 doses of MNU. The epithelium was hyperplastic but no tumors were present. A prominent glyocalyx of beaded filaments has developed on the luminal membrane. × 76,000.

Fig. 6. A profuse glyocalyx is shown on the free face of a bladder epithelial cell from a rat receiving a carcinogenic regimen of weekly s.c. injections of DBN. × 35,000.

Fig. 7. The glyocalyx on the luminal membrane of a transitional cell tumor of rat bladder, induced by maintaining the animal for 30 weeks on a diet containing 2 g saccharin per kg body weight per day after a single intravesicular dose of 2.0 mg MNU (cf. Fig. 2). × 40,000. Micrograph taken by J. Chowaniec.

Fig. 8. Beaded, filamentous glyocalyx on surface cell membrane of transitional cell tumor of the human bladder. Tumor of unknown etiology. × 60,000.
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