Squamous and Transitional Elements in Rat Bladder Carcinomas Induced by N-Butyl-N-4-hydroxybutyl-nitrosamine (BBN)

A Study of Cytokeratin Expression

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Three hundred rat bladders bearing tumors induced by N-butyl-N-4-(OH)butyl-nitrosamine (BBN) were examined by routine histologic study and immunohistochemical staining of intermediate filament types. Smaller lesions were similar to human urothelial dysplasia histologically and immunohistochemically. Progression of the lesions demonstrated large exophytic papillomas with extensive endophytic epithelial growth into abundant stroma. These lesions showed increasing predominance of squamous over transitional elements. Immunohistochemical findings confirmed these results and also demonstrated that morphologically indifferent cells, even in early lesions, express heavier cytokeratins characteristic of keratinizing squamous epithelium. These results demonstrate that BBN-induced bladder tumors show marked quantitative and qualitative differences from the most common, purely transitional, human bladder carcinomas. However, the development in BBN-treated rat bladders of two tumor types, squamous and transitional, from an altered urothelium may serve as an attractive model for further study of the molecular genetics of keratin expression. (Am J Pathol 1985, 120:419-426)

HUMAN transitional-cell cancer is a difficult clinical problem because morphologic features of the lesion give only general guidance to the expected clinical behavior, with wide individual variations. In addition, although transitional-cell carcinoma is clearly a lesion of the entire urinary tract epithelium, morphologic features of biopsy specimens of urothelium give few clues as to the expected behavior of clinically and histologically normal-appearing urothelium. In order to investigate these problems further, a suitable animal model of bladder cancer is most desirable, especially for study of morphologically normal but predictably carcinogenic bladder epithelium.

Bladder carcinogenesis induced by BBN shows a predictable time course with a long lag period after BBN administration but before morphologically neoplastic lesions appear. Although extensive experience with this carcinogen has been reported in the literature, there remains considerable confusion as to the histologic type(s) of the neoplasm(s) produced, with most authors concluding that both squamous and transitional-cell tumors result from BBN administration.

For investigation of this problem of tumor differentiation after BBN administration, expression of cytokeratins by the tumor offered a useful marker, as an extension of previous studies. For a general review of intermediate filament proteins and their expression in normal tissues and tumors, see Ramaekers et al. Specifically pertinent to the present study, the cytokeratins are a family of intermediate filament proteins expressed only by epithelium. The different cytokeratins are immunochemically distinguishable and differ in molecular weight and isoelectric point.

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Individual epithelial tissues express several different cytokeratins, the pattern of expression being characteristic of a specific tissue or tumor type. Cytokeratin 18, for example, is a 45-kD protein expressed normally in "simple," generally glandular, epithelium. In the normal bladder, cytokeratin 18 is detected in the superficial (umbrella) cells of the urothelium but not in cells deeper in the urothelium. In human bladder tumors, cytokeratin 18 is expressed by increased numbers of tumor cells with progression of the lesion to higher grade and higher stage. In contrast, keratinizing squamous epithelium does not express cytokeratin 18 but does express higher molecular-weight, more basic cytokeratins, as recognized by a monoclonal antibody, RKSE 60, developed in our laboratory. In normal bladder epithelium we have so far not found RKSE 60-positive cells, whereas isolated RKSE 60-positive tumor cells are occasionally found in bladder carcinomas (unpublished observations). Thus, RKSE 60 and a monoclonal antibody to cytokeratin 18, RGE 53, were chosen to further delineate the tumor type(s) produced in the rat bladder by BBN administration.

Materials and Methods

Animals

Male inbred ACI rats were used in the present study, housed 4 per cage with a balanced 12-hour light/dark cycle. Throughout the study they were allowed water and food (RMH-TH 1110, Hope Farms, Woerden, Netherlands) ad libitum.

The rats were divided into 10 groups of 30 rats, one group serving as a control (no BBN). Nine groups of rats received BBN (N-butyl-N-4-hydroxybutyl-nitrosamine; Deutsches Krebsforschungszentrum, Heidelberg, West Germany) as a 0.05% solution in the drinking water. Administration began at the seventh postnatal week and continued until the end of the 18th postnatal week (12 weeks' administration). Beginning at the 39th postnatal week (20 weeks after termination of BBN administration) one or two groups of rats were sacrificed weekly until postnatal week 43.

The rats were sacrificed by ether overdose, and the urinary tract was removed for study. The bladder and both kidneys, with approximately 5 mm of ureter, were divided in half, the bladder sagittally and the kidneys through the hilus. One-half of each organ was embedded in paraffin and used for routine histologic study with hematoxylin and eosin (H&E) staining. The other half was frozen in liquid nitrogen or cut immediately into frozen sections. Macroscopic examination of remaining organs, except brain and spinal cord, for evidence of tumor metastases was carried out at the time of sacrifice.

Immunohistochemistry

Frozen tumor material was cut on a cryostat and 5-7 μm-thick frozen sections were fixed with methanol (−20 C, 5-10 minutes) and acetone (room temperature, 10-30 seconds). Air-dried sections were rehydrated with phosphate-buffered saline (PBS) and incubated with the first antiserum. Incubation procedures for single- and double-labeling indirect immunofluorescence have been described before. The following antibody preparations were used in this study.

Rabbit Antiserum to Human Callus Keratins
Affinity-Purified on Human Skin Keratins (pKer)

This antiserum has been shown to react with most epithelial tissues, including keratinizing and non-keratinizing squamous epithelia, transitional and glandular epithelia, as well as exocrine and endocrine epithelial tissues. When applied to cultured epithelial cells, this antibody shows a fibrillar staining pattern, typical for intermediate filaments. Nonepithelial tissues or cell cultures are negative with this antiserum. The serum was diluted 1:10 to 1:20.

Mouse Monoclonal Antibody RGE 53,
Specific for Cytokeratin 18

This antibody reacts with glandular epithelia of the gastrointestinal tract and the genital system, with columnar and glandular cells of the respiratory tract, and with exocrine and endocrine glandular tissues. The antibody does not stain either keratinizing or nonkeratinizing squamous epithelium. Correspondingly, the antibody only reacts with adenocarcinomas and mesotheliomas, but not with squamous-cell carcinomas or nonepithelial tissues or tumors. In the human bladder, this antibody has been shown to stain only umbrella cells, while in human bladder carcinomas variable numbers of RGE 53-positive cells may be found, depending on the grade of the tumor.

Mouse Monoclonal Antibody RKSE 60,
Directed Against Keratin From Keratinizing Squamous Epithelium

This antibody stains suprabasal cell layers in human and rat skin, Hassall's corpuscles in the thymus, and occasionally some keratinizing cells in cervical squamous epithelium. The antibody does not react with glandular or columnar epithelia, nonkeratinizing squamous epithelia, or nonepithelial tissues or cells. In immunoblotting assays in our hands this antibody reacted with two high-molecular-weight keratin bands specific for human epidermis but not with other types of intermediate filament proteins. Preliminary studies indicate that RKSE 60 is directed against cytokeratin 10 (to be
published; personal communication by Dr. T. T. Sun, New York).

When tested on human tumor tissues, RKSE 60 reacted only with keratinizing areas in squamous-cell carcinomas from different sites, as well as with keratinizing cells in premalignant or borderline malignant lesions of the cervix. Occasionally some RKSE-60-positive cells were found in human bladder carcinomas (rare cells in 2 of 12 cases examined; unpublished observations).

**Rabbit Antiserum to Calf Lens Vimentin**

Preparation and specificity of this antiserum were described earlier. Briefly, the antiserum generally does not stain epithelial cells (for exceptions see, for example, Herman et al⁴) but strongly stains mesenchymal tissues and mesenchymally derived tumors.

Normal bladder epithelium, either human or rat, does not react with the antivimentin antiserum, but subepithelial fibroblasts, endothelial cells, inflammatory cells, and some vascular smooth-muscle cells are strongly positive with this antiserum. (All antiserum described above are available through Euro-Diagnostics BV, Apeldoorn, The Netherlands.)

Corresponding fluorescein isothiocyanate and rhodamine-conjugated second antibodies were obtained from Nordic, Tilburg, The Netherlands. In control experiments either second antibodies alone or primary antibodies to desmin and GFAP were used.

**Results**

**Normal Rat Bladder**

The normal rat bladder (Figure 1A) is lined by 4–6 layers of transitional epithelium, of which the most superficial layer is composed of flattened, so-called umbrella cells. The entire epithelium stains positively with the polyclonal antiserum to cytokeratins (pEer, Figure 1B). RGE 53, specific for the Mr 45,000 cytokeratin 18 polypeptide, stained only the superficial (umbrella) cells (Figure 1C). The entire epithelium was negative for vimentin and RKSE 60 staining (results not shown). None of the control rats in the present study showed deviation from this normal urothelium, either in bladder or in renal pelvis and ureters.

**BBN-Induced Bladder Tumors: Histology**

In rat bladders following BBN administration, the smallest deviation from normal urothelium is a proliferation of transitional cells producing a thickened mucosa composed of normal or atypical urothelial cells, analogous to human urothelial dysplasia. The next largest lesion is the development of papillary growth (Figure 2A and B). However, even at this stage, marked devia-
Figure 2—Bladder epithelial lesions induced by BBN. A—Papilloma with branching endophytic epithelial growth in abundant stroma. (H&E, x12) B—Higher magnification of a lesion similar to that in A. (H&E, x32) In A and B, note the normal epithelium in the upper left corner. Note the formation of solid epithelial nests in stroma. C—Atypia of epithelial nests with squamous metaplasia in nests and on the surface (luminal epithelium). (H&E, x32) D—Transformation of a massive papilloma to a predominantly necrotic acellular keratin mass. (H&E, x12) E and F—Invasive carcinomas. In E, note the squamous differentiation in the left half of the field and less differentiated tumor in the right half of the field. (H&E, x80) F—A solid exophytic tumor mass with invasion as small nests almost through the bladder wall. Morphologically, these cells show no clear squamous or transitional differentiation. (H&E, x32)
tion from the pattern of human neoplastic lesions is apparent. In contrast to human papillary transitional-cell carcinomas, BBN-induced lesions have an abundant fibrovascular stroma and the surface epithelium is generally either no thicker or only slightly thicker than normal rat urothelium.

The larger papillary structures show numerous invaginations of urothelium-lined channels throughout the stroma (Figure 2A). The urothelium lining these channels is thinner than normal urothelium, usually consisting of one or two cell layers in the larger papillary structures (Figure 2B). In addition to the ductlike structures, solid nests of transitional cells are seen. These nests of urothelium are morphologically reminiscent of inverted papillomas in the human bladder. Concomitant with the appearance of these cell nests, marked squamous differentiation is noted in both the surface epithelium covering the papillary structures and the epithelium of the solid cell nests and channels (Figure 2C). This squamous epithelium appears to replace the urothelium and is heavily keratinizing from the first point that it is recognizable as squamous epithelium. Thus, in bladders where large and/or multiple tumors are noted, the remaining lumen of the bladder may be completely filled with acellular keratin (Figure 2D).

Rats which had survived longer after beginning to develop papillary structures (42 or 43 weeks from beginning of BBN administration) began to show extensive necrosis of the papillary structures with increased production of keratin (Figure 2D). The largest tumors in fact contained primarily large masses of acellular keratin with only very few viable-appearing cells, mostly mature squamous cells.

Occasional nests of atypical cells were noted both in the stroma of the papillary structures and in the wall of the bladder (Figure 2E). These atypical cells are only rarely seen invading the muscle layer and may be clearly urothelial, clearly squamous, or of uncertain differentiation (Figure 2E and F).

These changes are analogous to those described by Druckrey and illustrated in subsequent studies. They were demonstrable exclusively in the bladders. Lesions were not seen in other urothelium-lined segments of the urinary tract, i.e., renal pelvis, ureter, or urethra. All BBN-treated animals in the present study showed at least focal urothelial dysplasia and some papillary lesions. No metastatic lesions were found.

Expression of Cytokeratin Types in BBN-Induced Tumors

The persisting normal urothelium as well as all the neoplastic urothelial lesions described above contained cytokeratin, as demonstrated by the polyclonal antikeratin antiserum. In contrast, vimentin was expressed only by stromal elements. None of the urothelial cells expressed vimentin, nor was evidence of cytokeratin expression found in stromal cells.

The minimal, dysplastic, lesions expressed a cytokeratin pattern similar to normal rat urothelium. In the superficial (luminal) layer of cells, cytokeratin 18 was detected, whereas deeper cell layers were negative by immunofluorescence for this cytokeratin type. In these lesions, no cells could be identified which expressed the high-molecular-weight cytokeratin recognized by RKSE 60. The polyclonal antiserum pKer showed accentuated staining of the most superficial and deep cell layers with less intense staining of the intermediate layers. The development of the smaller and simpler papillary lesions was not accompanied by changes in the pattern of keratin expression. The most superficial cell layers both on the surface of the papillomas and lining the ducts and tubes forming deeper in these papillary projections expressed cytokeratin 18. Cytokeratin 18 was not detectable in deeper cell layers, but the polyclonal antibody stained all epithelial cells.

Further growth of the papillomas with development of occasional solid urothelial cell nests produced the first clear deviations of cytokeratin expression from the pattern described above. Although the entire urothelium continued to stain with the polyclonal antikeratin (Figure 3A), cytokeratin 18 containing cells were identifiable lying singly and as small clusters within the cell nests, without relationship to a luminal surface (Figure 3B). At this stage, occasional areas of morphologically recognizable keratinizing epithelium were also seen both on the surface of the papillomas and lining ducts in the body of these structures. This squamous epithelium was strongly positive with the RKSE 60 antibody. In addition, individual cells not morphologically identifiable as squamous epithelium were found to express RKSE 60 (Figure 3C).

Further progression of tumor development with proliferation and massive growth of the papillary lesions was characterized by predominance of squamous over urothelial elements as determined by comparing staining with RKSE 60 with staining with RGE 53 (Figure 4A-C). The epithelium covering the surface of the papillomas as well as the bladder wall was predominantly keratinizing squamous epithelium, as were the cell nests within the papillomas, all strongly positive for RKSE 60. Morphologically indifferent cell nests, not clearly identifiable as squamous epithelium, were found which contained clusters of cytokeratin-18-positive cells, RKSE-60-positive cells, and occasionally mixtures of both cell types. Both cytokeratin-18- and RKSE-60-positive cells were found to cover the entire morphologic spectrum from mildly atypical to frankly
malignant. The invasive carcinomas (invasion into or through muscle) identified in the present study consisted of both cytokeratin-18- and RKSE-60-positive cells, generally without convincing morphologic squamous differentiation (Figure 2E and F).

Discussion

The present study has used antibodies to cytokeratin intermediate filament proteins to define the relationship of transitional and squamous cell elements in the bladder lesions induced by BBN administration to rats. The routine histologic features of our material were qualitatively similar to those reported in other studies. However, important quantitative differences were observed which were supported by the immunofluorescence findings.

In our material as in that described by Kunze and others, the histologic features of the smallest lesions mimicked those of human urothelial dysplasia. Immunofluorescence showed that these lesions were exactly analogous to human urothelial dysplasia, retaining the pattern of cytokeratin expression seen in normal urothelium.

The next stage of BBN-induced rat bladder carcinogenesis showed important differences from human neoplasms. Development of very large papillomas showed much more stromal proliferation than in human papillary lesions. Extensively branching urothelial nests showed endophytic growth within the stroma of these papillary lesions. Development of squamous metaplasia was early and extensive, such that squamous metaplastic epithelium with variable atypia predominated over recognizable urothelium in larger lesions.

These findings were supported and extended by immunofluorescence. The squamous metaplastic areas showed strong staining with RKSE 60, as expected. However, even in areas which appeared morphologically to be purely urothelial, cells lying singly or in small groups were shown to stain with the RKSE 60 antibody. This finding indicates that some neoplastic cells express an important protein marker of keratinizing squamous cell carcinoma without being morphologically identifiable as such. When compared with human transitional-cell carcinomas of the urinary bladder, which only occasionally show scattered RKSE 60-positive cells, these BBN-induced rat bladder tumors show proportionally a very high degree of keratinization. In addition to these cells showing keratinizing squamous features, the urothelial cell nests also showed single cells and cell nests expressing cytokeratin 18. This pattern of cytokeratin expression is indeed analogous to the development of higher-grade, higher-stage human transitional-cell carcinoma of the purely urothelial

Figure 3—Smaller, predominantly papillary lesion produced by BBN. Polyclonal antikeratin serum stains the entire epithelium (A). Monoclonal antikeratin 18 stains predominantly surface cells and occasional cells deeper in the papillary epithelium (B). A and B are double-immunofluorescence-stained from the same field. A similar lesion (C) stained with RKSE 60 shows a nest and several individual morphologically indifferent cells positive for this marker of keratinizing differentiation. (A-C, ×200)
type. Similarly, by both morphologic and immunofluorescence criteria, the few superficially invasive carcinomas in the present study all showed a mixture of both urothelial and squamous components, with squamous differentiation predominant.

The relative predominance of squamous metaplasia and squamous-cell carcinomas in the present series, compared with other reported studies, may be due to at least three factors: strain of rats, age of rats at induction, and differences in interpretation of the lesions. Very little information is available detailing strain-related differences in the histologic features of BBN-induced lesions. However, these lesions have been claimed to be predominantly urothelial in a number of strains, including an ACI strain such as was used in the present study.9

It has been reported that increased age of the rats at the time of initiation of BBN correlates with a higher proportion of squamous metaplasia.7 However, the rats in the present study, 7 weeks old at the time of beginning BBN exposure, should have had a very low incidence (approximately 15%) of squamous metaplasia, compared with the 100% which we actually observed.

Probably the most important difference in relative incidence of squamous and urothelial lesions in various studies can be attributed to differences in interpretation. The first description of BBN-induced neoplasia in the rat bladder characterized the resulting tumors as squamous-cell carcinomas.3 Subsequent authors chose to consider as squamous-cell carcinomas only those lesions showing no transitional-cell areas.11 This practice has led to the very high proportion of urothelial lesions reported in the later literature.12,13

The use of antibodies to cytokeratin types in the present study has further clarified the relationship of transitional and squamous cell elements. Although the earliest and smallest lesions are purely urothelial, analogous to human lesions, later lesions show patterns of cytokeratin expression consistent with a mixture of squamous and transitional carcinoma. This suggests that the later and larger neoplastic lesions produced by BBN administration are a mixture of two tumor types, transitional and squamous, both having originated from a transformed urothelium.

The predominance of squamous differentiation in BBN-induced neoplasms and the demonstration by immunofluorescence that morphologic characteristics alone are insufficient to allow recognition of early squamous differentiation casts doubt on the suitability of BBN-induced carcinogenesis as a model for the most common type of human bladder cancer. However, this model system does show the same changes in cytokeratin 18 expression originally described in human bladder cancer.19 Additionally, a second tumor line in the BBN-induced neoplasms shows the expression of

Figure 4A—A larger lesion induced by BBN, showing nests of predominantly indifferent cells with focal keratinization (upper left corner), stained with polyclonal antikeratin antibodies. Similar lesions, while continuing to show keratin-18-positive single cells and cell nests (B, RGE 53 stain) are predominantly stained with RRSE 60 as a marker of keratinizing squamous differentiation (C). (× 200)
cytokeratin characteristic of squamous-cell carcinoma. Thus, this model system may prove to be a useful tool in investigating the molecular genetics of cytokeratin expression and its relation to carcinogenesis. Further quantitative definition of these two tumor cell lines by flow cytometry and application of other specific (monoclonal) antibodies to cytokeratin should allow a more detailed analysis of their interactions, especially in early lesions.

References

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