A Long-Term Study of Reversible and Progressive Urinary Bladder Cancer Lesions in Rats Fed N-[4-(5-Nitro-2-furyl)-2-thiazolyl]formamide

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Summary

Hyperplasia and, ultimately, neoplasia of bladder epithelium were produced by feeding 0.2% N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT) to weanling male Fischer rats. Hyperplasia induced by FANFT feeding for 2, 4, or 6 weeks, followed by feeding control diet until the end of the 84-week experiment, was reversible, and at the end of the experiment bladder epithelium in these animals was normal by light and scanning electron microscopy. Hyperplasia produced by 8 or more weeks of FANFT feeding was irreversible and by 84 weeks had resulted in bladder tumors in all animals fed FANFT for 12 or more weeks and in 4 of 5 and 6 of 7 animals fed FANFT for 8 and 10 weeks, respectively. The epithelial changes after 6 and 8 weeks of FANFT were similar by light microscopy but different by scanning electron microscopy. Pleomorphic microvilli seen with scanning electron microscopy are the hallmark of irreversible, and possibly progressive, epithelial proliferative change.

Introduction

In 2 recently published papers (2, 5), we described the epithelial lesions in the urinary bladders of Fischer rats fed FANFT at a dose of 0.2% of the diet for various time periods up to 20 weeks and then maintained on control diet. Those papers presented light microscopy, TEM, and SEM findings after 20 and 50 weeks. This report covers the finding in animals maintained for a total of 84 weeks, at which time this experiment on the reversibility or irreversibility of lesions induced by various periods of feeding FANFT was terminated.

According to our previous studies, FANFT feeding for 2, 4, or 6 weeks produced epithelial hyperplasia of the bladder, which regressed to normal within 4 weeks after animals were placed on the control diet. The epithelial hyperplasia produced by FANFT feeding for either 8 or 10 weeks regressed somewhat within 2 weeks of receiving control diet, but focal areas of slight hyperplasia persisted in all animals in both groups up to 20 weeks. At 50 weeks rats fed FANFT for 8 weeks and control diet for 42 weeks had moderate to marked hyperplasia, but no tumors were found. Rats fed FANFT for 10, 12, 14, or 20 weeks followed by control diet all had transitional cell tumors at 50 weeks.

SEM of the luminal surface in rats fed FANFT for 2, 4, and 6 weeks demonstrated a mild to marked cobblestone appearance. Luminal cell surfaces are covered with microridges and/or small uniform microvilli. Bladder epithelial abnormalities in rats fed FANFT for 2 to 4 weeks regressed to normal when fed control diet for an additional 2 to 4 weeks, and in animals fed FANFT for 6 weeks the epithelial hyperplasia regressed to normal by 50 weeks. In animals fed FANFT for 8 weeks, followed by control diet for 42 weeks, the marked cobblestone pattern was still present at 50 weeks, with surface cells in some focal areas covered with pleomorphic microvilli. Rats fed FANFT for 10 to 20 weeks followed by control diet had papillary or polyoid tumors by 50 weeks, with pleomorphic microvilli seen on tumor cells and on adjacent epithelial cells.

This investigation was originally planned to extend over a 2-year period, but after 50 weeks it became apparent that a shorter time would permit us to assess the results of feeding FANFT for various time periods followed by control diet, since tumors were grossly palpable in their bladders, and the experiment was terminated at 84 weeks. Our interest was focused on the groups fed FANFT for 6 and 8 weeks, but animals fed FANFT for shorter or longer periods were continued to 84 weeks on control diet as well.

Materials and Methods

Eight groups of 4-week-old male Fischer rats, 40/group, were fed FANFT in the diet at a dose of 0.2% by weight for 2, 4, 6, 8, 10, 12, 14, or 20 weeks, respectively. Following the period of FANFT feeding, each group of test animals was maintained on the carcinogen-free control diet (Charles River rat chow) until the end of the experiment (Chart 1). Details of the experimental design, including consumption of food and carcinogen, can be found in an earlier paper (2). Rats were housed 5/cage, maintained at 24° and 50% humidity on a 12-hr light-dark cycle, and given water and food ad libitum. They were weighed every 2 weeks up to 6 days.
and thereafter every 4 weeks. At the end of 84 weeks, all animals remaining in the experiment were anesthetized with a lethal dose of 1.0 ml Nembutal i.p. (50 mg/ml; Abbott Laboratories, North Chicago, Ill.). The abdominal wall was opened, and the bladder was exposed and inflated by transurethral injection of 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, under a constant pressure of 50 mm Hg to a volume of 0.4 to 0.5 ml, depending on the age of the rat. After bladders were examined macroscopically for tumors or hyperplasia, the animals were killed and complete autopsies were performed. Details of light microscopy, TEM, and SEM procedures have been reported elsewhere (2, 5).

Results

Autopsy revealed no evidence of primary tumors in major organs other than the bladder, except for occasional interstitial cell tumors of the testis that were found in equal numbers in control and experimental groups.

Rats fed FANFT for up to 6 weeks developed a mild to moderate hyperplasia (Fig. 1), which regressed quickly to a normal 3-cell-layer mucosa and remained so until the end of the experiment (Fig. 2). Rats fed for 8 and 10 weeks developed a moderate to marked hyperplasia, with focal nodular or papillary lesions (Fig. 3). These rats showed no regression of their lesions and, after 76 and 74 weeks, respectively, of control diet, all rats had marked papillary or nodular hyperplasia (Fig. 4), and all except 1 rat in each group had transitional cell tumors. All rats fed FANFT for 12, 14, and 20 weeks had transitional cell tumors associated with marked hyperplasia of the bladder epithelium. Squamous cell components were present in some of the tumors. Stromal changes associated with tumor formation included increased fibrous tissue, increased numbers of blood vessels pushing up into the epithelium, and increased numbers of mast cells located near the sites of neovascularization.

At 84 weeks the TEM studies of rats having tumors revealed that luminal membrane asymmetrical plaques have been replaced by unit membrane and the luminal surface membrane is organized into a pleomorphic microvillous border. These pleomorphic microvilli usually have a core of microfilaments (Fig. 5), which is absent in the short uniform microvilli of normal renewal cells of animals fed FANFT for 6 weeks or less (Fig. 6) and is also absent from the surface crests that we have labeled leafy and rounded microridges from rats fed FANFT for 8 weeks or longer. Fusiform vesicles are absent and tonofilaments are reduced in surface cells but are increased in intermediate and basal cells. The rough endoplasmic reticulum cisternae are diluted, and there is increased aggregation of polysomes. The Golgi complex is unusually prominent in tumor cells, and there are increased numbers of lysosomes. Nuclei are irregular and contain 2 to 3 nucleoli. Intercellular space is increased, and there appears to be an increase in the number of desmosomes and a decrease in the number of tight junctions. The subbasal capillary endothelium is fenestrated, and the basement membrane is discontinuous and herniated by tumor cells. The stroma has increased numbers of fibroblasts, fibrocytes, collagen fibers, and mast cells. The detailed TEM findings in this experiment will be published separately.

When viewed with SEM the bladder epithelium of rats fed FANFT for up to 6 weeks and then control diet until the end of the experiment appears normal, with uniform, flat, polygonal superficial epithelial cells covered with microridges. Renewal intermediate cells are seen at irregular intervals on the epithelial surface (Fig. 7). Seam-like folds between cells appear as fine, slightly elevated lines, and the subepithelial blood vessels are as described for normal bladders (5). Rats fed FANFT for 8 weeks or longer and examined at 84 weeks have a diffusely cobblestone bladder epithelium. Cells tend to be round or spherical and are not uniform in size or shape. Although some surface cells are smooth, most of them are covered with pleomorphic microvilli, with or without associated uniform microvilli, or leafy or rounded microridges (Fig. 8). Table 1 lists the cell surface features found in this study with SEM and relates the presence of specific surface features to duration of FANFT feeding.

Leafy or rounded microridges should not be confused with the microridges found on normal cells. Normal microridges are made up by crests of symmetrical (9 to 10 nm) membrane which surround each 11- to 12-nm asymmetrical plaque. The leafy and rounded microridges that appear in TEM as microvilli are 9- to 10-nm symmetrical membrane structures that do not surround asymmetrical plaques but rather appear to cluster and fuse in a mosaic pattern. Cells with these surface features are not found in rats fed FANFT for less than 8 weeks. Papillary and polypoid lesions in rats fed FANFT for 8 weeks or longer and examined at 84 weeks have diffuse areas of proliferating cells. These cells are in grape-like clusters and appear to be tenuously connected. Cells may be either smooth or covered with pleomorphic microvilli.

Discussion

Rats fed FANFT for 6 weeks develop moderate epithelial hyperplasia of the bladder, but this proves to be reversible when they are placed on a control diet. At the end of the experiment, the bladder epithelium in all animals is normal. Rats fed FANFT for 8 and 10 weeks develop irreversible lesions. All animals in each group have moderate to marked...
epithelial hyperplasia at 84 weeks, and all except 1 animal in each group have at least 1 bladder tumor. All rats fed FANFT for 12, 14, or 20 weeks develop transitional cell tumors.

By light microscopy it is not possible to distinguish between the epithelial hyperplasia after 6 and 8 weeks of FANFT feeding. Such a distinction is possible with SEM. Pleomorphic microvilli as visualized by SEM are the hallmark of irreversible, but not necessarily progressive, proliferative epithelial lesions of the rat urinary bladder in this particular experimental model.

Basal and intermediate cells and those at the luminal surface have pleomorphic microvilli after 8 weeks of FANFT feeding. Not all epithelial cells, however, have pleomorphic microvilli. Cells that eventually become luminal cells may have uniform microvilli and normal microridges if asymmetrical plaques are being formed in the intermediate cells, but generally there are multifocal areas of the bladder epithelium where cells are covered with both uniform and pleomorphic microvilli. Because asymmetrical plaques are not being synthesized, the uniform microvilli on the surface cells can fuse and aggregate in a variety of shapes we have termed leafy or rounded microridges (5). These uniform microvilli when seen with TEM do not have a microfilament core, reflecting their transient nature. Pleomorphic microvilli, on the other hand, do have a microfilament core and are fixed structures similar to the microvillous border found in the gut (L. M. Franks, personal communication). Microfilament cores are not found in normal microridges of rat urinary bladder epithelium nor are they present in the epithelial cells lining the esophagus and oral cavity of the cat and rat (1). The absence of microfilaments in uniform microvilli may reflect their destruction by osmium fixation (8). The presence of microfilaments in pleomorphic microvilli may be due to protection by tropomyosin (8). Lazarides (7) has shown that, while binding to a class of stable microfilaments in cultured fibroblasts, tropomyosin does not bind to microfilaments in a dynamic cell surface state such as ruffling. Thus, although the absence of microfilaments from uniform microvilli may be a fixation artifact, their presence in pleomorphic microvilli in cells fixed under identical conditions indicates a more stable microfilament structure in the latter.

As indicated in "Results," stromal changes include neovascularization of altered epithelium associated with increased numbers of mast cells. Alterations in vascular pattern associated with experimental bladder carcinogenesis have recently been studied (4, 10). It seems possible that neovascularization may precede, as well as follow, tumor formation.

Tiltman and Friedell (11) noticed an increase in mast cells at foci of tumor development in FANFT-fed Fischer rats, and our results confirm their findings. In this study mast cells are characteristically found at sites of neovascularization of developing papillary tumors. Mast cell function in tumors is not known, but it has been suggested that the cells may encourage vascular proliferation in the skin (9). Kessler et al. (6) believe that mast cells play an intermediate role in tumor angiogenesis and suggest that mast cells may modify the adjacent stroma thus allowing for capillary growth. Counter to their findings on tumor angiogenesis factor, however, Folkman and Cotran (3) were unable to demonstrate an angiogenic response with mast cells on chick embryo chorioallantoic membrane.

### Table 1

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<td>Uniform and pleomorphic microvilli</td>
<td>Pleomorphic microvilli, leafy and rounded microridges</td>
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### References

Fig. 1. Moderate hyperplasia of the bladder epithelium after 6 weeks of FANFT feeding. H & E, × 400.
Fig. 2. Normal 3-cell-layer bladder epithelium at 84 weeks. Rats were fed FANFT for 6 weeks followed by control diet. H & E, × 400.
Fig. 3. Marked hyperplasia of the bladder epithelium after 8 weeks of FANFT feeding with early nodular hyperplasia. H & E, × 400.
Fig. 4. Transitional cell tumor of the bladder epithelium at 84 weeks. Rats fed FANFT for 8 weeks followed by control diet. H & E, × 100.
Fig. 5. Pleomorphic microvilli on surface tumor cell. Note central core of microfilaments (arrows). Rats fed FANFT for 8 weeks and control diet for 44 weeks. × 15,000.
Fig. 6. Uniform microvilli on surface bladder epithelial cells in an area of hyperplasia. No microfilament core is present. Rats fed FANFT for 6 weeks. × 15,000.
Fig. 7. Luminal surface of the urinary bladder of a rat fed FANFT for 6 weeks. Normal microridges are present on the surface cells in the upper portion of the micrograph. The renewal cells in the remainder of the micrograph are covered with uniform microvilli. × 8,000.

Fig. 8. Luminal surface of the urinary bladder of a rat fed FANFT for 8 weeks. Note cobblestone appearance of the cells. Pleomorphic microvilli are present on the surfaces of cells in the lower portion of the micrograph whereas rounded microridges are present on the cells in the upper portion. × 8,000.
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