Neovascularization in Benign and Malignant Urinary Bladder Epithelial Proliferative Lesions of the Rat Observed in Situ by Scanning Electron Microscopy and Autoradiography

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INTRODUCTION

The growth of solid tumors beyond a small size is generally accompanied by neovascularization (1), and a diffusible factor released from the tumors, tumor angiogenesis factor (9-12, 15, 29, 31), has been postulated as the mechanism for this phenomenon. Several normal, embryonic, and benign and malignant tissues have been assayed for angiogenesis potential with a variety of experimental techniques including grafting in s.c. pockets (1), ear chambers (21), cheek pouch chamber of hamsters (18, 19), anterior chamber of the eye (15, 17), intracorneally (14), and into the chorioallantoic membrane of the chicken (3, 4). In vitro tissue culture systems have also been attempted (8). Detection of neovascularization in situ has relied on light microscopic methods and autoradiographic techniques (6), which have the limitations of sampling and of estimating 3-dimensional processes from 2 dimensions. Scanning electron microscopy provides a 3-dimensional view of relatively large specimens and permits examination of tissues at high magnification and resolution, but it is limited to observation of surfaces. Murakami (27), however, has devised a method for the 3-dimensional observation of fine vascular arrangements utilizing scanning electron microscopy and plastic casting. We have used this technique to examine in situ neovascularization in various benign and malignant proliferative lesions of the urinary bladder in rats.

BBN4 is a potent carcinogen with selective organotropism for the urinary bladder in several species including the rat (13, 20, 22-24). The histogenesis of bladder carcinogenesis induced by BBN has been demonstrated to progress through stages from normal to simple hyperplasia, nodular hyperplasia, papillomas, and cancer (22, 24). The presence of vascular proliferation is readily detected in situ at each of these stages according to the techniques of Murakami (27), and differences in the pattern of proliferation can also be determined (32).

Angiogenesis has been demonstrated readily for several solid tumors, whereas normal tissues have generally lacked this activity (4, 10, 17, 19). Angiogenesis has even been suggested as a possible marker of neoplastic transformation (5). Reversible hyperplasias of the urinary bladder have been induced by a variety of methods (25, 26, 28, 30), and comparison of the vascular response of these benign proliferations with that of bladder carcinomas was attempted. We produced reversible hyperplasias by the i.p. injection of cyclophosphamide and also by ulceration of the mucosa by

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Vascular changes during carcinogenesis and during reversible regenerative hyperplasia of the rat urinary bladder were studied in situ by scanning electron microscopy of vascular casts and by autoradiography of the bladder. Carcinomas were induced in male Wistar rats by administration of 0.05% N-butyl-N-(4-hydroxybutyl)-nitrosamine in the drinking water for 8 weeks; rats were observed for a total of 40 weeks. Regenerative hyperplasia was produced in male Wistar rats by ulceration with a frozen steel rod; rats were observed for 15 days. It was produced in male Fischer rats by a single injection of cyclophosphamide (200 mg/kg body weight); rats were observed for 21 days.

The subepithelial capillaries of the bladder were arranged as a loose plexus of vessels of relatively uniform diameter, of low density, and connected to larger vessels in the deeper layers. Type 1 vascular proliferations, consisting of a high-density plexus of narrow capillaries with short terminal branches, were first observed at 2 weeks during N-butyl-N-(4-hydroxybutyl)nitrosamine carcinogenesis, increased in number through Week 8, and then decreased. Histologically, hyperplasia was present after 2 weeks, but there was no correlation between epithelial hyperplasia and vascular proliferation. Type 2 foci, larger-diameter vessels with long terminal branches, were observed only during Weeks 7 and 8. Type 3 foci, highly tortuous capillary loops, appeared after 6 weeks and were present with foci of papillary and nodular hyperplasia, papilloma, and cancer.

After ulceration or cyclophosphamide treatment, type 1 foci were observed in areas of granulation tissue and repair in the bladders, and type 3 foci were observed with lesions of papillary and nodular hyperplasia of the epithelium. The epithelial hyperplasia and foci of vascular proliferation were reversible so that the bladders appeared normal by 2 to 3 weeks. Thus, the formation and the reversibility or irreversibility of the type 3 vascular proliferations depend on the extent and reversibility or irreversibility of the epithelial proliferations.

1 The abbreviation used is: BBN, N-butyl-N-(4-hydroxybutyl)nitrosamine.
the application of a frozen metal rod to the serosal surface. The latter method avoids the presence of a retained foreign body and the administration of a highly toxic chemical. Sequential observation of ulceration, regeneration, and repair were performed to determine the presence and pattern of proliferation at different stages.

Light microscopic and autoradiographic studies were also performed for each type of lesion for comparison with the results observed by the scanning electron microscopy technique.

**MATERIALS AND METHODS**

**Vascular Casts.** The rats were anesthetized with ethyl ether. The abdomen was opened with a midline incision from xiphoid to pubis and then extended to the knee bilaterally. If urine was present in the urinary bladder, the urethra was immediately clamped at the base of the penis. If the bladder was not partially inflated by urine, 0.2 to 0.3 ml of 0.9% NaCl solution was injected into the bladder via a urethral catheter and the urethra was then clamped. A cannula was inserted into the aorta distal to the renal arteries and tied in place. The pelvic vasculature was perfused with warmed 0.9% NaCl solution, drainage being from a small incision of the inferior vena cava at the junction of the iliac veins. The femoral arteries and seminal vesicles were ligated within 1 min after perfusion began. Once the perfusate was clear, Mercox CL-2B (prepared immediately before use because of rapid polymerization; Dainippon Ink and Chemicals, Inc., Tokyo, Japan) was injected through the aortic cannula at a pressure of less than 120 mm Hg until the perfusate showed evidence of polymerization. Perivesical fat and prostate were trimmed from the bladder without holding of the bladder with forceps. The bladder was removed after ligation of the bladder neck, and a small portion was excised for histological examination. The remainder of the bladder was digested with 25% NaOH until only the vascular cast remained. The cast was dried, coated with gold by the ion-spattering technique, and examined at 20 kV with a Hitachi HHS-2R scanning electron microscope. The small pieces of bladder for histological examination were fixed in ice-cold 3% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4. The specimens were washed with cacodylate buffer, dehydrated through an ascending alcohol series, and embedded in Epon 812. Sections 1 μm thick were stained with toluidine blue and examined for histological appearance and for the presence of leakage of the vascular cast technique were the same for both strains of rats and for the different ages examined. The normal bladder vasculature.

**Autoradiography.** Rats to be examined by autoradiography were given 1 i.p. injection of [3H]thymidine (New England Nuclear, Boston, Mass.) at 9:30 to 10:30 a.m. at a dose of 1 μCi/g body weight and were sacrificed 1 hr later (33). The bladders were inflated, fixed with 10% phosphate-buff ered formalin, and embedded in paraffin. Serial 5-μm-thick sections were coated with NR-M2 emulsion (Konishiroku Photo Industrial Co., Ltd., Tokyo, Japan) by the dipping method; the exposure time was 2 weeks. After development the sections were stained with hematoxylin and eosin. The labeling index for epithelial cells was calculated by counting 2000 epithelial cells in each of 3 areas of each bladder lesion or normal-appearing bladder. The results were compared statistically with the use of Student's t test.

**BBN Carcinogenesis.** One hundred male Wistar rats (Nihon Rat Co., Saitama, Japan) weighing approximately 200 g were given 0.05% BBN in the drinking water for 8 weeks and then maintained on water without BBN for an additional 32 weeks (40 weeks, total observation period) at which time carcinomas of the bladder were present (23). The rats had food (Oriental MF; Oriental Yeast Co., Tokyo, Japan) and water available ad libitum, and they were housed in a temperature- and humidity-controlled room. The rats were weighed periodically, but no growth retardation was noted. Eight rats were killed each week for the first 8 weeks and, at Week 40, 3 were used for preparation of vascular casts and 5 were used for autoradiography. In addition, 3 rats were killed each week for Weeks 9 to 12, 16, and 20 for preparation of vascular casts of the bladder. Histological appearances of the urinary bladder of rats were classified as follows: simple hyperplasia showing a diffuse proliferation of bladder epithelial cells increasing the mucosa to 4 to 6 cell layers; papillary and nodular hyperplasia with an obvious fibrovascular core by light microscopy; papilloma showing marked papillary proliferation with slight cellular atypia; and papillary transitional cell carcinoma with cellular atypism and occasional mitoses.

**Ulcer-Induced Hyperplasia.** Ulceration of the bladder mucosa with subsequent regenerative hyperplasia and repair was produced by applying a steel rod frozen at ~78° in dry ice-acetone to the serosal surface for 2 sec, twice, with 5 sec between applications as described previously (30). Thirty-two male Wistar rats weighing approximately 120 to 140 g were examined, and 8 rats were killed at each of Days 2, 5, 10, and 15 after ulceration. Five rats were examined by autoradiography, and 3 were used for preparation of vascular casts.

**Cyclophosphamide-Induced Hyperplasia.** Sixty male Fischer rats (Clea Inc., Osaka, Japan) weighing approximately 150 g were given a single i.p. injection of cyclophosphamide (Shionogi and Co., Ltd., Osaka, Japan) (25, 26, 28) 200 mg/kg body weight. Eight rats were sacrificed at each of Days 2, 5, 8, 10, 12, and 21 after injection; 5 of the rats were examined by autoradiography and 3 were used for preparation of vascular casts. The remaining rats died during the course of the experiment due to cyclophosphamide toxicity as evidenced by growth retardation and severe hematuria. Rats not treated with BBN, cyclophosphamide, or ulceration from both strains were also examined at different ages to determine the normal appearance of the bladder vasculature.

**RESULTS**

**Normal Urinary Bladder.** The histological appearance and the results obtained by autoradiography and by the vascular cast technique were the same for both strains of rats and for the different ages examined. The normal bladder epithelium consisted of 2 to 3 cell layers by light microscopy with a capillary network present immediately subjacent to the epithelial basement membrane (Figs. 1 and Neovascularization in the Urinary Bladder
Type 2 foci consisted of larger-diameter capillaries with extent or location of epithelial hyperplasia. When BBN was instilled, types 2 and 3, after Weeks 7 and 6, respectively. Decreased, and foci were rarely observed at Weeks 16, 20, and 40. Two other types of neovascularization were observed; however, almost all type 3 foci still lie beneath the basement membrane. Leakage of Mercox CL-2B from vessels was occasionally detectable, usually as spheres and less commonly as diffuse clumps of cast material which were easily distinguishable from the various patterns of neovascularization. Leakage was observed to a greater extent in casts prepared from ulcerated or cyclophosphamide-treated bladders.

BBN Carcinogenesis. Two weeks after administration of BBN, simple hyperplasia was present through the fifth week of BBN administration. Papillary and nodular hyperplasias were observed from the sixth week of BBN administration and thereafter. By Week 20 there were marked papillary proliferations with cellular atypia present. Papillary transitional cell carcinomas occupying the entire bladder lumen were present at 40 weeks.

Autoradiography showed rare labeled nuclei in epithelial or endothelial cells after 1 week of BBN. The labeling index in epithelial cells in areas of simple hyperplasia, nodular or papillary hyperplasia, and carcinomas was significantly higher than in the control bladder but not significantly different between these various lesions (Table 1). Occasional endothelial cells were labeled in the submucosa and increased slightly from Weeks 3 to 8 and in carcinomas after 40 weeks.

Small foci consisting of a high-density plexus of small-diameter capillaries with multiple short terminal branches (type 1) were detectable in vascular casts after 2 weeks of BBN treatment (Fig. 3). Although the number of type 1 foci per bladder increased through Week 8, the number was quite variable and appeared to have no relationship to the extent of epithelial hyperplasia. When BBN was discontinued after 8 weeks, the number of type 1 foci decreased, and foci were rarely observed at Weeks 16, 20, and 40. Two other types of neovascularization were observed, types 2 and 3, after Weeks 7 and 6, respectively. Type 2 foci consisted of larger-diameter capillaries with numerous long terminal branches projecting toward the lumen (Fig. 4) and usually arose from type 1 foci. Type 2 foci were infrequently present and were observed only after Weeks 7 and 8 of BBN administration. Type 3 foci consisted of highly tortuous capillary loops without terminal branches, and they arose from normal-appearing capillaries, not from type 1 foci (Figs. 5 and 6). The diameter of capillaries in type 3 foci was generally larger than that for normal capillaries and was generally uniform throughout 16 weeks (Fig. 7). By 20 weeks the diameter varied from area to area and was occasionally irregular; these variations in type 3 foci were more pronounced in carcinomas, but the vascular pattern was that of type 3 (Fig. 8). The number and extent of type 3 foci increased gradually, and the extent of papillary and nodular hyperplasia corresponded to the extent of type 3 foci (Fig. 6). The predominant pattern of neovascularization in papillomas and carcinomas was type 3. All type 3 foci observed at papilloma and papillary and nodular hyperplasia lie beneath the basement membrane. Microdestructions of basement membrane by invasive growth of transitional cell carcinomas were occasionally observed; however, almost all type 3 foci still lie beneath the basement membrane. Leakage of Mercox CL-2B from vessels was occasionally detectable, usually as spheres and less commonly as diffuse clumps of cast material which were easily distinguishable from the various patterns of neovascularization. Leakage was observed to a greater extent in casts prepared from ulcerated or cyclophosphamide-treated bladders.

Ulcer-Induced Hyperplasia. The entire thickness of the bladder wall was necrotic, with hemorrhagic ulceration of the mucosa. Regenerative hyperplasia of the mucosa followed ulceration with the most marked proliferation occurring 5 days after ulceration when there was papillary and nodular hyperplasia. The ulcer was completely covered with epithelium by Day 10, and the bladder returned to normal by Day 15. Subjacent to the ulcer was marked edema of the connective and muscular tissue and granulation tissue formation, which was most marked at Day 5.

The labeling index of epithelial cells in the hyperplastic areas surrounding the ulcer increased after ulceration with maximum labeling at Day 2 (Table 2). Nonhyperplastic epithelium also showed increased labeling at Days 2 and 5. Endothelial cells in submucosal vessels were labeled at Days 2 and 5 but were rare at later times. Fibroblasts, smooth muscle cells, and serosal cells were also labeled. The vascular casts demonstrated considerable leakage at the central areas of the ulcer at Days 2 and 5, but type 1 foci were identifiable at Days 2 and 5 (Fig. 9), became less frequent by Days 7 and 10, and were rarely seen at Day 15. Type 3 foci were observed at Day 5 (Fig. 10) but had disappeared by Day 15. No type 2 foci were observed.

Cyclophosphamide-Induced Hyperplasia. By the second day after injection, there is extensive hemorrhagic ulceration of the bladder mucosa with necrosis of the subjacent tissues. Slight hyperplasia was present by Day 2 and was most marked at Days 8 to 12 when the epithelium was 10 to 15 cell layers thick and papillary formation occurred. Focal areas of ulceration remained through Day 12 but were covered with epithelium by Day 21. Twenty-one days after injection foci of simple hyperplasia remained, with the mucosa 5 to 8 cells thick, and there was mild fibrosis in the submucosa.

The labeling index of the mucosa was greatest on Day 5.
and decreased thereafter but remained above normal levels at Day 21. Endothelial cells in the submucosa were most frequently labeled on Days 5 to 10 and then decreased, but labeled endothelial cells were present on Day 21 also. Fibroblasts, smooth muscle cells, and serosal cells were also labeled.

As in the bladders with ulceration due to freezing, there was extensive leakage from the ulcerated areas of the bladder, particularly submucosally, 2 to 10 days following cyclophosphamide treatment of the rats. Nevertheless, type 1 foci were easily identifiable at Day 2 and increased in number through Day 8 (Fig. 11). They gradually decreased and were infrequently observed on Day 21. Type 3 foci were first observed on Day 8, were most common on Day 12 (Fig. 12), but were rarely observed on Day 21. No type 2 foci were observed.

DISCUSSION

Several types of tissue have been examined for angiogenic potential by a variety of bioassay methods (1-4, 6, 8-12, 14-19, 21, 29, 31), but these methods do not demonstrate directly the interaction between epithelium and vascularity in situ. The preparation of vascular casts for examination by scanning electron microscopy makes possible the 3-dimensional study of vascular proliferation in situ, and in the case of the rat urinary bladder, a hollow viscous, the entire organ can be studied. This is of particular importance considering the multifocal nature of chemically induced bladder carcinogenesis. When used in conjunction with light microscopic examination of epoxy-embedded sections from Mercox-injected bladders before NaOH digestion, a correlation can be established between the histology of the epithelium and the type of vascularization present. For BBN carcinogenesis (20, 22-24), particularly the early stages, and for ulcer (30) and cyclophosphamide (25, 26, 28)-induced regenerative hyperplasia, there have been extensive studies on the types of epithelial lesions present at various times allowing further correlation between epithelial proliferations and the types of neovascularization observed by the vascular cast method at those times. Autoradiographic studies also provide information on the relationship of vascular proliferation with epithelial lesions.

Three patterns of vascular proliferation were detectable in the urinary bladder by the vascular cast method. Type 1 foci were observed during BBN carcinogenesis beginning at Week 2, increasing to Week 8 when BBN administration was discontinued, and decreasing subsequently. Type 1 foci at first appeared to be related to simple epithelial hyperplasia. However, during the first 5 weeks of BBN carcinogenesis, there was diffuse simple epithelial hyperplasia whereas type 1 foci were small and focal; some bladders, especially during Weeks 2 and 3, had simple hyperplasia but no type 1 foci were present. By light microscopy of epoxy-embedded tissues, occasional submucosal foci of increased vascularity were observed corresponding in size and frequency of occurrence to type 1 foci observed by scanning electron microscopy. In bladders with hyperplasia induced by ulceration or cyclophosphamide, type 1 foci were extensive and were observed in relationship to foci of granulation tissue during the process of repair. In addition, type 1 foci were only infrequently observed in bladder carcinomas, and they were related to foci of necrosis and inflammation present in those tumors. In a transplantable bladder carcinoma, type 3 foci were also the predominant pattern of vascular proliferation, but type 1 foci were observed around areas of necrosis (unpublished observation). Thus, it appears that type 1 foci correspond to direct injury to blood vessels due either to necrosis and inflammation or to a direct action of BBN. Occasional hemangiomas of the bladder have been observed in animals that received BBN, a more extreme reaction of the vasculature to BBN (13).

Few type 2 foci were observed and only at Weeks 7 and 8 of BBN carcinogenesis, but not in rats given cyclophosphamide or ulcerated with a frozen rod. Papillary and nodular hyperplasia were already present, but the relationship of the type 2 foci to these lesions could not be ascertained. Type 2 foci arose from type 1 foci but were comprised of vessels of larger diameter with long terminal branches. New vessels are fragile, and slight damage would result in leakage. Although type 2 foci might represent leakage from type 1 foci, it is very unlikely since type 1 foci were observed...
at several time intervals in all 3 groups of rats, but type 2 foci were only rarely observed. Also, leakage was observed frequently in casts of bladders with ulcerated lesions and regenerative hyperplasia and less frequently during BBN carcinogenesis and in normal rats, and the leakage always took the shape of spheres or sheet-like structures. Leakage also could be excluded by examination of sections from the epoxy-embedded material.

In contrast to type 1 foci, type 3 foci were always observed in relationship to marked epithelial proliferative lesions, papillary or nodular hyperplasia, papillomas, or carcinomas. They arose from normal capillary areas, and the endothelial cells demonstrated no cellular atypia. If the epithelial lesions regressed as in the rats treated with cyclophosphamide or ulceration by a frozen rod, then the vascular proliferations also regressed. Progression of the lesions to carcinoma resulted in maintenance and progression of the vascular proliferations. Although proliferating epithelium is not a requirement for the induction of angiogenesis (2), epithelial proliferation is one of the important factors in the induction of type 3 neovascularization. The labeling index during BBN carcinogenesis increased by Week 2 and remained elevated, but type 3 lesions did not appear until Week 6. The labeling index in bladders ulcerated by freezing or cyclophosphamide increased rapidly, reaching a maximum on Days 2 and 5, respectively, but also decreased rapidly, approaching normal values by Days 10 and 21, respectively. Type 3 foci appeared at Days 5 and 8, respectively, when epithelial hyperplasia was at a maximum.

The relationship between neovascularization and the growth of cancer has been emphasized by several investigators (9), and it has been suggested that a tumor angiogenesis factor may be secreted by tumor cells to stimulate neovascularization (9–12). Thus, angiogenic potential has been demonstrated for several tumours. However, various normal adult and embryonic and benign proliferative tissues also have demonstrated angiogenic activity (4, 31). The results of our experiments indicate that neovascularization generally is not detectable in the normal urinary bladder but that it occurs in both reversible and irreversible epithelial proliferative lesions. One type of response (type 1) appears to be related to direct damage or stimulation of blood vessels, whereas the type 3 response appears to be dependent on epithelial proliferation. The in situ method of studying the interaction between epithelium and endothelium only allows correlations of each other. However, the type 3 foci appeared after epithelial proliferation had begun. The type 3 foci could possibly have been caused by a factor secreted by proliferating epithelial cells that stimulated vascular proliferation. Interactions between epithelium and mesenchyme are necessary for development and differentiation of tissues (7). Angiogenic factor from epithelial cells may be one of the factors of epithelio-mesenchymal interactions.

Because vascular proliferation occurred whether the epithelial cells were benign or malignant, it would be more reasonable to assume that these proliferating epithelial cells secrete an angiogenesis factor by activation of a gene already coded in the normal cell DNA than to assume that the cell has produced a new factor requiring a new gene.

REFERENCES


Fig. 1. Vascular cast of normal urinary bladder demonstrating loose subepithelial capillary plexus. × 100.

Fig. 2. Relationship of subepithelial capillaries and epithelium in normal urinary bladder embedded in epoxy after intravascular injection of Mercox CL-2B. Toluidine blue, × 400.

Fig. 3. Vascular cast of bladder after 2-week BBN administration showing a focus of type 1 vascular proliferation consisting of a high-density plexus of narrow capillaries. × 150.

Fig. 4. Type 2 vascular proliferation in a rat after 8 weeks of BBN administration. × 400.
Fig. 5. Early stage of type 3 vascular proliferation arising from an area of normal capillaries in a rat after 8 weeks of BBN administration. × 180.

Fig. 6. Increased number of capillaries in subepithelial area in an area of nodular hyperplasia. No leakage from vessels is seen. Specimen was prepared by intravascular injection of Mercox and embedding in epoxy. Toluidine blue, × 400.

Fig. 7. More extensive type 3 vascular proliferation in a rat after 8 weeks of BBN followed by 8 weeks of observation (16 weeks). × 100.

Fig. 8. Type 3 vascular proliferation in a urinary bladder carcinoma induced by BBN (40-week total observation period), showing variation in size and shape of the capillary loops. × 180.
Fig. 9. Type 1 vascular proliferation 5 days after ulceration of the bladder by freezing. × 450.
Fig. 10. Type 3 vascular proliferation in the bladder 10 days after ulceration by freezing. × 300.
Fig. 11. Type 1 vascular proliferation in the bladder 5 days after i.p. cyclophosphamide injection. × 250.
Fig. 12. Type 3 vascular proliferation in the bladder 12 days after cyclophosphamide injection. × 250.
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