Heterotopic Urinary Bladders in Rats Produced by an Isograft Inoculum of Bladder Fragments and Air

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Summary

A pseudobladder is formed by inoculating rats s.c. with a combination of rat bladder fragments and air. Within 1 month a cyst is produced that is lined completely by normal-appearing urothelium. The heterotopic pseudobladder persists for as long as 6 months. In some 6-month-old bladder walls, foci of bone formation are found in the stroma underlying the urothelium. The lining epithelium at all stages from 1 to 6 months is characteristic of normal urothelium by light and electron microscopic criteria.

Introduction

The search for an in vivo model with which to study the genesis, neoplastic progression, and spread of bladder cancer led to development of the model described in this paper in which is created a cyst with a volume several ml that is lined by normal urothelial cells. Our early work was directed toward the use of the granuloma pouch developed by Selye (4), but this pouch proved unsatisfactory for the establishment of a lining of normal urothelium. Modifications were tried using autotransplants of urinary bladder wall, minced and injected into preformed granuloma pouches. This approach was not successful possibly because of the environment of the inflammatory pouch itself or the small number of urothelial fragments injected. After these initial failures we discovered that a mince of bladder from sibling rats survived, proliferated, and formed a gross pouch completely lined by transitional epithelium when the tissue fragments were injected with a large volume of air s.c. in the backs of Lewis-Wistar rats.

Huggins (2, 3), in his experiments on osteogenesis, was able to grow cysts completely lined by transitional epithelium in the dog using intact portions of bladder mucosa and succeeded in his aim of achieving bone formation. In similar osteogenesis experiments, Fahrer et al. (1) worked with guinea pigs using flaps of bladder mucosa. Recently, Yalciner and Friedell (5) have reported the production of very small cysts, 2 to 3 mm in diameter, lined by transitional epithelium. These occurred following s.c. inoculation by trochar in the rat of a 1- to 2-cu mm fragment of bladder wall.

Our present model is inexpensive and easy to produce in a short period of time. Large pouches are completely lined by a transitional epithelium in 3 or 4 weeks and, with their epithelial lining intact, have persistend for as long as 6 months, the longest interval studied at this time. The lining is normal by both light and electron microscopic criteria.

Materials and Methods

Rats used in this experiment were of the Lewis-Wistar strain, approximately 8 weeks old and averaging 240 g. Three rats were used as donors for each recipient rat. Donors were killed by an i.p. injection of pentobarbital sodium. Recipient rats were anesthetized with i.p. pentobarbital sodium in a dose of 5 mg/100 g of rat. Immediately after killing, the urinary bladders of 3 rats were removed intact with as little prosthetic and perivesical fat as possible and placed in a glass Petri dish containing 2 drops of Hanks' balanced salt solution. The bladders were cut with 2 No 11 knife blades on No 7 scalpel handles until the mince was fine enough to pass through a 16-gauge needle. Approximately 1 ml of the mince was drawn up into a 20-ml plastic syringe fitted with a 16-g x 1.5-inch B-D Yale needle. Fifteen ml of air were added to the contents of the syringe.

The back of each recipient was shaved and cleaned with 70% alcohol. The needle was introduced s.c. into the back from caudad to cephalad for its full length. The air and bladder mince were then injected, and an additional 2 cu cm of air were passed through the needle to flush out any remaining mince. A wire animal clip was placed over the entrance wound as the needle was withdrawn to prevent escape of air and mince. Coincident with injection a large bubble appeared under the skin of the rat. By 48 hr after inoculation most of the air had been absorbed, leaving a palpable fluctuant cyst approximately one-fourth of the initial size. In most animals the cyst remained this size for the duration of the experiment.

All animals were inspected daily for the presence of the pouch. Pouches were harvested at 7 and 11 days and at 2, 4, 8, and 24 weeks. Animals were killed with pentobarbital sodium, and the cyst was excised with a wide cuff of contiguous skin. Cysts were carefully dissected free of the s.c. tissue and fixed in 10% alcoholic formalin for 24 hr before being cut for histological study (Fig. 1). Sections of tissue were stained with hematoxylin and eosin.

1 Supported by Research Grant CA 14137 from the National Cancer Institute. Reprint requests to any of the authors should be addressed to Cancer Bioassay Laboratory, Department of Pathology, The Medical College of Pennsylvania, 3300 Henry Avenue, Philadelphia, Pa. 19129.
2 Presented April 29, 1974, to Delta Chapter, Alpha Omega Alpha, National Medical Honor Society.
3 Institute for Environmental Medicine, New York University Medical Center, New York, N. Y.
Specimens from donor bladder and pouches of 4, 8, and 24 weeks were processed for electron microscopy. Fragments measuring 1 to 2 cu mm were fixed at room temperature in 3.5% glutaraldehyde in phosphate buffer containing 1% CaCl₂. The tissues were washed in phosphate buffer and post-fixed in 1% osmium tetroxide for 1 hr in the refrigerator. The specimens were then dehydrated in graded ethyl alcohol, brought back to room temperature, processed through Epon 812-propylene oxide mixture, and finally embedded in Epon 812. Sections 1 μm thick were cut and stained with toluidine blue. Ultrathin sections of appropriate fields were cut with a diamond knife on a Porter-Blum (MT-2) ultramicrotome. These were stained with uranyl acetate and lead citrate, and examined with a Hitachi HS-7S electron microscope at an acceleration of 50 kV.

RESULTS

Gross Examination and Light Microscopy. Our observations are summarized in Table 1. After 7 days we found a well-formed single cyst filled with hemorrhagic fluid. The connective tissue wall of the cyst was 2 to 3 mm thick and lined on its inner surface by scattered patches of viable transitional epithelium (Figs. 2 and 3).

By the 11th and 14th days one-half to three-fourths of each cyst was lined by urothelium. The wall by the 14th day was still 2 to 3 mm thick and was composed mainly of fibrous tissue with some inflammatory cells. The cyst fluid was dark brown. At both early stages a few specimens showed foci of necrotic epithelium within their walls. One 14-day-old pouch contained in the wall a focus of glandular tissue resembling prostate. A few bundles of smooth muscle were also noted in the wall of most cysts.

By 2 weeks, and thereafter, the pouches appeared to be multilocular. On careful examination we found that the multiple cystic structures were composed of a single large chamber that communicated directly with a number of smaller cavities. In addition a few small completely isolated cysts were sometimes found. The central cavities varied in fixed tissues from 11 x 8 x 2 mm to 5 x 5 x 2 mm. The volume of fluid that could be aspirated from the cysts varied from 2 to 4 ml.

At 4 weeks, 7 of 9 specimens contained pouches completely lined by transitional epithelium from 2 to 5 cell layers thick (Figs. 4 to 6). The other 2 specimens were primarily composed of necrotic tissue in clumps with many inflammatory cells. The fluid in the cysts was much clearer than at earlier stages. In several of the pouches the wall adjacent to the skin surface was thin, although the urothelial lining of the whole cyst was a uniform 2 or 3 cell layers thick. In 1 rat the pouch lining was complete but the wall had areas of necrosis and inflammation. Smooth muscle bundles were again consistently seen (Fig. 7). These appeared to be located in the connective tissue just beneath the urothelial surface.

Of the 8-week-old pouches, 4 of 5 were excellent with a complete lining of transitional epithelium characteristic on light microscopy. In 1 pouch the lining of epithelium was interspersed with foci of necrosis. Smooth muscle persisted in this group and appeared in its usual pattern, as bundles of muscle cells beneath the urothelium. The cystic fluid here was light brown.

By 24 weeks pouches were still present in all rats (Figs. 8 and 9), but 2 of the 9 pouches showed much chronic inflammation and necrosis. The others maintained a complete

<table>
<thead>
<tr>
<th>Wk after preparation</th>
<th>No. of rats inoculated</th>
<th>No. of sacs identified</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>Scattered foci of transitional epithelium line the cyst. Cyst fluid is sanguineous. Cyst measures 8 x 8 x 3 mm. Cyst wall measures 2 or 3 mm maximum.</td>
</tr>
<tr>
<td>1.5</td>
<td>2</td>
<td>2</td>
<td>There are several communicating cavities. Cysts are one-half to three-fourths lined by transitional epithelium. Cyst fluid is dark brown. Largest cyst measures 8 x 8 x 2 mm. Bundles of smooth muscle are noted in cyst wall.</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>5</td>
<td>All cysts are approximately four-fifths lined by transitional epithelium. One cyst is completely lined and has a small focus of necrotic tissue in wall. One pouch contains what appears to be prostatic tissue in the wall. Fluid is serosanguineous. Largest cyst measures 9 x 8 x 2 mm. Smooth muscle persists in wall.</td>
</tr>
<tr>
<td>4º</td>
<td>9</td>
<td>7</td>
<td>Two specimens are composed of necrotic tissue and inflammatory cells. Seven pouches are completely lined by transitional epithelium. In one the lining has interspersed areas of inflammatory cells. Cyst fluid is light brown. Largest cyst measures 11 x 8 x 2 mm. Smooth muscle bundles are present in wall underlying the epithelium.</td>
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<tr>
<td>8º</td>
<td>5</td>
<td>5</td>
<td>All cysts are completely lined by transitional epithelium. One pouch contains an area of inflammatory cells and necrotic tissue. Fluid is dark brown to yellow. Largest cyst measures 5 x 5 x 2 mm.</td>
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<tr>
<td>24º</td>
<td>9</td>
<td>9</td>
<td>Cysts are completely lined by transitional epithelium. Two pouches have much necrosis and inflammation. Three pouches contain considerable bone in the wall underlying the epithelium. Cyst fluid is serous. Smooth muscle is present in some of the pouch walls. Largest cyst measures 6 x 5 x 3 mm.</td>
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* Electron microscopy done on one specimen. In every case superficial epithelium contained discoid vesicles lined by asymmetrical membranes characteristic of urothelium.
lining of urothelium, some with small foci of necrotic tissue and hemosiderin-laden histiocytes in the wall. Pouch fluid was pale yellow. The largest cyst at this stage measured approximately 6 x 5 x 3 mm. Three of the specimens showed bone formation in the pouch wall, which in certain areas was closely apposed to the urothelial surface (Fig. 10).

Electron Microscopy. A comparison was made of the ultrastructural features of the donor urinary bladder epithelium and the transplanted epithelium after 4, 8, and 24 weeks. The transplanted epithelium had the characteristic features of transitional epithelium. The luminal surface was irregular with alternating projections and depressions. The superficial cells were darker than the intermediate and basal cells (Fig. 11). The characteristically asymmetrical unit membrane, unique to the bladder, was found in its typical locations, on the luminal surface and around discoid, fusiform, or round vesicles in the cytoplasm of the cells in the 2 or 3 superficial cell layers. The vesicles were randomly oriented with the most superficial ones generally perpendicular to the luminal surface. The asymmetrical unit membrane consisted of a thick luminal component overlying a thinner, darker cytoplasmic component. The 2 components were separated by an electron-lucent zone (Fig. 12). The multilayered transitional epithelial cells were resting on a continuous basement membrane that separated them from the underlying collagen fibers (Fig. 13).

DISCUSSION

The clinical and experimental literature on chemical carcinogenesis of the urinary bladder is large, but for postcarcinogenesis phases hard facts about the progression and the spread of bladder cancer are meager. From clinical experience we know that low-grade papillary tumors sometimes "recur" with the same features and are effectively managed by local destruction of recurrences. At other times recurrences are found to be invasive cancers requiring major surgical procedures and radiation therapy. It is not known what distinguishes the 2 patterns of recurrence from one another, whether the populations of transformed cells that give rise to each tumor are unique from the very beginning, or whether over a period of time populations of cells of lower malignancy evolve to give rise to progeny of higher malignancy.

We developed the heterotopic urinary bladder in the rat as a 1st step in a long-term experimental study of the biology of urothelial cancer. We are studying the implantation of established transplantable rat bladder cancers on the normal lining of the experimental bladder. We are also concerned with the effect of carcinogens applied topically in the urothelial pouch with particular interest in the course of the neoplasm and the host from the diagnostic onset to the death of the animal. Unlike the intact urinary bladder, urinary obstruction and infection leading to death of the host is not a problem early or late in the experiment. We are thus able to anticipate longer periods of study for individual animals, providing a better opportunity to study the complex phenomena of progression.

As a refinement of the model we are determining the best means of producing a fistula to connect the sac with the skin surface. Such a "pseudourethra" would permit the insertion of chemicals or the removal of samples of the fluid contents at appropriate intervals. Changes in the mucosal epithelium in response to inocula may be monitored with a small cystoscope in the same manner as in the practice of urology.

This technique could be applied in other areas of cancer research. Cavities lined by epithelia from various organs might be produced since there is no reason to assume that the formation of heterotopic cysts is limited to rats or to urothelium.

ACKNOWLEDGMENTS

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REFERENCES

Fig. 1. A, dorsal view of a rat 2 months after the preparation of a heterotopic "urinary bladder" which appears as an oval cyst. B, view of the pouch in A. A flap of skin was folded back to expose the deep aspect of the adherent pouch, and the pouch was incised. There were 2.5 ml of brown fluid in the pouch. The lining of the pouch has a trabecular pattern.

Fig. 2. View of a segment of the 7-day-old pouch at low magnification. A single cavity is lined by a thin patchy layer of urothelium. Most of the wall is composed of dense connective tissue. H & E, X 35.

Fig. 3. Higher magnification of the 7-day-old pouch. A representative segment from a wide patch of urothelium demonstrates the overall normal appearance. Deep to the epithelial layer there is a dense connective tissue. H & E, X 320.

Fig. 4. View of a portion of a 4-week-old pouch at low magnification. In the upper part of the figure is a portion of the large central cavity, now collapsed following incision and fixation. In other planes the lining epithelium was seen to be continuous between the main chamber and its accessory chamber in the lower right. H & E, X 35.

Fig. 5. Higher magnification of a 4-week-old pouch. A representative area shows the normal urothelium characteristic of the entire pouch. H & E, X 320.

Fig. 6. Greater detail of another representative area from a 4-week-old pouch. As in Fig. 5, normal urothelium is seen, including the "umbrella" cells constituting the most superficial layer of the normal stratified transitional epithelium. H & E, X 525.

Fig. 7. View of smooth muscle in the wall of a 4-week-old pouch. Smooth muscle persisted for 6 months and appeared normal. H & E, X 320.

Fig. 8. View of part of a 6-month-old pouch at low magnification. An hour-glass communication (upper left) joins 2 contiguous large cavities both completely lined by urothelium. H & E, X 35.

Fig. 9. At higher magnification, the 6-month-old urothelium looks completely normal. H & E, X 320.

Fig. 10. View of a focus of bone formation in the stroma immediately below urothelium 6 months after preparation of the pouch. H & E, X 320.

Fig. 11. Low-magnification electron micrograph of the cells lining the heterotopic bladder 6 months after preparation. The cells bordering the lumen (upper right-hand corner) stain darkly and contain numerous discoid, fusiform, or round vesicles characteristic of rodent urothelium. Uranyl acetate and lead citrate, X 5,000.

Fig. 12. High-magnification electron micrograph of luminal surface (upper left-hand corner) and a portion of a vesicle from a superficial cell of a 6-month-old pouch. These structures are delineated by an asymmetrical membrane that consists of a luminal thicker component and a cytoplasmic thinner component. The 2 components are separated by an electron-lucent zone. Uranyl acetate and lead citrate, X 124,000.

Fig. 13. A portion of a basal cell of a 6-month-old pouch is resting on a continuous basement membrane that follows the basal infolding of the urothelial cell. The membrane separates the epithelium from the underlying collagen fibers. Uranyl acetate and lead citrate, X 16,000.
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