Establishment and Characterization of a Transplantable Dibutylnitrosamine-induced Mouse Bladder Tumor Line FCB

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SUMMARY

Bladders were removed from IF × C57BL F₁ hybrid mice that had been treated for 36 weeks with dibutylnitrosamine and implanted s.c. into similar hybrid mice to await the appearance of tumors. One of the tumors induced by this method has been serially transplanted for 42 generations. This has been designated FCB and is a rapidly growing transitional cell carcinoma with a stable morphology, as shown by light and electron microscopy. The tumor grows in a variety of sites and can be transplanted to either C57BL or IF mice. It will grow rapidly in vitro as a monolayer and re-forms solid tumor tissue upon reimplantation. The growth characteristics and morphology of the FCB tumor are described and discussed.

INTRODUCTION

Although a number of studies have been made of experimental bladder carcinogenesis in rats (9, 11, 12, 23, 24, 39), rather less attention has been paid to chemically induced murine bladder tumors (20, 38). In part this may be due to the fact that few agents are known to be potent bladder carcinogens in mice (1, 2, 8, 10, 19). However, DBN, which is a recognized bladder carcinogen (28), has recently been shown to be very active in the mouse (4, 42). While it is known that mouse bladder tumors can be transplanted (20), no studies appear to have been made of their fine structure during serial transplantation, and no established tumor line appears to be available.

The aim of the present investigation was to study the transplantability of DBN-induced murine bladder tumors and to attempt to establish and characterize a stable transplantable bladder tumor line. Since the induction of mouse bladder tumors by DBN requires prolonged treatment and is preceded by a lengthy latent period (42), a method was used in this study which involved the s.c. implantation, into young isogeneic mice, of bladders removed from animals that had been treated with the carcinogen. This was an adaptation of a technique used previously in studies of murine pulmonary tumor induction (14, 16, 17, 25).

MATERIALS AND METHODS

Induction of Bladder Tumors

The animals used throughout this investigation were male IF × C57BL F₁ hybrid mice, approximately 2 months old. These had been bred in this laboratory and received Oxoid 42B diet and water ad libitum during the experiment.

DBN was synthesized by R. Turner in this laboratory from dibutylamine (Koch-Light, Colnbrook, England) by the method of Vogel (40). The purity of the product was established by its traveling as a single peak on gas chromatography (Perkin Elmer F11: Apiezon L, and Carbowax and KOH, coated capillary columns).

A total of 18 s.c. injections of 5 µl, at 14-day intervals, were administered to each of 6 mice. Three weeks after the final injection, the mice were killed by cervical dislocation and their bladders were removed and implanted s.c. into the flanks of a further six 2-month-old IF × C57BL F₁ mice. Three of these implanted bladders subsequently gave rise to tumors.

The tumors were examined histologically and 2 were identified as malignant transitional cell carcinomas, the remaining tumor being a hemangioma. All the tumors were transplanted into s.c. sites in similar F₁ hybrid mice. One of the transitional cell carcinomas, designated FCB, was selected for further study and was maintained by serial s.c. transplantation in these mice.

Study of Bladder Tumor FCB

Growth Rate of s.c. Implants in Isogeneic Mice. After the 7th transplant generation, tumor tissue was removed from host mice and finely minced. Each of 18 mice received a standard 0.1-ml s.c. inoculum of tumor tissue. Two further groups of mice were similarly treated, the tumor inocula, however, being 0.5 and 1.0 ml, respectively. After 19 days all the animals were killed, except for one in the final group that had died previously, and their tumors were weighed and measured with calipers.

Growth of s.c. Implants in C57BL and IF Mice. After 20 transplant generations, the FCB tumor was implanted into 18 mice of each of the 2 strains, C57BL and IF. The
implants, approximately $2 \times 2 \times 3$ mm, were implanted s.c. by means of a trocar into the flanks of the recipients.

**Growth in Vitro.** Tumor tissue was teased out into Medium 199 (Biocult Laboratories, Paisley, Scotland) and then grown as monolayers in glass bottles, using the same medium.

**Implantation into Various Sites.** Single pieces of the solid tumor, approximately 1 to 2 mm in diameter, were implanted surgically into the lumina of the bladders of 12 mice.

After 48 days of growth in tissue culture, the tumor cells were made into a suspension. Three groups of mice, each consisting of 12 male 1F x C57BL F1 hybrids, were inoculated with this cell suspension. Each mouse received a single injection of 0.2 ml (approximately $2 \times 10^4$ cells). The 1st group of mice were given s.c. injections into the flank, the 2nd were treated i.p., and the 3rd received injections into the pelvis of the kidney.

**Electron Microscopy.** Tumor-bearing mice were killed by cervical dislocation when the tumor was in its 20th, 34th, and 42nd transplant generations. The tumor tissue was rapidly excised and samples, taken from the solid peripheral areas, were placed immediately into ice-cold 4% glutaraldehyde, buffered with 0.067 M cacodylate buffer (37) at pH 7.2. The tissue was minced into 1-cu mm pieces and fixation continued for 4 hr, at 0-4°. It was then washed for 16 hr at 0-4°in 0.25 Mr sodium cacodylate and postfixed in Millonig's tartrate (TAAB Laboratories, Reading, England) in 0.1 N sodium hydroxide, buffered with 0.067 M cacodylate buffer and examined in a Philips 301 electron microscope at an accelerating voltage of 80 kV.

Sections were cut with glass or Ge-Fe-Ri diamond knives, on a Sorvall MT-1 Porter-Blum ultramicrotome. They were mounted on uncoated copper grids, stained with 0.2% lead tartrate (TAAB Laboratories, Reading, England) in 0.1 N sodium hydroxide, and examined in a Philips 301 electron microscope at an accelerating voltage of 80 kV.

For light microscopy the tumor tissue was fixed in 10% buffered formol-0.9% NaCl solution and paraffin embedded; the sections were stained with hematoxylin and eosin.

**RESULTS**

**Light Microscopy.** The FCB tumor originated, in the bladder implant, as a moderately well-differentiated stage 1 (36) transitional cell carcinoma (Figs. 1 and 2). This exhibited numerous mitotic figures and was seen to be invading the submucosa on a broad front. Some evidence of squamous metaplasia was present. After the 15th transplant generation, the FCB tumor presented a stable histological appearance, which was that of an anaplastic tumor (Figs. 3 and 4), with a very high incidence of mitoses. The cells were more elongated and some giant cells were seen.

**Electron Microscopy.** The FCB tumor appeared, in the electron microscope, to be composed of loosely packed cells which were irregular in outline (Fig. 5). The cells failed to exhibit any marked degree of polarity. Their nuclei appeared to be round or irregularly oval and possessed few deep indentations. However, nuclei that contained abnormal inclusions and pseudoinclusions were occasionally observed (Fig. 6).

The cells possessed abundant cytoplasm, which contained moderate numbers of mitochondria. These mitochondria were small in size and variable in shape and possessed relatively few cristae.

In addition, the cytoplasm contained variable quantities of the granular endoplasmic reticulum. This organelle was sometimes present as short cisternae, dispersed throughout the cytoplasm (Fig. 5). However, it also frequently appeared in more bizarre forms (Figs. 7 and 8), which included elongated, sinuous cisternal profiles and simple or complex annular structures. In some cells the granular endoplasmic reticulum was widely distended and its lumen contained a finely granular material (Fig. 9).

The Golgi apparatus was well developed (Fig. 10) and some tumor cells contained numerous dictyosomes (Fig. 11).

An abundance of free ribosomes was observed in the cytoplasm. In some of the tumor cells the majority of the ribosomes were present as single particles (Fig. 7); in others they were present as "polysomal" groups (Fig. 8). In any single cell the state of aggregation of the ribosomes attached to the granular endoplasmic reticulum was similar to that of the free particles.

The cell surface was irregular but lacked any apparent specialized structures such as microvilli. However, in spite of the absence of readily discernible cellular polarity, the presence of desmosomes at the adjoining surfaces of the tumor cells betrayed their epithelial character (Fig. 10).

In all the tumor samples that were examined by electron microscopy, a substantial proportion (approximately 10%) of the cells contained virus-like particles. The particles appeared to be exclusively present within the cisternae of the granular endoplasmic reticulum (Fig. 11).

Immature particles were also occasionally observed, in the process of budding into the cisternal lumen (Fig. 11). These particles had the characteristic morphological appearance of a type A virus.

**Growth Rate of s.c. Implants of Solid FCB Tumor.** The s.c. tumor implants grew rapidly, killing their hosts in approximately 3 weeks. Measurements of tumor growth are summarized in Table 1. A 15- to 30-fold increase in volume was noted after 19 days of growth.

**Growth in C57BL and 1F mice.** The FCB tumor implants

<table>
<thead>
<tr>
<th>No. of mice</th>
<th>Size of tumor inoculum (ml)</th>
<th>Mean dimensions of tumor implants (mm)</th>
<th>Mean wt of tumor implants (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>1.0</td>
<td>41 x 18</td>
<td>10.62</td>
</tr>
<tr>
<td>18</td>
<td>0.5</td>
<td>34 x 21</td>
<td>5.47</td>
</tr>
<tr>
<td>18</td>
<td>0.1</td>
<td>15 x 11</td>
<td>1.47</td>
</tr>
</tbody>
</table>
grew in both the parent strains. Growth was rapid and the tumors reached large proportions within 3 weeks, the majority of the hosts dying shortly thereafter.

**Growth in Vitro.** The tumor cells grew well in tissue culture. The cultured cells grew as monolayers on glass and appeared to be epithelioid in their morphology. The monolayers became confluent approximately 6 days after each culture was “split,” the size of the inoculum being $5 \times 10^6$ cells, using 4-ounce culture flasks. After the 7th passage *in vitro*, s.c. reinoculation of the cells into the flanks of male IF × C57BL F, hybrid mice gave rise to solid tumors. These grew as rapidly as the original FCB tumor and were histologically indistinguishable from it.

The culture medium in which the cells were growing became somewhat viscous and sticky after 3 days of growth.

**Growth in Other Sites.** Of the 12 mice with solid FCB tumor tissue implanted into their bladder lumina, all developed large tumors, arising from the implants. The injection of tissue-cultured tumor cells into the pelvis of the kidney also invariably resulted in their rapid growth in that site. After 21 days the recipient mice had to be killed, owing to the presence of large FCB tumors in the kidney. However, an attempt to grow the FCB tumor in ascites form, by injecting cultured cells into the peritoneal cavity, was unsuccessful. In all the recipients the tumor grew well as disseminated nodules of solid tumor tissue attached to the peritoneum. However, only 1 mouse out of 12 also showed the presence of i.p. fluid when sacrificed. When this fluid was passaged in further recipients no tumors were obtained.

**DISCUSSION**

The present investigation has demonstrated that DBN can be used to induce transplantable transitional cell carcinomas of the bladder in mice. The tumor line established, FCB, is less well differentiated than the original tumor. This is not unexpected in view of the known capacity for progression in mouse bladder tumors (20). However, by the 15th transplant generation the morphology had become stable, no further progression being evident thereafter, in histological or electron microscope preparations, until the 42nd generation.

It is evident that the FCB tumor will grow rapidly, both *in vivo* and *in vitro*. *In vivo* it will grow readily in a variety of sites, although not in ascites form. Both the solid tumor and the monolayer growing in cell culture produce copious amounts of mucin-like material. In addition, the FCB tumor can be transplanted not only into the IF × C57BL F, hybrids in which it originated but into either of the parent strains.

The ability of the cell cultures of FCB, upon reimplantation, to produce solid tumors with a morphology identical to those transplanted normally was noted. This is in accord with the findings of Rigby and Franks (35) that human bladder tumor cell lines behaved similarly when implanted into the hamster cheek pouch.

Electron microscopy of the FCB tumor revealed that it was composed of loosely packed, irregularly shaped cells. The tumor cells lacked the fusiform cytoplasmic vesicles that are characteristic of the normal transitional epithelial cells of mice and other species (21, 31, 33, 34, 41). This represents a loss of differentiation which is correlated with the anaplastic histological appearance of the tumor and the apparent loss of polarity of the cells. However, the presence of adhesion plaques between adjacent tumor cells (11, 32) provided clear evidence of their epithelial cell origin. The abundance of free ribosomes observed in the cytoplasm is characteristic of the cells of rapidly growing tumors.

The well-developed granular endoplasmic reticulum and Golgi apparatus possessed by the FCB tumor cells suggest a capacity for secretory activity. This is reinforced by the observations of granular endoplasmic reticulum cisternae distended with material and the ability of the tumor to produce an abundance of mucin-like material, both *in vivo* and *in vitro*. The possibility arises, therefore, that the FCB tumor originated from the bundle cells (21, 30) of the transitional epithelium. However, no other fine structural evidence was available to support this view, and a well-developed granular endoplasmic reticulum has been observed in human transitional cell carcinoma (15).

Many of the fine structural features of the FCB tumor cells, including the variable appearance of the mitochondria and the granular endoplasmic reticulum, were those common to many tumors, whatever their origin. The nuclear inclusions observed appeared to be derived mainly from pseudoinclusions (3, 5, 7, 22, 26) or cytoplasmic invaginations, rather than the true inclusions observed in some tumors (6, 18). The development of these types of inclusion has been discussed by Kendrey (22). However, the constant presence of A-type virus particles was of some interest, in view of the reported presence of cross-reacting tumor-associated antigens in chemically induced mouse bladder tumors (38), although there is no evidence of any causal relationship between the virus and the induction of the FCB tumor.

The establishment and characterization of the FCB tumor line provides a new model for studies of tumor behavior, since such transplantable murine bladder tumor lines are relatively unknown. A particular advantage of FCB is that it is equally suitable for *in vivo* or *in vitro* studies.

Finally, it appears that the 2-stage method of tumor induction used in this study may provide a useful technique, which could be applied with advantage to studies of any type of tumor that requires a long period of time to develop.

**ACKNOWLEDGMENTS**

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**REFERENCES**

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Fig. 1. Photomicrograph of mouse bladder containing DBN-induced transitional cell carcinoma. H & E, × 40.

Fig. 2. Higher-power photomicrograph showing detail of Fig. 1. The invading edge of the bladder tumor is shown. H & E, × 290.

Fig. 3. Survey photomicrograph of FCB transplantable bladder tumor. H & E, × 40.

Fig. 4. Photomicrograph, at higher magnification, of the FCB tumor. H & E, × 290.

Fig. 5. Survey electron micrograph of the FCB tumor, showing several cells that are irregular in shape and loosely packed. The nuclei are characteristically round or oval. × 11,500.

Fig. 6. Electron micrograph showing detail of nucleus (N) of FCB tumor cell. Note the presence of numerous abnormal inclusions. × 20,000.

Fig. 7. The electron micrograph shows elongated profiles of the granular endoplasmic reticulum (ER) in the cytoplasm of an FCB tumor cell. The ribosomes, whether free or attached to the membranes, are present as single particles. × 26,000.

Fig. 8. Detail of the cytoplasm of an FCB tumor cell. The granular endoplasmic reticulum (ER) forms complex annular profiles. The ribosomes in this cell are present as polysomal groups. × 26,000.

Fig. 9. Electron micrograph of FCB tumor cell cytoplasm showing the distension of the granular endoplasmic reticulum (ER) by its finely granular contents. The mitochondria are irregular in shape. × 26,000.

Fig. 10. A large Golgi zone (G) is shown in this electron micrograph. Junctional structures are present at the surfaces of 2 adjacent tumor cells (arrow). × 20,000.

Fig. 11. Electron micrograph of a portion of a FCB tumor cell showing the presence of numerous dictyosomes of the Golgi apparatus (G). A number of virus-like particles are also shown (V). × 26,000. Inset, electron micrograph of immature virion budding into the granular endoplasmic reticulum of an FCB tumor cell (arrow). × 31,000.
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