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Establishment of a transplantable carcinoma arising from the intrahepatic bile duct in Syrian golden hamsters

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Abstract. A subcutaneously transplantable cancer line from the intrahepatic bile duct (IHBD) induced by *N*-nitrosobis(2-oxopropyl) amine was established in Syrian golden hamsters. The doubling time of this tumour was 2.6 days when 2×10^5 tumour cells were inoculated subcutaneously (take-up rate was 100%). Growth of the tumour was significantly faster in male hamsters but neither oestrogen nor androgen receptors were detected in the tumour. The primary and all allograft tumours were tubular adenocarcinomas with fibrosis and a scirrhous pattern resembling human IHBD carcinoma of the peripheral type. Transmission electron microscopic findings showed irregular glands covered with numerous microvilli. Blood-group-related antigens including A, B and H were positive. P-Glycoprotein, which is an indicator of multidrug resistance, was also positive. Carcinoembryonic antigen and CA19-9 as general tumour markers of the biliary tract were negative. The deoxyribonucleic acid (DNA) pattern of this transplantable carcinoma was diploid. This newly established animal model of a transplantable IHBD carcinoma can be used to examine the mechanisms of synthesis and secretion of tumour-associated antigens and to study potential therapeutic agents.

Key words: Intrahepatic bile duct carcinoma – *N*-Nitrosobis(2-oxopropyl) amine – Hamster

Introduction

The treatment of clinical intrahepatic bile duct (IHBD) carcinoma is challenging since early detection is rare and routes of spread of the tumour make effective approaches to treatment difficult. An animal model would be valuable.

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In our laboratory, primary IHBD carcinoma was induced in Syrian golden hamsters following cholecystoduodenostomy with dissection of the distal common bile duct. *N*-Nitrosobis(2-oxopropyl)amine (BOP) was then administered. The study addressed the induction of bile duct carcinoma related to reflux of pancreatic juice into the biliary tract. BOP is well recognized as a pancreatic carcinogen in hamsters and also induces a bile duct carcinoma (Pour and Thorkatla 1978). The tumours induced in the IHBD in our study were morphologically similar to those found in man. The biological and morphological characteristics of this transplantable IHBD tumour line are described.

Materials and methods

Eighty Syrian golden hamsters of both sexes (non-inbred) were supplied by the Shizuoka Laboratory Animal Center (Shizuoka, Japan). All were over 6 weeks of age and were housed in plastic cages on sawdust bedding and given a CE2 pelleted diet (Japan Clea, Tokyo, Japan) and water ad libitum. The standard laboratory conditions were: temperature, 22° C; relative humidity, $40 \pm 5\%$; light/dark cycle 12 h/12 h (at the Laboratory Animal Center for Biochemical Research, Nagasaki University School of Medicine). All experiments were done following the guidelines for animal experimentation.

Histologically diagnosed IHBD cancer was induced by subcutaneous injection of BOP into Syrian golden hamsters. A primary tumour is shown in Fig. 1, where it can be seen that the glands have a small lumen and mucin is produced with a uniform pattern. The glandular epithelium is composed of small cuboidal cells with round nuclei. Some tumours have predominantly small cells with scanty cytoplasm, but abundant sclerosis. Tumours were removed following sodium pentobarbital anaesthesia (50 mg/kg body weight, intra-peritoneal injection), rinsed in sterile saline, and transferred to a sterile plastic Petri dish to which Hanks' solution was added. The tissue was sliced and cut into pieces about 0.5 mm^3 using a sterile scalpel, scissors and forceps, and the cut tissue was placed in Hanks' solution. Some cells were tested for viability, using the trypan blue dye exclusion method. Viable tumour cells were inoculated subcutaneously into the dorsum of anaesthetized hamsters and histological confirmation of the tumours which developed was recorded.

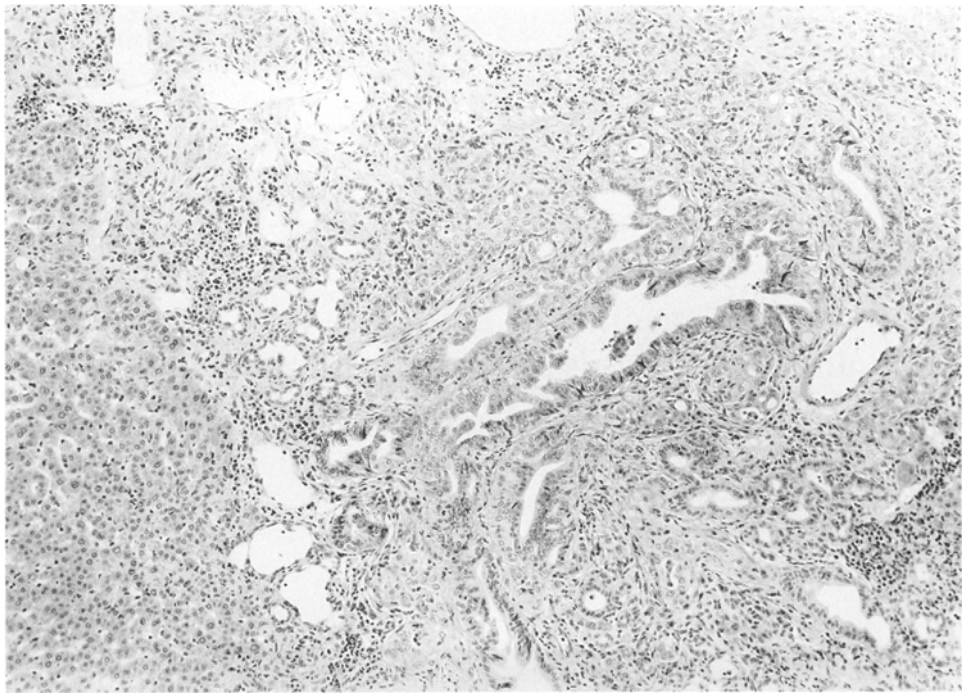

Fig. 1. Histological appearance of a primary intrahepatic bile duct tumour with a well-differentiated adenocarcinoma. The glands have a small lumen and epithelium is composed of small cuboidal cells with round nuclei. H&E stain, ×100

Viable 2×10^5 (0.2 ml, 10^6 ml) tumour cells were injected into the subcutaneous tissue of ten recipient female hamsters and a growth curve was estimated by measuring the longest and shortest diameters of the tumours at the 7th, 11th, 14th, 18th and 21st days after inoculation. Measurements were made following anaesthetization of the animals with ether and the estimated tumour weight was calculated using the method of Ovejera et al. (1978): tumour weight $= a^2 \times b/2$ (g) where a is the shortest diameter (cm) and b the longest diameter (cm).

In order to determine the effects of sex on growth, viable 1×10^5 (0.1 ml, 10^6 ml) tumour cells were injected into the subcutaneous tissue of the five male and five female, 8-week-old hamsters. Measurement of tumour size was made on the 7th, 14th and 21st days after inoculation. Samples for analysis for oestrogen receptors were taken from females and samples for androgen receptors from males. The resected specimens were immediately placed in a dry-ice box and receptors were examined by radioimmunoassay.

We used the 10th generation subcutaneous tumours for histological examination. Pieces of each tumour were fixed in 10% formalin and embedded in paraffin, using standard techniques. Each 4-μm section was stained with haematoxylin and eosin. For transmission electron microscopy (TEM) and scanning electron microscopy (SEM), tumour tissues were fixed in 2% buffered glutaraldehyde with 10% formalin. After fixation, these tissues were sliced about 1 mm thick. After repeated washing in phosphate buffer solution (PBS), the samples were fixed in 2% osmium tetroxide, and dehydrated in an ascending series of ethanol. Samples for TEM were embedded in Epon, sliced into ultra-thin sections (60–80 nm), stained on the grid with 2% uranly acetate and 0.4% lead citrate, and observed using a JEM-1200EX (Japan Electron Optics Laboratory, Tokyo, Japan) with 60–80 kV accelerating voltage.

After dehydration, samples for SEM were dried by the critical point method and spattered with gold. Observation was made using a JSM-35CLaB6 (Japan Electron Optics Laboratory) with 15 kV accelerating voltage.

In immunohistochemical studies, monoclonal antibodies (mAbs) against the synthetic trisaccharide of blood groups A, B and H determinants (Dako, Tokyo, Japan) were used and CEA (Dako), CA19-9 (Toray-Fuji Bionics, Tokyo, Japan), P-glycoprotein (P-glycoCHEK-c219, Centcore, Malvern, Pa., USA) were examined by an avidin-biotin peroxidase complex (ABC) immunohistochemical method, using Vectastain ABC kit (Vector Laboratories Burlingame, Calif., USA). The concentration of each mAb was 40 μg/ml. Samples for examination were obtained from the subcutaneous tumours. The staining score was made according to a classification based on the number of positive staining cells, and samples were categorized into one of the following: 0% (−); <5% (1+); 5–30% (2+); 30–70% (3+); and >70% (4+). The pattern of cellular staining was categorized as glycocalyx (luminal), diffuse (granular) cytoplasmic and Golgi pattern (Egami et al. 1989).

Specimens taken from the subcutaneous tumours were put into PBS for DNA analysis. Tumour tissues were sliced and cut into pieces about 0.5 mm^3, using a sterilized scalpel, scissors and forceps. After the addition of PBS, the preparation was passed through wire mesh. An equal volume of 1% ribonucleic acidase was added and DNA was stained with propidium iodide in TRIS-EDTA buffer (50 μg/ml). Analysis of the stained solution was made using a flow cytometer (FACScan, Becton and Dickinson, Mountain View, Calif., USA).

Comparisons between mean tumour weight in male and female hamsters were made using Student's t-test.

Results

Serial changes in tumour weight are illustrated in Fig. 2. The mean tumour weight was approximately 1.5 g and 3.0 g on the 14th and 18th days after inoculation and the doubling time was calculated to be 2.6 days. Cystic change in the tumour was evident on the 14th day. The take-up rate was 100%. There were no metastatic lesions.

When comparisons of tumour weight on the 21st day after inoculation were made, we found a statistically significant difference between male and female hamsters ($P < 0.05$), as shown in Fig. 3. The doubling time was 2.8 days, in both sexes. Based on this evidence, we

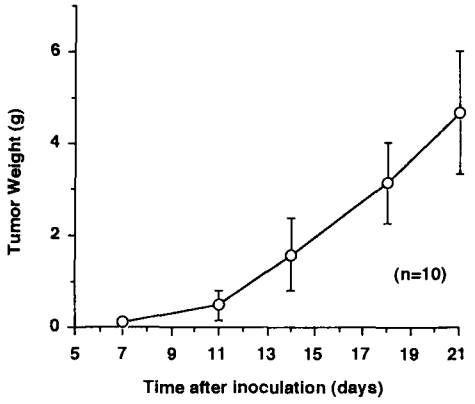

Fig. 2. Growth curve when 2×10^5 ($n = 10$) tumour cells were inoculated into subcutaneous tissue. Tumour weight was calculated using the following method: tumour weight = (shortest diameter)^2 × (longest diameter)/2

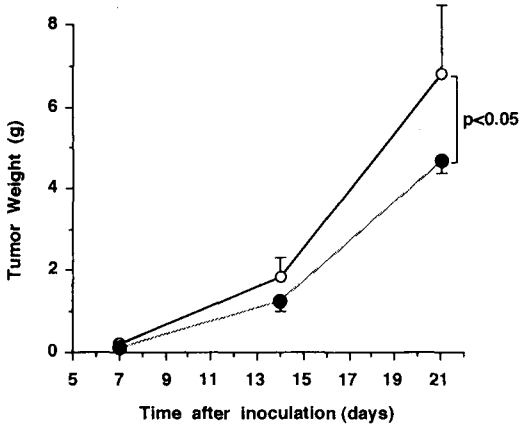

Fig. 3. Influence of sex on tumour growth; 10^5 tumour cells were inoculated subcutaneously into male (⊢○⊣, $n = 5$) and female (⊢●⊣, $n = 5$) hamsters. At the 21st day after inoculation, there was a significant difference between tumour weight between the sexes ($P < 0.05$)

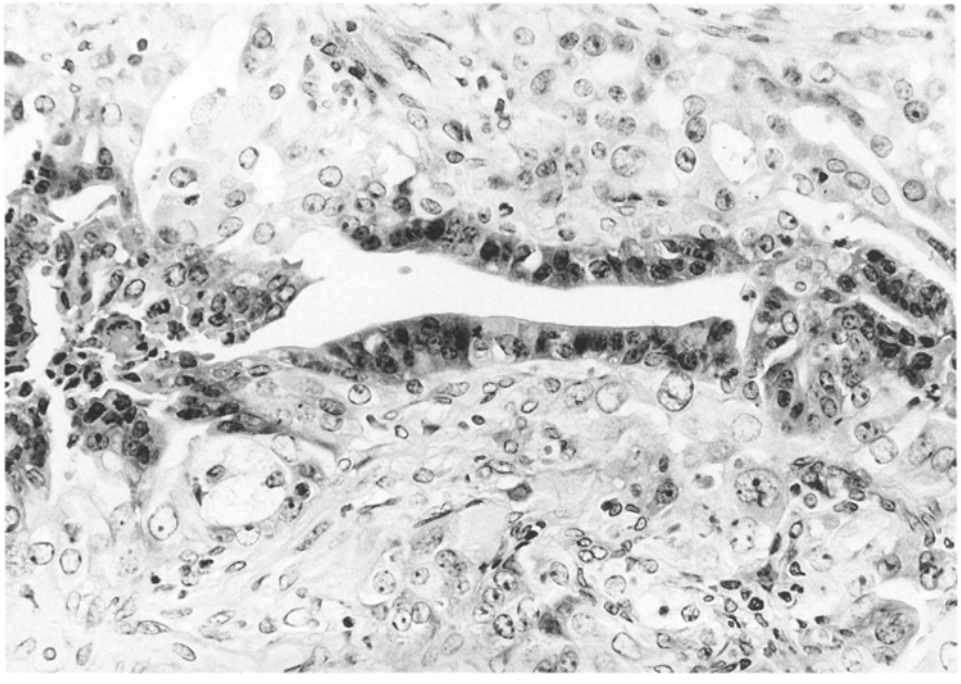

Fig. 4. Histological appearance of a transplanted subcutaneous tumour with a moderately differentiated adenocarcinoma. H&E stain, ×200

searched for receptors for oestrogen and androgen in the tumour tissue but all findings were negative.

Transplanted subcutaneous tumours were firm and round when small but when they grew rapidly, cystic change was progressive. The cut surface of the tumour was a yellowish white. The primary was a well-differentiated adenocarcinoma, but tumours at the 10th generation were moderately and poorly differentiated (Fig. 4). We also evaluated the tumours histologically up to the 20th generation; the main structure of all tumours was a tubular adenocarcinoma and had not changed from the primary lesion.

Expression of antigens is listed in Table 1. Both A-antigen and antibody against P-glycoprotein were highly

Table 1. Expression of antigens in subcutaneous allografts (avidin biotin peroxidase complex method)

Antigens	Staining score
A-antigen	(4+)
B-antigen	(2+)
H-antigen	(1+)
P-glycoprotein	(4+)
CEA	(−)
CA 19-9	(−)

CEA, Carcinoembryonic antigen

Staining score on the basis of the number of cells showing: (−), 0%; (1+), <5%; (2+), 5–30%; (3+), 30–70%; (4+), >70%

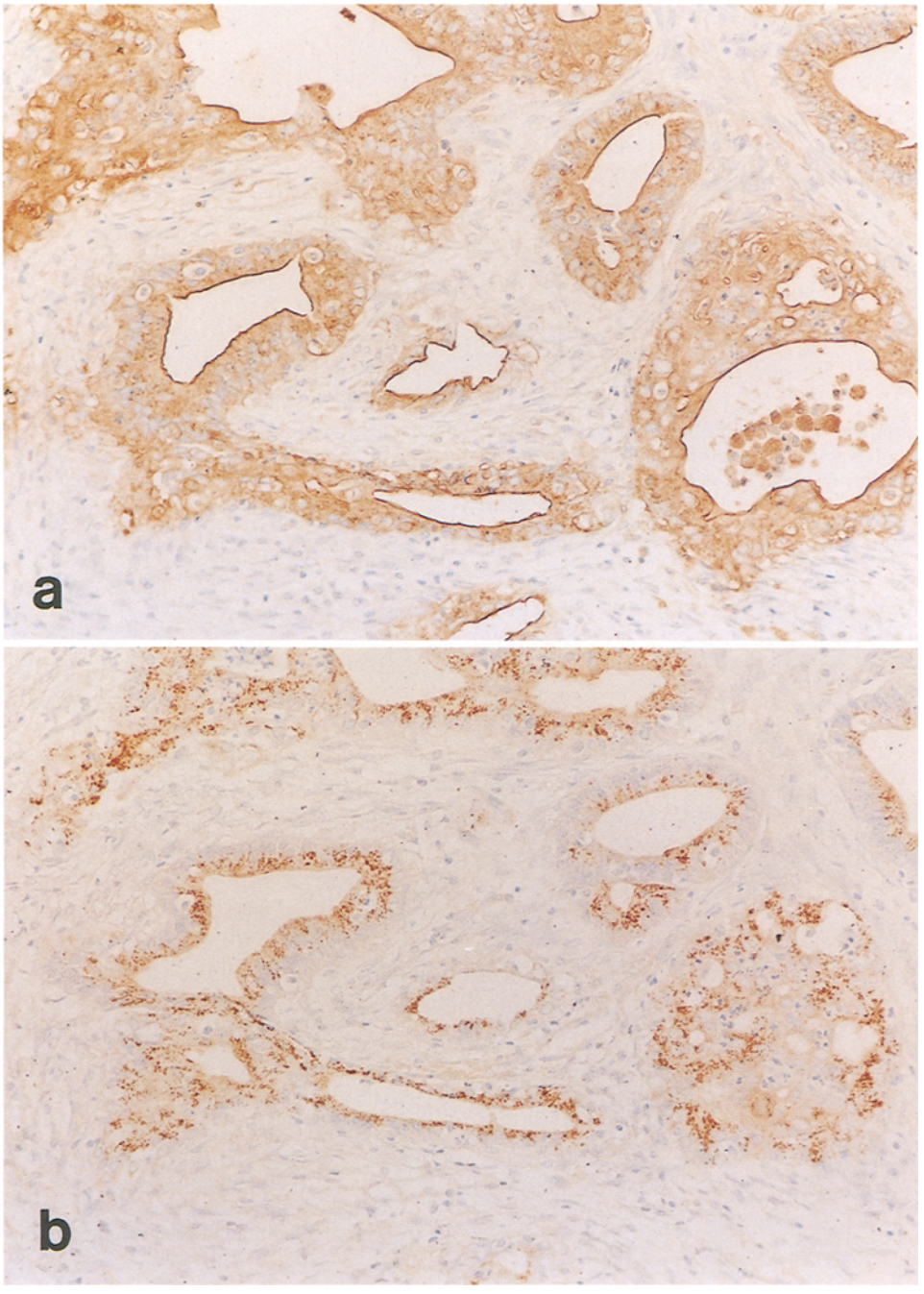

Fig. 5. a Expression of A antigen in the transplanted tumour. Gland-forming tumour cells showing diffuse and glycocalyx patterns. ABC method, ×200. **b** Expression of antibody against P-glycoprotein in the transplanted tumour. Tumour cells have the Golgi pattern. ABC method, ×200

stained. A different staining pattern was observed between mAbsA and mAbsP-glycoprotein. Staining of mAbsA was diffuse and of a glycocalyx pattern (Fig. 5a), whereas the pattern of mAbsP-glycoprotein was Golgi (Fig. 5b). Both mAbsB and H showed a moderate staining score. Antibodies against CEA and CA19-9 were negative.

On TEM tumour cells were composed of an irregular glandular structure with numerous microvilli. Variously sized lysosome granules were present near the lumen. Cells were connected by primitive junctions in a scirrhous area. The nuclei were almost oval but some were irregularly shaped and notches were present. Marginal chromatin was coarse and a few nucleoli were noted (Fig. 6). SEM showed the lumen of glands to be lined with a single layer of tumour cells and the glands were irregularly shaped (not illustrated). These findings corresponded to the histological findings in gland formation. These tumour cells had a columnar epithelium and the cell boundaries were clearly distinguished. Cell surfaces were slightly rounded and covered with numerous microvilli.

A DNA histogram is shown in Fig. 7. The DNA pattern was diploidy. Coefficient of variation (CV) was 3.9%.

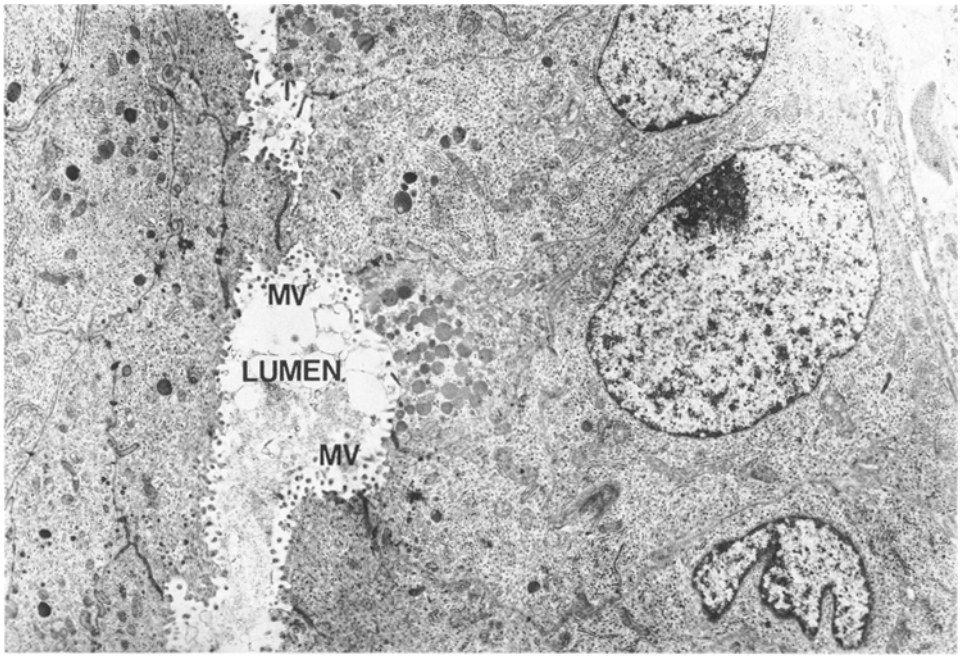

Fig. 6. Transmission electron microscopical appearance showing an irregular gland with a well-developed microvillus (*MV*). The nuclei were almost oval, but notches were sometimes present. × 5000

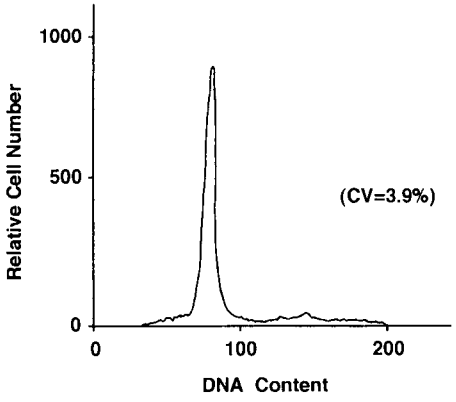

Fig. 7. DNA histogram, showing a single and a diploidy pattern. Coefficient of variation (*CV*) was 3.9%

Discussion

Since the 1980s, improved understanding of basic pathophysiology, new approaches to diagnosis by developments in imaging and novel medical and surgical treatment have been used in the treatment of hepatobiliary and pancreatic malignant disease. However, an animal model would be useful in determining sensitivity to anticancer drugs and in elucidating events related to metastasis.

BOP-induced experimental pancreas tumours are useful in this context. These are adenocarcinomas, which in immunohistochemical studies express the A-antigen strongly.

In our experiments, the primary tumour induced was located within the portal area and was a tubular adenocarcinoma with a fibrosis, scirrhous pattern. Subcutaneous inoculation of the IHBD carcinoma cells produced locally growing tumours in all the recipient hamsters (take-up rate was 100%). Histologically, all allografts also showed tubular adenocarcinoma with a fibrous, scirrhous pattern. The findings are generally consistent with those of human IHBD, where the most common histological pattern of the peripheral type of human IHBD carcinoma is the small glandular adenocarcinoma with abundant sclerosis (Roy 1979). The pancreatic tumours induced by BOP were also adenocarcinomas and these tumours readily metastasized to regional lymph nodes (Egami et al. 1991). The IHBD tumours in our study did not metastasize in the early phase.

In experimental pancreatic carcinoma in hamsters, the A-antigen and the pancreatic tumour antigen are considered to be effective tumour markers (Matsuzaki et al. 1987). In our experimental IHBD carcinoma model, the A-antigen was strongly stained. The so-called blood-group-related antigens are a family of cell surface carbohydrate structures (John and Ronald 1986) which have been used as tumour markers (Kenneth 1987; Ten 1985) and positive reactions have been observed in human carcinoma of the stomach (John et al. 1982), colon (Sakamoto et al. 1986), pancreas (Steven et al. 1987), lung (Laura et al. 1983) and urinary tract (Kay and Wallace 1961). These antigens are also tumour markers in neoplastic lesions in Syrian golden hamsters (Egami et al. 1991). The A-antigen discriminates between benign and malignant lesions far better than do other antigens (Tomioka et al. 1991). Our experimental IHBD carcinoma model may be useful in evaluating diagnostic studies of blood-group-related antigens.

The staining score of P-glycoprotein in this transplantable carcinoma was high. P-glycoprotein is a product of the *mdr*-1 gene and is considered to be a drug pump which carries anti-tumour agents out of the cell (Joshua and Bart 1989; Ronald et al. 1990). For this reason, this antigen plays an important role in multi-

drug resistance and our model will also be useful for studies on the effects of drug therapy.

MAbc219 is not completely characterized as a mAbsP-glycoprotein, as it also reacts with *mdr*-3 gene product (Alfred et al. 1991). This *mdr*-3 gene is not well understood and the significance of this finding is uncertain. Both A-antigen and P-glycoprotein were strongly stained. We used mAbc219 as a mAbsP-glycoprotein, and c219 cross-reacts with blood type A substance (Connie et al. 1991). However, a different staining pattern was observed between mAbsA and mAbc219; diffuse for mAbsA and in a glycocalyx pattern, whereas the pattern of mAbc219 was Golgi. The c219 probably reacts to mAbsP-glycoprotein.

The doubling time from the 7th to 21st days was identical in both sexes (2.8 days), but on the 21st day after inoculation there was a significant difference between tumour weight in the males and females ($P < 0.05$). These findings suggest that inoculated cells have a latent phase and that this is different (shorter) in males. A similar sexual difference was seen in the azaserine-induced rat pancreatic carcinoma model (Daniel and Sumi 1990; Pour 1989; Sumi et al. 1989). Tumour growth was faster in intact than in castrated males (Sumi et al. 1989). In our IHBD carcinoma model, neither oestrogen nor androgen receptors were detected, and no such receptor has been detected in drug-induced pancreatic carcinoma in rats (Lhoste et al. 1987).

The present newly established transplantable IHBD carcinoma will aid in elucidating mechanisms involved in the synthesis and secretion of tumour-associated antigens, and will be of use in evaluating possible related therapies.

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