ESTABLISHMENT AND CHARACTERIZATION OF CELL LINES FROM LIVER FLUKE-ASSOCIATED CHOLANGIOCARCINOMA INDUCED IN A HAMSTER MODEL

Tasanee Tengchaisri[1], Nalinee Prempracha[2], Witaya Thamavit[3], Somnate Boonpucknavig[3], Siriporn Sriurairatana[4] and Stitaya Sirisinha[1,2]

[1]Laboratory of Immunology, Chulabhorn Research Institute; [2]Department of Microbiology; and [3]Department of Pathobiology, Faculty of Science, Mahidol University; [4]Department of Pathology, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand

Abstract. Cholangiocarcinoma (CCA) is a relatively rare tumor that occurs primarily in tropical countries and particularly in those with a high incidence of liver fluke infection. A hamster model for a liver fluke-associated CCA has been described previously. In the present study, hamster cholangiocarcinoma cell lines were established and characterized in order to obtain information regarding diagnostically useful tumor markers which could shed light for a future investigation for human cholangiocarcinoma. Two related cell lines, one from the original intrahepatic bile duct tumor and one from an allotransplanted tumor, were established. The established cell lines were found to have population doubling times of 31 and 26 hours respectively, and were maintained in Ham's F12 medium supplemented with 10% fetal bovine serum for over 80 passages. The cell monolayers were subjected to scanning and transmission electron microscopic study and found to have ultrastructural characteristics, including cytoplasmic lumens, consistent with those of adenocarcinoma cells of epithelial origin. An immunoperoxidase study using monoclonal antibodies (MAbs) specific for tumor antigens showed the cytoplasm and membrane of both cell lines to be positive. These antigens were also secreted in soluble form into the culture medium, judging from polyacrylamide gel electrophoresis in the presence of SDS and from immunoblot analyses. Different lines of evidence presented suggested that a 200 kDa glycoprotein produced and secreted by the tumor cell lines could be considered a cholangiocarcinoma-associated marker which has diagnostic potential.

INTRODUCTION

Although cholangiocarcinoma (CCA) occurs only sporadically in most parts of the world, it is still one of the more common cancers in many tropical countries, particularly in areas where liver fluke infection is still endemic (Flavell, 1981). In these countries, it remains one of the more important public health problems (Haswell-Elkins *et al*, 1992; Parkin *et al*, 1991). Because there is no specific and practical diagnostic method currently available (Nakajima and Kondo, 1989; Sirisinha, 1989), the patients are often diagnosed much too late for any successful management. The prognosis is poor, and there is a very high mortality rate. Identification of a specific marker(s) for CCA should facilitate development of specific methods for its detection. The availability of cholangiocarcinoma cell lines should speed up the progress in this area of investigation and give some insights into this problem. A few CCA cell lines have thus been established from tumors occurring in patients not known to have been infected with liver fluke (Miyagiwa *et al*, 1989; Shimizu *et al*, 1992; Storto *et al*, 1990; Yamaguchi *et al*, 1985). However, there is some evidence suggesting that the carcinogenic process might be different for CCAs that occur with or without associated liver fluke infections (Tsuda *et al*, 1992; Sirisinha, 1994). A few years ago, we therefore established a new CCA cell line that originated from the tumor of a patient with a medical history compatible with being infected by liver flukes (Sirisinha *et al*, 1991).

A hamster model for a liver fluke-associated cholangiocarcinoma with characteristics similar to that which occurs in humans has been previously described (Thamavit *et al*, 1978). This experimental model provides us with an alternative investigative approach. In the present study, CCA cell lines were establised and subsequently characterized from the experimentally induced hamster CCA. The cell lines

Correspondence: Prof Stitaya Sirisinha, Department of Microbiology, Faculty of Science, Mahidol University, Rama VI Rd Bangkok 10400, Thailand. Fax 662-247 1222.

secreted a number of tumor-associated products, among which was a 200 kDa glycoprotein that appeared to have features compatible with being a diagnostically useful tumor marker.

MATERIAL AND METHODS

Induction of liver fluke-associated cholangiocarcinoma in hamsters

Liver fluke-associated cholangiocarcinoma histopathologically similar to that found in humans was induced in Syrian golden hamsters (Armed Forces Research Institute of Medical Sciences, Bangkok) (Thamavit *et al*, 1978). In brief, one month old hamsters were infected by intragastric route with 100 metacercariae of *Opisthorchis viverrini* (Ov) obtained from naturally infected cyprinoid fish. Two months later the animals were given 0.0025% dimethylnitrosamine (DMN) in drinking water for 10 weeks. The tumors that subsequently developed (usually within 2-4 months) were removed and verified by microscopic examination. The intrahepatic bile duct tumor that developed was propagated serially *in vivo* by intramuscular injection of small fragments into the hindleg of adult hamsters. The recipient animals developed detectable tumors within 1-3 months. The epithelial cell nature of the original intrahepatic tumors (OT) and the allotransplanted tumors (AT) was confirmed microscopically and used for the establishment of HaLCCA-1 and HaTCCA-1 cell lines, respectively.

Primary cell cultures

Tissues from OT and AT hamsters were minced into fine fragments by the crossed scalpel technique using Ham's F12 medium (Gibco Laboratories, Grand Island, NY) containing penicillin (100 U/ml) and streptomycin (100 µ/ml) (Sirisinha *et al*, 1991). The thoroughly washed fragments (10-12 pieces) were seeded into 25 cm^2 plastic tissue culture flasks (Nunc, Roskilde, Denmark) containing one ml of Ham's F12 culture medium supplemented with 10% fetal bovine serum (FBS, Flow Laboratories), 10 ng/ml epithelial growth factor (EGF, Gibco), and a mixture (ITS) of insulin (10 µg/ml), transferrin (5.5 µg/ml) and selenium (6.7 ng/ml) (Gibco). The cultures were incubated in 5% CO_2 atmosphere at 37°C. Cells were allowed to grow out from the tumor fragments and allowed to attach to the surface for 2-3 days before 2 ml of fresh culture medium was added and subsequently replenished every 3-5 days.

Establishment of permanent tumor cell lines

Within 4 weeks after the start of the primary cultures, aggregated tumor cell colonies surrounded by fibroblasts could be observed. Contaminating fibroblasts were removed by exposing the culture to 0.05% trypsin (Gibco) and 0.02% EDTA solution for a few seconds, followed by immediate rinsing with phosphate buffered saline (PBS). The procedure was repeated several times until contaminating fibroblasts were eliminated, leaving behind slightly pleomorphic epithelial cell monolayers attaching to the plastic surface. After a confluent cell growth was obtained, serial subcultures were made every 7 to 10 days using a split ratio of 1 : 5 and treatment of monolayers with 0.25% trypsin and 0.1% EDTA in PBS. The cells were subsequently cultured in Ham's F12 containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin in the absence of EGF and ITS. Contaminating fibroblasts were periodically removed by differential trypsinization as described above. Tests for mycoplasma contamination were performed by bacteriological culture and biochemical assays (Freundt *et al*, 1984).

Growth studies

The growth curves of these cell lines (HaLCCA-1: passage 15; HaTCCA-1 passage 25) were constructed as described previously (Sirisinha *et al*, 1991). In brief, 2×10^5 cells were added to a series of 60-mm plastic Petri dishes (Nunc) containing approximately 5 ml of Ham's F12, 10% FBS and antibiotics. The cells were incubated and the culture medium was replaced with fresh medium every 2 days. The number of viable cells obtained by trypsinization of the monolayers was counted at days 1 and 2, and thereafter every other day for 2 weeks. The population doubling times of the cell lines were calculated from the growth curves when the cells were in exponential growth.

To determine the colony-forming efficiencies, 100, 500 and 1,000 tumor cells in 10 ml of the culture medium were cultured in 100 mm plastic Petri dishes and incubated at 37°C in a 5% CO_2 atmosphere for

14 days. The dishes were then washed with PBS and stained with methylene blue. Colonies consisting of 10 or more cells were counted under a phase contrast microscope.

Production of monoclonal antibodies

Monoclonal antibodies (MAbs) against the tumor antigens were produced by immunizing BALB/c mice subcutaneously with crude aqueous extracts of AT tumor homogenate. The initial immunization consisted of 50 μg of tumor proteins in complete Freund's adjuvant, and this was followed by one or two subcutaneous injections of the same amount of antigen in incomplete Freund's adjuvant given at 3-week intervals. Three weeks after the last subcutaneous injection, a prefusion booster injection, consisting of 50 μg of the antigen in PBS, was given by an intraperitoneal route and fusion was performed 3 days thereafter (Billings *et al*, 1990).

The antibody-producing hybridomas were initially screened by indirect ELISA using crude tumor homogenates at a predetermined concentration of 5 μg/ml. Clones with positive OD readings against the homologous antigen were analyzed for specificity using different normal hamster tissues and hamster serum. Only the CCA-positive clones that failed to react with other antigens were used for single cell cloning, clone expansion and immunoglobulin isotyping.

Immunochemical and immunohistochemical techniques

Tumor antigens present in crude tumor and cell monolayer homogenates and in spent culture medium were analyzed by SDS-PAGE and by immunoblotting using polyclonal prefusion mouse serum and MAbs (Laemmli, 1970). Molecular weights of interesting components were determined from a standard curve established with pre-stained molecular weight markers (Gibco): lysozyme (14.3 kDa), β-lactoglobulin (18.4 kDa), carbonic anhydrase (29.0 kDa), ovalbumin (43.0 kDa), bovine serum albumin (68.0 kDa), phosphorylase B (97.4 kDa) and myosin (200 kDa). The indirect ELISA used for the screening of MAbs was essentially the same as described previously (Dharmkrong-at *et al*, 1986). The concentrations of all reagents used were predetermined by checkerboard titration.

For histological localization of the tumor antigens, cell monolayers cultured on cover slips were fixed with either acetone or 1% glutaraldehyde in PBS. These specimens were exposed to the MAbs at room temperature for 30 minutes, followed by thorough washing with PBS. The reaction was detected by biotin-streptavidin-peroxidase staining using a commercial kit (Immunon™, Shandon, Inc., Pittsburgh, PA) essentially as described (Warnke and Levy, 1980). The peroxidase staining was also used for the identification of cytokeratin in these 2 hamster cell monolayers, employing monoclonal antibody from DAKO-CK1 clone LP34 reactive against human keratin (Sirisinha *et al*, 1991).

Electron microscopy

Ultrastructural characteristics of the cell lines were studied by both scanning (SEM) and transmission (TEM) electron microscopic techniques. For the SEM, the cells were first cultured on cover slips in a Leighton tube until a confluent growth was obtained. The monolayers were then briefly washed with 0.1 M sodium cacodylate buffer (SCB) pH 7.2 and fixed with 2.5% (w/v) glutaraldehyde in SCB or with glutaraldehyde-ruthenium red fixative for demonstrating mucopolysaccharides (Luft, 1971). The fixed cells were washed and then post-fixed with 1% (w/v) osmium tetroxide or osmium tetroxide-ruthenium red in the same buffer and again washed with distilled water. The specimens were dehydrated in ethanol, critical-point dried in a Hitachi HCP-2 apparatus using liquid CO_2 as a transitional medium, sputtered with gold (IB-2 coater, Giko Engineering Co) and viewed under a scanning electron microscope (Hitachi S-570) operating at 15 KV.

For the TEM, the confluent cell monolayers in plastic flasks were first washed, fixed and post-fixed as described for the SEM. The fixed specimens were then removed very gently with a silicon-rubber policeman, pelleted by centrifugation at 1,000 rpm for 10 minutes and embedded in EM bed-812 (Electron Microscopy Science, Washington, PA). Ultrathin sections were cut, stained with uranyl acetate and lead citrate and viewed with a transmission electron microscope (Hitachi HU-12A) set 10 operate at 75 kV.

RESULTS

Histology of the original intrahepatic bile duct tumors and allotransplanted tumors

Microscopic examination of hematoxylin and eosin stained sections of tumors removed from these animals revealed the presence of proliferating neoplastic bile duct epithelium with glandular formation and abundant fibrous stroma typical of cholangiocarcinoma described for humans. The nuclei were round to oval and contained one or more prominent enlarged nucleoli. A large number of the neoplastic cells, particularly those of the original intrahepatic tumor, were mucin secreting as demonstrated by positive mucicarmine staining. The tumor cells exhibited ultrastructural characteristics typical of adenocarcinoma, namely, desmosomes, cytoplasmic intermediate microfilaments, apical microvilli and intracytoplasmic lumens. Microvillus-lined intercellular lumens could be readily observed. Of particular interest was the presence in many tumor cells of lamellar body inclusions (Fig 1) similar to surfactant granules previously described for alveolar pneumocytes type II (Ghadially, 1988).

Fig 1–A transmission electron micrograph of the original intrahepatic bile duct tumor illuststrating microvilli (V), lamellar body inclusions (arrow head), indented nuclei and intercellular lumens (L). bar = 2 μm.

Establishment of cell lines

Within 24-48 hours of incubation, polygonal-to-spindle shaped cells with large nuclei grew out from the periphery of the tissue fragments and firmly adhered to the bottom of the plastic tissue culture flasks. By differential trypsinization, contaminated fibroblasts gradually disappeared during the one month of primary culture in a fully supplemented medium. During serial subculture in medium supplemented with 10% FBS only, the cells were uniformly polygonal with a cobblestone-like appearance. Both the HaLCCA-1 and HaTCCA-1 cell lines were maintained for over 80 passages during the 2-3 years period of *in vitro* culture. However, these two cell lines could be maintained in a relatively healthy state for only 2-3 days in a protein-free unsupplemented Ham's F12 medium. Tests for mycoplasma were negative.

Morphology and growth characteristics of the cell lines

Under the phase contrast microscope, the cells exhibited prominent round nucleus with large and occasionally multiple nucleoli and a cytoplasm filled with dense granules (Fig 2). Cells occasionally piled up, and tubule-like structures were noted on occasion.

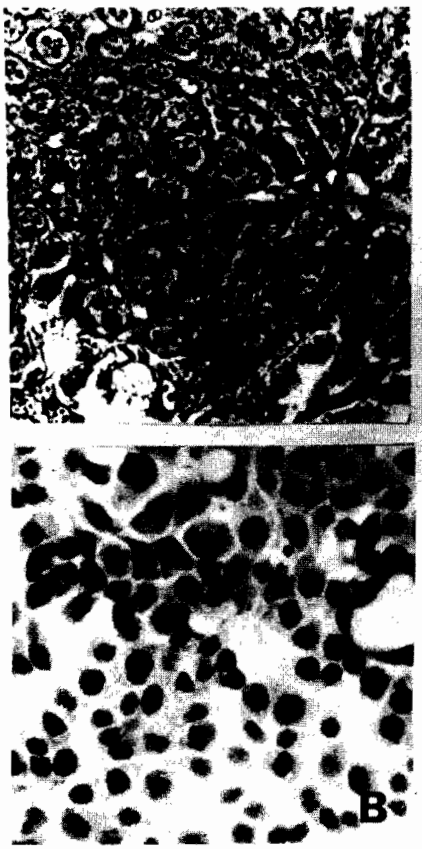

Fig 2–Photomicrographs of HaTCCA-1. (A) Phase contrast micrograph of the cell line at passage 14 illustrating epithelial cells with large nucleus containing multiple nucleoli. (B) Haematoxylin and eosin staining of the cells at passage 40. Note a large number of cells undergoing active multiplication. X200.

To ascertain whether these cell lines were adenocarcinoma of epithelial cell origin, the cell monolayers were stained with monoclonal antibody against the 45 and 56 kDa components of human epithelial cell cytokeratin (Dako) and the results are compatible with those obtained with other histochemical and ultrastructural studies. Sporadically, mucin-producing cells could also be observed particularly with the HaLCCA-1 line, judging from positive mucicarmine staining. These features were typical of mucin-secreting epithelial cell cancers. The growth curves of these two cell lines were similar with population doubling times of 31 hours for the HaLCCA-1 and 26 hours for the HaTCCA-1. The mean colony forming efficiencies were 11.33 and 34.32% respectively. After the establisment and characterization of the two cell lines described in this communication, we now have several other hamster cholangiocarcinoma cell lines with characteristics identical to these two cell lines.

Ultrastructural studies of the cell lines

The ultrastructural appearance of these two cell lines were almost indistinguishable from one another. With the SEM, the cells were found to be rather uniform in size and shape, but on occasion multinucleated giant cells could be noted. Fig 3 clearly demonstrates abundant microvilli on the surfaces of a large majority of these cells. However, one could see that the density, shape and size of these microvilli varied from cell to cell (Fig 3A). Some cells exhibited long and thin microvilli, while others had short and thick microvilli. When the cells were fixed with ruthenium red, the presence of glycoproteins or mucopolysaccharides on the microvilli could be clearly observed (Fig 3B). On occasion, ruthenium red-positive secretions were also found deposited on the cell surface.

The TEM revealed the presence of polygonal cells having large, finely granular nucleus with one or more prominent nucleoli. Under high magnification, cytoplasmic microfilaments typical for cytokeratin fibers found in other epithelial cells could be easily visualized (Fig 4). Consistent with the SEM, different shapes and sizes of microvilli could be observed. Ruthenium red stained mucopolysaccharides were found on the cell surface. Throughout the cytoplasm, one could find numerous inclusion bodies and vacuoles and some cells possessed microvillus-lined intracytoplasmic lumens, either lying empty or partially filled with secretory products. These features were similar to those described earlier for the liver tumor that developed in the hamsters (Fig 1). More than one-third of the cell line population, particularly

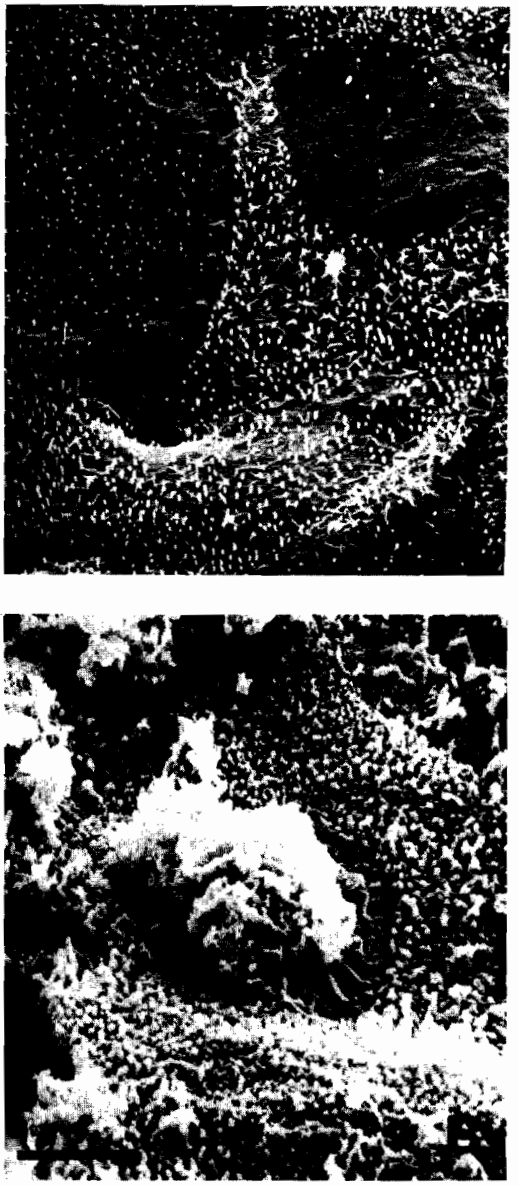

Fig 3–Scanning electron micrographs of HaLCCA-1 showing varying size, shape and density of surface microvilli. The cell monolayers at passage 62 were fixed with glutaraldehyde (A) or glutaraldehyde-ruthenium red (B) to demonstrate glycoprotein. bars = 5 μm.

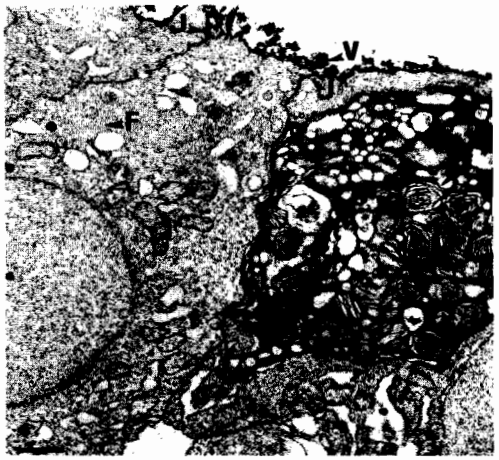

Fig 4–A transmission electron micrograph of HaLCCA-1 showing lamellar body inclusions (arrow head) and intermediate microfilaments (F). The cells at passage 21 were fixed with glutaraldehyde-ruthenium red to demonstrate glycoprotein on surface microvilli (V) and terminal tight junction (J). bar = 1 μm.

from the HaLCCA-1 cell line, exhibited lamellar body inclusions (Fig 4). Well developed junctional structures typical of epithelial cell linings were also observed among adjacent cells. On occasion, intercellular lumens with intruding microvilli could be seen.

Immunohistochemical studies

Both the tumor sections and the cell lines derived from these tumors were allowed to react with various MAbs against tumor antigens by the immunoperoxidase and immunofluorescent technique. These MAbs gave a different degrees of positive staining of proliferating biliary cells. Different areas of the cytoplasm of the tumor cells bound different MAbs. For example, one MAb (6E5) invariably stained positively the apical border area and the contents of the glandular lumen in the tumor sections. This MAb also reacted strongly with the cytoplasm and the membrane of both tumor cell lines (Fig 5). The membrane staining was verified when the glutaraldehyde-fixed cells were studied. Similar conclusion was reached using the immunofluorescent technique.

Fig 5–Immunoperoxidase staining of HaLCCA-1 cell line with MAb 6E5. The HaLCCA-1 cell line at passage 62 was fixed with acetone and allowed to react with MAb 6E5. Note strong cytoplasmic and membrane stainings by a majority of tumor cell adjacent to cells that failed to take up the MAb X400.

Identification and characterization of tumor antigens

Crude homogenates prepared from OT and AT as well as from their respective cell monolayers were found to have highly complicated protein profiles by SDS-PAGE and could not be distinguished from normal cell homogenate. However, Fig 6 illustrates results of immunoblotting analysis using MAb 6E5 (lane A) showing a band at the 200 kDa position which stands out distinctively, as it was not detectable in normal tissue extracts. When the extracts were electrophoresed, blotted onto nitrocellulose membranes and stained with different lectins, a strong positive staining with wheat germ agglutinin could be observed at the 200 kDa position (Fig 6, lane B), suggesting the presence of a glycoprotein most likely containing large amounts of sialic acid and/or N-acetylglucosamine. In order to identify the immuno reactive epitopes of this glycoprotein, an experiment was carried out in which the tumor homogenate was treated with periodate prior to reacting with the MAbs. Probing with MAbs 6E5, 3B9 and 4A4, a positive immunoblotting reaction at the 200 kDa position could not be observed after periodate treatment, suggesting that the epitopes for these MAbs were associated with the carbohydrate moiety (Fig 7). With MAbs 6C12 and 5B10, the reactive epitopes appeared to be associated with the protein core because periodate treatment did not completely abolish the positive reaction.

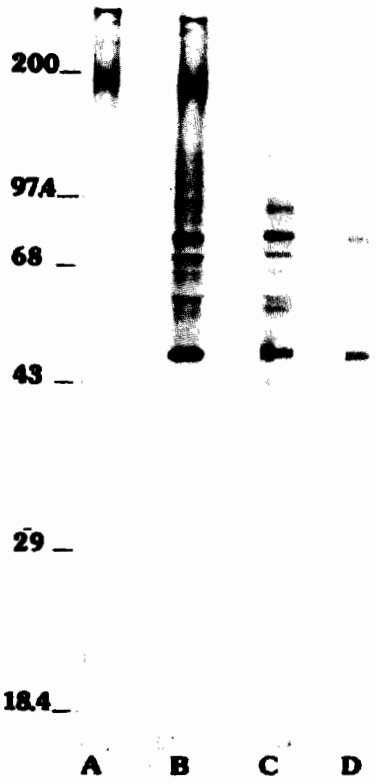

Fig 6–Identification of the 200 kDa tumor antigen. Crude homogenate from allotransplanted tumors was subjected to SDS-PAGE, blotted onto nitrocellulose membrane and probed with MAb 6E5 (lane A), wheat germ agglutinin (lane B), concanavalin A (lane C) and Vicia villosa (lane D). The immunoreactive 200 kDa component reacted strongly with wheat germ agglutinin. Molecular weight markers in kilodaltons are shown on the left.

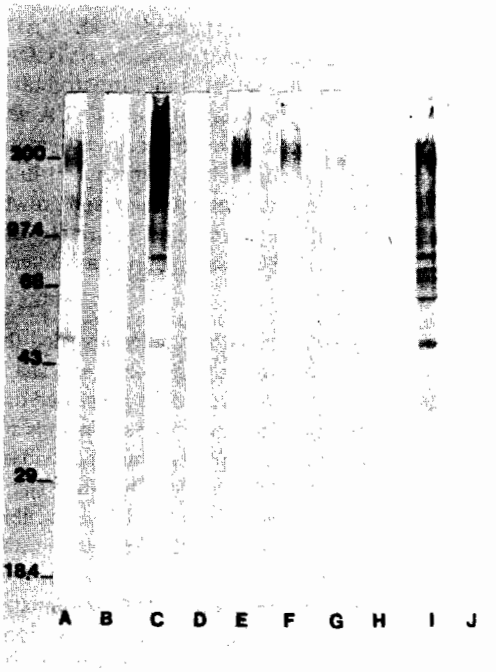

Fig 7–Effect of periodate oxidation on the immunoreactive epitopes of the 200 kDa antigen. Crude homogenate from allotransplanted tumors were electrophoresed, blotted onto nitrocellulose membrane, allowed to react with periodate (lanes B, D, F, H and J) or buffer (lanes A, C, E, G and I) as untreated controls and then probed with various MAbs; 5B10 (lanes A and B), 6E5 (lanes C and D), 6C12 (lanes E and F), 3B9 (lanes G and H) and 4 A4 (lanes I and J). The immunoreactivity of the 200 kDa component with the MAbs 6E5, 3B9 and 4A4 was completely abolished following periodate treatment.

Secretion of tumor-associated products

In this experiment, cells were maintained in the supplemented medium until a confluent cell growth was obtained. The cell monolayers were then washed twice with unsupplemented Ham's F12 medium and incubated overnight in the same medium to further remove any serum protein that might have adhered to the cell surface. Fresh unsupplemented medium was then added and the spent culture medium was collected 48 hours later. The culture fluid was dialyzed and then concentrated by lyophilization before it was subjected to SDS-PAGE and immunoblotting analyses. When the gel was stained for protein, a weak band at the 200 kDa position could be noted, particularly in the supernatant fluid obtained from HaLCCA-1. Immunoblotting analyses employing MAbs again illustrated a strong immunoreactive component at the 200 kDa position. On the other hand, the supernatant fluid from neither of these two cell lines gave positive results in tests for detecting the carbohydrate markers (*eg*, CA 19-9, CA 125 and CA 153) previously demonstrated for a number of human CCA cell lines.

DISCUSSION

Altogether the data presented in this study

showed that the two cell lines, one derived from an original intrahepatic bile duct tumor (HaLCCA-1) and one from an allotransplanted tumor (HaTCCA-1), exhibited morphological and ultrastructural characteristics similar to those of human CCA cell lines previously described by other investigators, including our own group. Both hamster cell lines were similar with regard to morphology and growth characteristics and they remained relatively unchanged for more than 80 passage during a 2-3 years' period.

Results from ultrastructural studies revealed the presence of microvilli of varying sizes and shape (Figs 1, 3). Moreover, adjacent cells possessed different densities of the microvilli. These findings are consistent with the results of the immunoperoxidase staining which showed positive and negative stainings adjacent to one another (Fig 5). These possibly represented different stages of cell differentiation and development. Stitnimankarn and his associates previously noted that cells from well differentiated human CCA tissue exhibited numerous, long microvilli, while poorly differentiated ones seemed to have only scanty and blunted microvilli (Stitnimankarn *et al*, 1978).

The presence of microvillus-lined, intracytoplasmic lumens was rather unique and it has not yet been noted in other CCA cell lines. Stitnimankarn *et al* (1978) reported the presence of similar structures in tumor sections from patients with moderately to poorly differentiated CCA. It is generally agreed that the presence of intracytoplasmic lumens is suggestive of adenomatous epithelial cell differentiation. Based on these studies, the presence of intracytoplasmic lumens should be a useful feather for the identification of cells or tissues derived from the bile duct. However, an unexpected finding was the presence in both the tumor cells and cell lines of structures morphologically similar to the lamellar body inclusions found in alveolar pneumocytes type II (Figs 1, 4). Why such structures should be present in cells derived from bile duct remains to be elucidated. The presence of lamellar body inclusions in two different types of cells, each performing different functions may give some clue to their biological significance.

The tumor cells synthesized and secreted a large number of proteins and glycoproteins, some of which may eventually be useful as diagnostic markers for CCA. Employing MAbs produced against the tumor, we identified a 200 kDa glycoprotein (by immunoblotting) not only in the tissue homogenates but also in the spent culture medium from both cell lines. Immunohistochemical analyses showed this glycoprotein to be present largely in the apical border area. This is consistent with the results obtained from electron microscopic examination of cells fixed with ruthenium red, which indicated the presence of glycoprotein(s) on the microvilli (Figs 3, 4) and in secreted droplets adhering to the cell surface. The large quantity of this antigen on the secretory surfaces is a logical explanation to our previous observations which demonstrated the presence of this component in the bile of tumor-bearing animals (Prempracha *et al*, 1994; Sirisinha *et al*, 1994). A relatively weaker but definitely positive staining of the extracellular matrix of tumor sections explains why this antigen is also detectable in the serum. The information obtained in the present study should give valuable clues needed for the elucidation of a corresponding tumor marker for the human counterpart. Should this be the case, it would then be possible to develop a reliable laboratory diagnostic method needed for the detection of a liver fluke-associated cholangiocarcinoma. Early diagnosis of cholangiocarcinoma is badly needed for countries like Thailand where as many as 8 million people currently are at risk of developing the tumor (Vatanasapt *et al*, 1990).

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