ULTRASTRUCTURAL CHARACTERISTICS OF LIVER FLUKE ASSOCIATED HUMAN CHOLANGIOCARCINOMA CELL LINES

Siriporn Sriurairatana[1], Tasanee Tengchaisri[2] and Stitaya Sirisinha[2,3]

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

Abstract. The ultrastructure of a cholangiocarcinoma cell line (HuCCA-1) originally established from an intrahepatic bile duct tumor of a patient seropositive for a liver fluke infection was studied by scanning (SEM) and transmission (TEM) electron miscroscopy. With the SEM, the surface of HuCCA-1 cells were found to be covered with microvilli. The size of these microvilli varied from cell to cell and they were irregularly distributed. The TEM clearly revealed the presence of cytokeratin filaments, an intracytoplasmic lumen, tight junctions at the apices and desmosomes at the lateral surfaces of neighboring cells, all of which are characteristics of adenocarcinoma cell origin. However, the tumor mass that developed in a nude mouse following subcutaneous injection of these cells was found to exhibit some morphological changes. Specifically, about 20-30% of the tumor cells, particularly those lining the base of the tumor tubules, exhibited electron dense tonofilaments typical of squamous cells. However, this alteration was reversible as the cell line (HuCCA-1Nu) derived from this nude mouse-passage did not exhibit any characteristics reminiscent of squamous cells. These observations are consistent with those occasionally found in human cases reported previously by other investigators. Altogether, the data showed that squamous transformation of adenocarcinoma cells can occur under appropriate conditions. It further showed that reversion to adenocarcinoma cells can occur when the microenvironment is changed.

INTRODUCTION

Cholangiocarcinoma (CCA) is a liver cancer of rather low incidence worldwide when compared to hepatocellular carcinoma. However, CCA is not uncommon in northeastern Thailand where the prevalence of liver fluke infestation is high. The association of *Opisthorchis viverrini* (*Ov*) infestation with the etiological development of CCA has been established from epidemiological, clinical, pathological and experimental animal studies (IARC, 1994). Establishment of a continuous CCA cell line is essential for characterizing the tumor cells and may pave the way to subsequent development of methods for CCA diagnosis and treatment. A few such cell lines have been described and characterized (Hayada 1975; Yamaguchi *et al*, 1985; Homma *et al*, 1987; Kusaka *et al*, 1988; Miyagiwa *et al*, 1989; Storto *et al*, 1990; Sirisinha *et al*, 1991; Iemura *et al*, 1992). However, most of these were derived from patients living outside endemic areas of liver fluke infection. One cell line, HuCCA-1, was established from a Thai patient whose serum was ELISA positive for antibody to *Ov* (Sirisinha *et al*, 1991). A variant of this HuCCA-1 cell line named HuCCA-1Nu has been recently established from tumors developed in a nude mouse which received a subcutaneous injection of trypsinized HuCCA-1 cells. In the present study, combined scanning (SEM) and transmission (TEM) electron microscopy was carried out to illucidate the ultrastructure of these two related tumor cell lines and to compare it with that of a hamster cholangiocarcinoma cell line that was described recently by our group (Tengchaisri *et al*, 1995).

Correspondence: Dr Stitaya Sirisinha, Department of Microbiology, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand. Fax: 662-247 1222, 662-6445411

MATERIALS AND METHODS

Cholangiocarcinoma cell lines

The HuCCA-1 cell line used in this study has been continuously passaged in our laboratory since 1989 when it was first described (Sirisinha *et al*, 1991). The HuCCA-1Nu cell line was established

more recently from a tumor that developed in a nude mouse that received HuCCA-1 tumor cells.

Nude mouse tumor

An HuCCA-1 monolayer at passage 60 was trypsinized to obtain a single cell suspension and 2×10^6 cells were injected subcutaneously into the hindleg of a 4-week old athymic nude mouse (National Cancer Institute). The tumor that developed 7-week later was removed and histopatho-logically confirmed by light and electron micro-scopy. The remaining tumor tissue was used to establish a new primary cell culture and a permanent cell line (HuCCA-1Nu).

SEM

The HuCCA-1 and HuCCA-1Nu cell lines were cultured on glass cover slips and were directly fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (CB) pH 7.4 for 1 hour, post-fixed in 1% osmium tetroxide in CB for 1 hour, dehydrated through a graded ethanol series and transferred to isoamyl acetate. The fixed cell monolayers were dried in a critical point dryer (HCP-2), Hitachi), and then ion-sputterred with gold. The samples were observed and photographed with a Hitachi SEM, model S-570, at 15 KV.

TEM

The HuCCA-1 and HuCCA-1Nu cell lines grown in culture bottles were fixed with glutaraldehyde and osmium tetroxide fixatives as described above for SEM or with the addition of 0.15% ruthenium red for preservation of mucopolysaccharides. After fixation, the cells were gently scraped off, centrifuged and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Hitachi TEM, HU-12A at 75 KV.

For comparison, tumor tissues removed from nude mice were also sectioned and similarly fixed in 4% paraformaldehyde and 1% glutaraldehyde in CB for 2 hours, post-fixed in 1% osmium tetroxide in CB for 1 hour, dehydrated and embedded in Epon 812. The tissue sections were thereafter processed for TEM as described above.

RESULTS

In general, ultrastructural characteristics of the HuCCA-1 and HuCCA-1Nu cell lines were essentially similar at all passages (passages 7 and 87 for HuCCA-1 and passages 6 and 44 for the HuCCA-1Nu). By SEM, the cells were bizarre in shape and size and grew in a pavement-like arrangement. Giant cells were regularly present at all passages (Fig 1). The surfaces of the tumor cells were lined with different densities of microvilli of varying shapes and sizes. The irregularly distributed microvilli varied from cell to cell and could at times be used to outline the borders of adjoining cells (Fig 2). There was a loss of contact inhibition, judging from cells piling up on top of one another, but at most for not more than 3 layers. Secretory products were often found adhering on the cell surfaces, particularly on cells with scanty microvilli (Fig 3).

With the TEM, the tumor cells were found to polarize with respect to the tissue culture plate. The cells exhibited a large, finely granular nucleus with one or more large nucleoli. The nucleus was sometimes deeply indented. The cytoplasm was relatively clear with scanty organelles. Microfilaments typical of cytokeratin found in epithelial cells were randomly distributed. Cells adhered to their

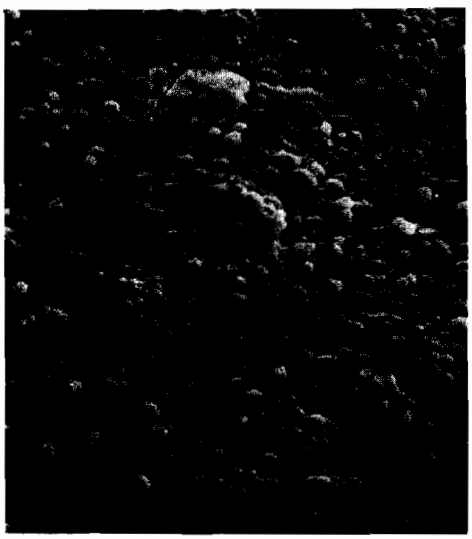

Fig 1–SEM of HuCCA-1Nu at passage 44 revealing cells with bizarre shapes and sizes in a pavement-like arrangement. Giant cells (G) were frequently present × 165.

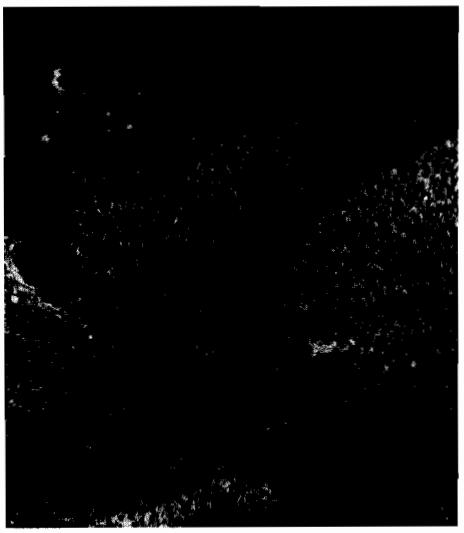

Fig 2–SEM revealing pleomorphic microvilli irregularly distributed on HuCCA-1Nu cell surfaces (passage 44) × 2,500.

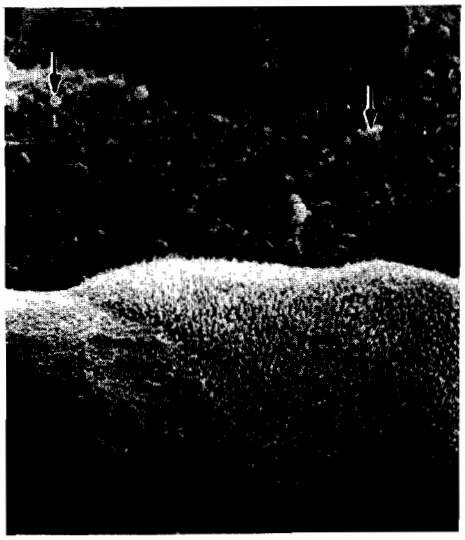

Fig 3–SEM of HuCCA-1Nu at passage 44. Secretory products (arrow) can be seen adhering to the surface of the tumor cells. A giant cell (G) with a surface densely populated by microvilli shows no adhesion of secretory products × 1,670.

neighbors by a tight junction at the apical border and by desmosomes laterally (Fig 4). Some were closely apposed whereas others were interdigitated laterally with intermittent desmosomes. A microvillus lined intracytoplasmic lumen was constantly seen and found to be partially or fully filled with secretory products as well as glycocalyceal bodies (Figs 4, 5). When compared with parental HuCCA-1 cells, HuCCA-1Nu showed an increase in secretory activity as evidenced by a relatively larger number of cytoplasmic secretory granules and by the quantity of secretions in the intracytoplasmic lumen and on the cell surface (Fig 5). Ruthenium red preserved mucopolysaccharide was

Fig 4–TEM of a HuCCA-1 cell (passage 67) showing an intracytoplasmic lumen and desmosomal (arrow) connection to other cells × 13,600.

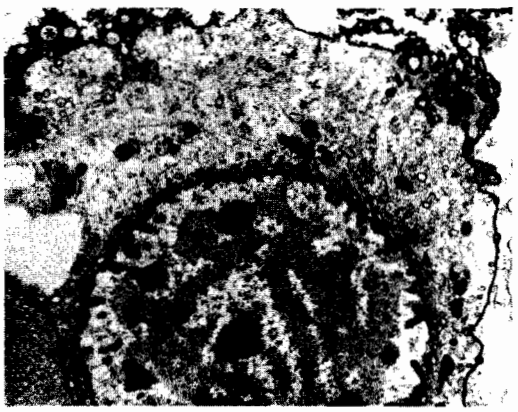

Fig 5–A HuCCA-1Nu cell (passage 44) showing a distended intracytoplasmic lumen filled with secretion and glycocalyceal bodies (GB). Ruthenium red-stained mucopolysaccharides (arrow) can be seen on the cell surface and associated with the cell membrane. In contrast, the dye failed to stain the intracytoplasmic luminal membrane and the luminal contents. Numerous secretory granules (★) can be found close to the cell surface × 17,000.

found associated with the cell surface. The protocol used for staining did not allow the dye to reach the intracytoplasmic lumen and therefore the latter failed to take up the stain. Fig 6 illustrates an intercellular gland-like structure the lumen of which is filled with secretions. All these features are similar to those described previously for the hamster cholangiocarcinoma cell lines.

It should be pointed out that while the microfilaments of both the original and nudemouse passage cell lines exhibited ultrastructural characteristics typical of well differentiated cholangiocarcinoma cells, a number of the tumor cells in the tumor sections obtained directly from tumor-bearing animals did not. Instead, it was found that 20-30% of the cells at the base of the tumor tubules formed small foci of cells in sheet-like arrangements. These cells showed cellular alterations characterized by electron dense tonofilament bundles resembling those found in squamous cells (Figs 7, 8). There were no secretory granules seen in these tumor cells.

DISCUSSION

The results presented in this study illustrated detailed ultrastructural features of human cholangiocarcinoma cell lines that were derived from an original intrahepatic bile duct tumor and after nudemouse passage. The morphological characteristics

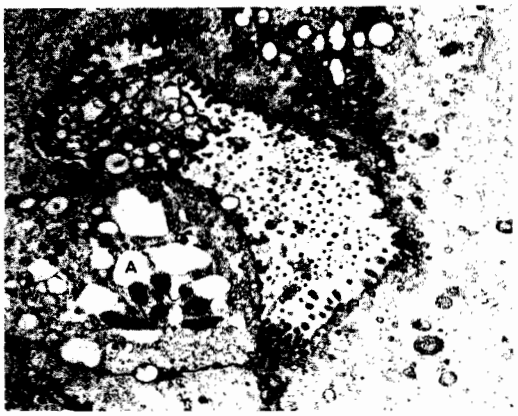

Fig 6–An intercellular lumen in HuCCA-1 at passage 87. The cell at the lower left is rich in amorphous secretory granules (A) × 12,500.

Fig 7–TEM section of a nude mouse tumor. Tumor cells show cellular alteration with electron dense tonofilament bundles in the cytoplasm (arrow) × 2,700.

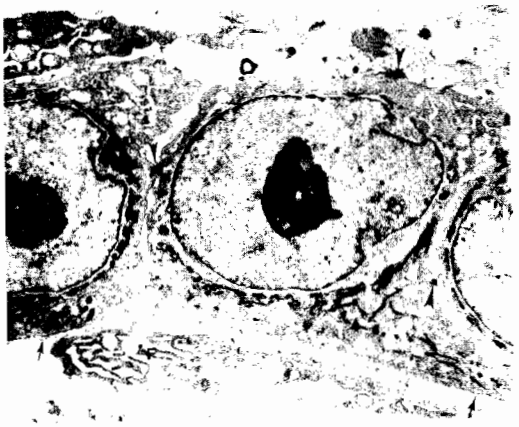

Fig 8–Cells with tonofilament bundles typical of the squamous cell type at the base of a tumor tubule. Desmosomes (arrow head) and fragmented basement membrane (arrow) can be readily observed. Part of a fibroblast (F) is seen at the bottom × 8,500.

of both cell lines were similar. Ultrastructure typical of the two cell lines included surface microvilli, cytoplasmic intermediate microfilaments, desmosomal attachments and apical tight junctions to adjoining cells. Microvillus lined intracytoplasmic lumens could be readily seen (Figs 4-8). These cellular structures were essentially identical with those of CCA cell lines established from an experimentally induced tumor in a hamster model (Tengchaisri *et al*, 1995). It should be noted, however,

that there were no lamellar body inclusions such as those frequently found in the experimentally induced CCA in hamsters (Tengchaisri *et al*, 1995). Although there is no satisfactory explanation for this difference, it may be related to the differences in the carcinogenesis induction process. However, association with a liver-fluke promoter does not appear to exert any influence on the morphology of these cell lines since morphological similarlities have been noted among human CCA cell lines developed from tumors of patients from endemic and non-endemic areas of liver fluke infection (Yamaguchi *et al*, 1985; Miyagiwa *et al*, 1989). These ultrastructural features were rather stable, as no differences were noted between cells taken from early and late passages (data not shown).

Although the ultrastructural features reported for these cell lines appeared to be rather stable, it is possible that they may be influenced and altered by the microenvironment under *in vivo* conditions. For example, to our knowledge, morphological alteration of intermediate microfilament bundles to those typical of squamous cells (Figs 7, 8) has never been reported following nude mouse passage of established adenocarcinoma cell lines. Results from the study of Iemura and associates (1992b) showing a gradual histological alteration from adenomatous to squamous cell carcinoma following serial subcutaneous transplatation of tumor fragments into nude mice are consistent with our results. Altogether, the data suggests that *in situ* alteration to a squamous cell type is due to metaplastic transformation of adenocarcinoma cells. The transformation could have come about from a variety of cytokines and host responses to tumor challenge. This conclusion is consistent with evidence from a clinicopathological study by Nakajima and Kondo (1990) of an intrahepatic cholangiocarcinoma with a squamous cell carcinoma component. However, until now there has been no evidence to show that squamous cell tumors can revert back to the adenocarcinomatous type as shown here. The reversion occurred under suitable microenvironmental conditions, *ie* during *in vitro* cultivation in enriched culture medium. It could be argued, on the other hand, that the original tumor may have been heterogeneous and may have consisted of cells with a morphology which somehow preferentially propagated in the nude mouse. However, the HuCCA-1Nu cell line which was established after nude mouse passage of the parental HuCCA-1 did not express any characteristics squamous cell type.

ACKNOWLEDGEMENTS

This study was supported by research grants from the Chulabhorn Foundation (Thailand) and the China Medical Board of New York (USA). Collaboration by staff at the National Cancer Institute (Ministry of Public Health, Bangkok, Thailand) particularly with regard to the supply and maintenance of nude mice is greatly appreciated. The authors are also grateful to Dr TW Flegel for his comments and suggestions during the preparation of this manuscript.

REFERENCES

Hayada Y. Cholangiocarcinoma-H-1 cell line. In : Ohboshi S. Sugano H, ed. Tissue Culture of Human Cancer Cells. *Japan: Asakura*. 1975; 181-2.

Homma S, Nagamori S, Fujise K, *et al*. Human bile duct carcinoma cell line producing abundant mucin *in vitro*. *Gastroenterol Jpn* 1987; 22 : 474-9.

Iemura A, Maruiwa M, Yano H, *et al*. A new human cholangiocarcinoma cell line (KMC-1). *J Hepatol* 1992a; 15 : 288-98.

Iemura A, Yano H, Mizoguchi A, *et al*. A cholangiocellular carcinoma nude mouse strain showing histologic alteration from adenocarcinoma to squamous cell carcinoma. *Cancer* 1992b; 70 : 415-22.

IARC. Monographs on the Evaluation of Carcinogenic Risks to Humans: Schistosomes, Liver Flukes and *Helicobacter pylori*, 1994; 61 : 121-75.

Kusaka Y, Tokiwa T, Sato J. Establishment and characterization of a cell line from a human cholangiocellular carcinoma. *Res Exp Med* 1988; 188 : 367-75.

Miyagiwa M, Ichida T, Tokiwa T, *et al*. A new human cholangiocellular carcinoma cell line (HuCCA-T1) producing carbohydrate antigen 19/9 in serum-free medium. *In Vitro Cell Dev Biol* 1989; 25 : 503-10.

Nakajima T, Kondo Y. A clinicopathologic study of intrahepatic cholangiocarcinoma containing a component of squamous cell carcinoma. *Cancer* 1990; 65 : 1401-4.

Sirisinha S, Tengchaisri T, Boonpucknavig S, *et al*. Establishment and characterization of a cholangiocarcinoma cell line from a Thai patient with intrahepatic bile duct cancer. *Asian Pacific J Allergy Immunol* 1991 : 9 : 153-7.

Storto PD, Saidman SL, Demetris AJ, *et al*. Chromo-

somal breakpoints in cholangiocarcinoma cell lines. *Genes Chromosomes Cancer* 1990; 2 : 300-10.

Tengchaisri T, Prempracha N, Thamavit W, *et al.* Establishment and characterization of cell lines form liver fluke-associated cholangiocarcinoma induced in a hamster model. *Southeast Asian J Trop Med Public Health* 1995; 26 : 231-9.

Yamaguchi N, Morioka H, Ohkusa H, *et al.* Establishment and characterization of the human cholangiocarcinoma cell line HChol-Y1 in a serum-free, chemically defined medium. *J Natl Cancer Inst* 1985; 75 : 29-35.