Establishment and Characterization of a Chicken Hepatocellular Carcinoma Cell Line, LMH

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ABSTRACT

A hepatocellular carcinoma cell line, LMH, has been established from a hepatocellular carcinoma induced in a male leghorn chicken by diethylnitrosamine. The cell line is characterized by well-differentiated morphological and biochemical features including the expression of glucose-6-phosphatase and canalicular ATPase activities and triplid karyotype with six marker chromosomes.

The cells have been continuously propagated in culture for 5 yr and are now at about the 120th passage. Morphological changes occurred in culture associated with gradual increase in growth rate at about the 40th passage. However, the biochemical and chromosomal features remained constant.

This is the first established avian cellular epithelial cell line and will allow comparative investigation of a number of parameters relevant to chicken hepatocarcinogenesis.

INTRODUCTION

Cell lines provide useful tools for a variety of studies including cellular metabolism and regulation, virus replication, and carcinogenesis under standardized conditions. For these purposes numerous cell lines from different species have been established. The difficulty of obtaining permanent culture cell lines from chicken tissues has long been known (1-5), and it is only relatively recently that lymphoblastoid cell lines from avian virus-induced lymphomas (6, 7) or leukemias (8, 9) and fibroblastic cells demonstrating long-term growth from normal, Rous sarcoma virus-treated or carcinogen-treated chicken embryo cells were established (10-13). Chicken epithelial cell lines have hitherto not been reported. We describe here the establishment of a hepatoma cell line from diethylnitrosamine-induced primary hepatocellular carcinoma in a male leghorn. The cells retain a number of differentiated phenotypic traits of chicken hepatocytes and are of potential usefulness for comparative investigation of biological parameters.

MATERIALS AND METHODS

Animals and Carcinogen Treatment. Male leghorn LM strain chickens, 20 days of age, were purchased from Nisselken Co., Tokyo, Japan. They were maintained in aluminum cages in an air-conditioned room. Starting from July 3, 1980, animals were given drinking water containing 100 ppm diethylnitrosamine (Tokyo Kasei Co., Tokyo, Japan) for 9 wk and then weekly or biweekly i.m. injections of 20 to 100 mg diethylnitrosamine/kg for 30 wk. About 24 wk after discontinuation of carcinogen treatment, an animal became moribund with a hepatic tumor and was sacrificed on Oct. 9, 1981. The liver was greatly enlarged and granular with multiple, up to bean-sized tan nodules. No morphological distinction could be made between the original and tumor-intrahepatic metastasis grossly.

Cell Culture. Tumorous tissues were taken from several nodules of the liver separately, transferred onto plastic plates (Nunc; 60 mm) containing 5 ml of Waymouth's medium supplemented with 10% fetal calf serum and kanamycin (6 μg/ml), minced, and cultured at 37°C in a CO₂ incubator. The medium was changed the next day and twice a wk thereafter.

Morphological Observation. The original hepatic tumors and tumors which developed in nude mice after inoculation of the cells were fixed in 4% formalin and used for routine histological observation. The cultured cells were examined under inverted phase-contrast microscopy.

Tumorigenicity Test. Athymic nude mice were inoculated s.c. with 1 × 10⁶/ml passage 30 cells suspended in Waymouth's MB 752/1 medium and then maintained in isolated sterile cages.

Histochemistry. Culture cells and frozen sections of the tumors which developed in nude mice after inoculation of the cells were stained for GGTase activity by the method described by Rutenburg et al. (14) and for G6Pase and ATPase activity by the methods of Wachtstein and Meisel (15). Fibronectin was detected by immunological reaction in culture fluid using anti-fibronectin serum (Bethesda Research Laboratories Inc., Philadelphia, PA).

Chromosome Studies. Logarithmic phase cells were treated for 2 h with Colcemid and harvested by ordinary trypsin treatment. They were then hypotonicallly treated with 0.075 M KCl for 20 min at 37°C and fixed with methanol-acetic acid (3:1), and chromosome counts were carried out on enlarged photographs of metaphase plates. Chromosomes were also examined by the trypsin-Giemsa banding technique (16).

Electron Microscopy. The cells obtained were seeded in a 100-mm plastic dish (Corning) and allowed to proliferate. The dishes were rinsed with Dulbecco's phosphate-buffered saline, and the cells were scraped gently with a rubber policeman from culture vessels, fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min at room temperature, and then postfixed in 2% osmium tetroxide-0.1 M phosphate buffer (pH 7.4) for 30 min at room temperature. They were dehydrated in series of alcohol and propylene oxide and embedded in Epon-812. Ultrathin sections were cut on an LKB microtome with a glass knife. Observations and photography were performed using a Hitachi HU-12 electron microscope, after contrastin sections with uranyl acetate.

Reverse Transcriptase Activity. The cell-free medium of the culture was assayed for endogenous reverse transcriptase activity according to the method described by Bauer and Temin (17).

RESULTS

Establishment of the Cell Line. Within a few days cells forming small foci began to grow slowly. From all plates containing cells from different nodules, very similar sheets of epithelial-looking cells were obtained. The first subpassage of the largest epithelial foci was carried out after 2 mo when the size reached 20 mm in diameter. The cells have subsequently been continuously subcultured for 5 yr, approximately once a mo for the initial 3.5 yr and once a wk now, at about the 120th passage level.

Fibroblastic cells and macrophages were observed as contaminating elements among epithelial-type cells in the initial few passages, but later they disappeared completely.

Chromosome Analysis. Chromosome analysis was performed

1 The abbreviations used are: GGTase, γ-glutamyl transpeptidase; G6Pase, glucose-6-phosphatase.

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4460
large chromosomes

small and micro chromosomes

sex chromosomes

marker chromosomes

Fig. 1. Trypsin produced G-banding karyotypes of chicken hepatocellular carcinoma cell line LMH with 116 chromosomes showing 6 marker chromosomes (30th passage). The chromosome number was determined by counting 39 metaphase cells.

Fig. 2. Growth curves of the LMH cell line at passage 120 with 10% fetal calf serum (O). Medium was changed every third day. Growth curve in serum-free medium (8). Medium was changed every 2 days. Bars, SE.

twice. The first analysis was carried out at about the 30th passage and the second at the 120th passage. The results of both analyses were essentially identical. The avian origin of the cells was confirmed by the presence of numerous microchromosomes.

The number of chromosomes ranged from 88 to 124, the modal number being 116 (normal diploid 2n = 78) (18–20). All the cells commonly possessed 6 marker chromosomes (Fig. 1), clearly indicating a clonal origin for the cells.

Concluding the establishment of a new cell line, we designated the culture line LMH.

Growth Characteristics. The LMH cells grew relatively slowly during the initial 3.5 yr requiring subpassage once a mo when 2 x 10^5/ml cells were plated onto 60-mm dishes. After about 40 passages, the cell line began to demonstrate change in cell morphology associated with gradual increase in the growth rate. At present, 5 yr from the beginning and at about the 120th passage, the cells are growing much faster, requiring subpassage once a wk. Growth curves of the cells at passage 120 are shown in Fig. 2. The population doubling time during the logarithmic phase was 25 h. The LMH cells proliferate well in serum-containing medium but also grow in serum-free medium with an approximate halving of the growth rate (Fig. 2).

Morphological Characteristics. All the hepatic nodules examined and the tumors which developed in the subcutis of nude mice after inoculation demonstrated a very similar histological appearance, i.e., well-differentiated trabecular hepatocellular carcinoma with occasional transition to adenocarcinomatous structures (Fig. 3, a and b).

Until about the 40th passage, the LMH cells grew in a typical cobblestone-like pattern, and cells were polygonal with abundant cytoplasm and large round nuclei with a few prominent nucleoli (Fig. 4). After this time, the cells began to adopt a dendritic-like appearance, and the typical cobblestone-like pattern was observed only in occasional high cell density areas of dishes (Fig. 5). This morphological change appeared irreversibly fixed. Electron microscopy at the 30th passage revealed a large number of mitochondria, prominent Golgi apparatus, abundant rough endoplasmic reticulum, free ribosomes, and glycogen granules in the cytoplasm (Fig. 6). The plasma membrane possessed numerous well-developed microvilli. Biliary canaliculus-like structures (Fig. 6), desmosomes, and tight junctions were formed between adjacent cells. No virus particles were identified.
CHICKEN HEPATOMA CELL LINE

Fig. 3. a, photomicrograph of the original liver tumor demonstrating a well-differentiated trabecular hepatocellular carcinoma. H & E, ×60. b, light microscopic appearance of a tumor that developed in a nude mouse after transplantation of LMH cells. The histological appearance is indistinguishable from the original tumor shown in a. H & E, ×140.

Fig. 4. Morphological features of the LMH cells in culture at passage 5. Polygonal cells with abundant cytoplasm and large round nuclei with prominent nucleoli are arranged in a typical cobblestone-like pattern. Phase contrast, ×14.

Fig. 5. LMH cells in culture at passage 120. Note dendritic-like appearance together with sheet-like pattern. ×30.

Fig. 6. An electron micrograph of LMH cells. Well-developed cell organelles, desmosomes, tight junctions, and biliary canaliculus-like structures are shown (30th passage). ×9360.

Enzyme Markers. In spite of the change in the morphology and growth rate, the biochemical features of the LMH cells at the 30th and 120th passages were essentially the same. G6Pase activity could be clearly demonstrated histochemically both in the cultured cells (Fig. 7a) and in tumor tissue which developed in nude mice (Fig. 7b). Weak Ca\(^{2+}\), Mg\(^{2+}\)-dependent, formalin-resistant ATPase activity was also evident in cultured cells (Fig. 8) and tumor tissue. No GGTase activity could be demonstrated histochemically in either cultured cells or tumors in nude mice. Fibronectin was detected in the culture fluid.

Reverse Transcriptase Activity. This was negative in the culture medium.

Tumorigenicity. Tumorigenicity was demonstrated in nude mice twice, utilizing 30th and 120th passage cells. When 10\(^7\) cells at the 30th passage were inoculated into the subcutis of the back, tumors reaching 2 cm x 1 cm developed after 7 mo. In the second inoculation experiment, similar tumors developed after 3 mo.
CHICKEN HEPATOMA CELL LINE

Fig. 7. a, cytochemical demonstration of G6Pase activity in cultured cells (passage 6). Diffuse cytoplasmic activity is evident. ×80. b, histochemical demonstration of strongly positive G6Pase activity in a tumor developed in a nude mouse after transplantation of cells. ×12.

Fig. 8. Cytochemical reaction for ATPase in the culture cells. The enzyme activity is demonstrated on the outer cellular membrane between the neighboring cells. ×30.

DISCUSSION

To our knowledge this is the first paper describing the establishment of an epithelial cell line of domestic fowl. This line was derived from a hepatocellular carcinoma induced by diethylnitrosamine after long-term treatment. Although not originally cloned, clonality of the cells comprising the LMH line is indicated by the presence of 6 marker chromosomes in all the cells.

The hepatocellular nature of the LMH line is evident from the morphology of the tumors developing in nude mice after inoculation and the expression of G6Pase activity. Morphological features including only mild cellular atypism, possession of abundant cellular organelles, and formation of biliary canalicular-like structures together with positive G6Pase and ATPase activities indicate a relatively high grade differentiation of the carcinoma cells, even after the morphological change and increased growth rate.

Since many important avian sarcoma and leukemia viruses have been isolated from mesenchymal malignancies of chicken and extensively contributed to oncogene studies, the availability of this cell line might be useful for studying chemical carcinogenesis, especially that of epithelial cells, in comparison with viral sarcomagenesis.

In addition to the problems of determining ideal culture conditions, one of the reasons for the difficulty in establishing epithelial cell lines of fowls has been the difficulty of inducing primary neoplasms in this animal by carcinogen treatment. As compared with the situation in rodent systems, much larger doses of carcinogenic agents and much longer latent periods have been necessary for inducing carcinomas of parenchymal organs in the domestic fowl (21–23). In the present experiment, the total dose of diethylnitrosamine and the latent period required to induce a hepatocellular carcinoma were roughly 4 to 5 times those required in the rat system. The presently described LMH cell line might prove useful for elucidating mechanisms of resistance to carcinogenesis of domestic fowl cells.

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