Establishment and Characterization of a Cell Line from a Chemically-induced Mouse Hepatoblastoma

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Abstract. We established a cell line (MHB-2) from a hepatoblastoma (HB) induced by diethylnitrosamine (DEN) and sodium phenobarbital (PB) in male B6C3F1 mice and examined the biological characteristics of MHB-2. MHB-2 cells grew as monolayers in culture and showed a spindle or polygonal shape. Immunohistochemically, the original tumor cells and MHB-2 cells were negative for keratin, alpha-fetoprotein and albumin. Electron microscopically, MHB-2 cells had irregular-shaped nuclei with prominent nucleoli, abundant free ribosomes, myelinosomes, desmosomes and surface microvilli. Growth of this cell line was significantly accelerated by hepatocyte growth factor (HGF) and expression of its receptor c-met was confirmed by the reverse transcription-polymerase chain reaction (RT-PCR). MHB-2, however, was not found to be tumorigenic when transplanted into the subcutaneous tissue of syngeneic, nude or scid mice. To our knowledge, this is the first report on the establishment of a cell line derived from a mouse HB. — Key Words: cell line, hepatoblastoma, HGF, mouse, phenobarbital.

Full Paper: Pathology

MATERIALS AND METHODS

Source of cell line: The original HB was induced in a mouse by administration of DEN and PB as described previously [9]. Briefly, male 6-week-old B6C3F1 mice (Charles River Japan, Yokohama, Japan) were given DEN intraperitoneally at a dose of 80 mg/kg, and then fed diet containing PB (Tokyo Kasei, Tokyo, Japan) at a concentration of 500 ppm until the end of the experiment. At the 52nd week of experiment, the animals were euthanized and necropsied under ether anesthesia. Some liver nodules were selected for culture after portions of the nodules were taken and fixed in 10% buffered formalin for diagnosis with paraffin embedded sections stained with hematoxilin and eosin (HE).

Cell culture: Fresh tumor tissues were cut into small pieces, minced in Dulbecco’s modified Eagle’s medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) with sterile scissors and washed with DMEM. The tumor pieces were treated with dispase (50 U/ml; Godo Shusei, Tokyo) for 30 min at 37°C and large tumor pieces were allowed to settle. Supernatant fluid containing cell clumps was collected after centrifugation at 100 g and cell pellets were resuspended in DMEM containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, U.S.A.) plus serum extender MITO (0.1%, Collaborative Biochemical Products, Bedford, MA, U.S.A.), 100 units/ml penicillin (Gibco), and 100 units/ml streptomycin (Gibco) and cultured on dishes coated with collagen type I (Iwaki, Tokyo) in a humidified 5% CO2 incubator at 37°C with weekly changes of the medium. After 2 months, growing colonies were harvested with trypsin/EDTA and passaged several times. Any remaining fibroblasts were removed by mechanical scraping and the differential attachment selection method, after which cultures of the cells forming epithelial-like monolayers were obtained (MHB-2 cell line). MITO contains insulin, transferrin, epidermal growth factor, endothelial growth supplement, dexamethasone and triiodo-17β-estradiol. The absence of Mycoplasma pulmoris contamination of the MHB-2 cell line was confirmed by the direct agar method.

Growth characteristics: To evaluate the influence of growth factors on cell proliferation of the cell line, DMEM with 10% FBS in the absence of MITO was used as a control medium. Cells were plated at 1 × 104 cells/dish in 35 mm plastic dishes in control medium alone or supplemented with MITO, hepatocyte growth factor (HGF; 10 ng/ml, Toyobo, Tokyo, Japan) or insulin/transferrin (each, 5 µg/ml, Sigma, Toyobo, Tokyo, Japan).
RESULTS

Original HBs in the livers of mice: The incidence of DEN-PB-induced HBs in this experimental protocol was 9/31 (29%). Primary tumors showed typical histologic features of mouse HBs. Briefly, tumor cells were elongated to spindle-shaped with moderately abundant eosinophilic cytoplasm and irregular hyperchromatic nucleus. The elongated cells were frequently arranged in pseudorosettes or ribbons around the central blood vessels (Fig. 1). Immunohistochemically, the primary tumors were negative for keratin (Fig. 2), AFP and albumin (data not shown).

Morphological and immunohistochemical characteristics of the cell line: MHB-2 cells exhibited a spindle to polygonal shape in dense cultures of epithelial cell-like monolayers (Fig. 3). Immunohistochemically, the cell line proved negative for keratin, albumin and AFP (data not shown).

Electron microscopy: MHB-2 cells demonstrated large, irregular shaped nuclei with one or more prominent nucleoli. Their cytoplasm contained small sized mitochondria, abundant free ribosomes, and myelinosomes. Microvillus cytoplasmic projections and desmosomes were occasionally observed (Fig. 4).

In vitro growth characteristics: In DMEM containing 10% FBS without MITO, no substantial increase in cell number on plastic dishes was observed. Growth of the MHB-2 cell line was significantly accelerated by HGF (P<0.01) but not by insulin/transferrin (Fig. 5).

RT-PCR analysis for c-met: Expression of c-met, a receptor of HGF, was detected at the mRNA level by RT-PCR (Fig. 6).

Tumorigenesis: The MHB-2 cell line was not tumorigenic when transplanted into the subcutaneous tissue of syngeneic, nude or scid mice (data not shown).

DISCUSSION

Human HB, the most common malignant tumor of the liver in childhood, exhibits a wide range of epithelial and mesenchymal lines of differentiation [2, 3]. Epithelial HBs include fetal and embryonal subtypes [8]. Mouse HBs occur spontaneously in aged mice and can be induced by certain chemicals in several strains [4, 15, 20, 21]. We have previously reported mouse HBs induced by DEN and PB [9], which are similar to those of embryonal type seen in man. In the present study, we succeeded in establishing a cell line in culture (MHB-2) from the mouse HB induced by DEN and PB with the same protocol as described in our previous report [9]. To the best of our knowledge, MHB-2 is the first established cell line derived from a mouse HB.

The histogenesis of mouse HB remains controversial [4, 15, 22]. Its frequent location within or adjacent to common hepatocellular tumors [2, 3] has lead to the suggestion of the origin from hepatocytes. However, reports [9, 15] of a lack of AFP indicate the origin from cholangiolar cells [2, 15]. In the present study, no HBs were observed in non-

Statistical analysis: The statistical significance of differences in growth was analyzed by Student’s t-test.

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promoted control group mice treated with DEN alone. Thus, it is possible that the present primary tumor arose from cells of the hepatocytic lineage known to be susceptible to PB promotion [16].

The present immunohistochemical and electron microscopic findings for MHB-2 cells are consistent with previous observations for mouse HBs [9, 15]. The characteristic myelinosomes observed in MHB-2 cells closely resembled the structures observed in hepatocytes exposed to certain kind of amphiphilic drugs [17], suggesting the origin from hepatocytes. On the other hand, MHB-2 cells did not bind anti-AFP serum, unlike hepatocellular carcinoma cells [15]. However, the mounts of rough endoplasmic reticulum for effective AFP-production might not have been sufficient as in the study of human HB cells by Horie et al. [6]. Thus, the negativity for AFP in MHB-2 cells does not necessarily preclude the origin from hepatocytes. As for keratin, it has previously been reported that mouse HB cells often show squamous metaplasia with immunohistochemical binding of anti-keratin antibodies [15]. In this study, however, squamous differentiation was not observed, and no keratin-positive reaction was seen in MHB-2 cells and primary HB cells in contrast to the positive staining of normal bile ducts. The poorly-differentiated characteristics of HB cells observed in this study, as well as in previous reports, suggest that there are two possible origins as previously suggested by Diwan et al. [2]; 1) malignant conversion of hepatocytic tumor with loss of differentiation, and 2) evolution from non-hepatocyte precursor cells.

It is noteworthy that the MHB-2 cell line required HGF for optimal growth, in addition to 10% FBS, and expressed the c-met, a protooncogene encoding the HGF receptor which is predominantly expressed in epithelial cell types and overexpressed in a variety of human and rodent neoplasms [5, 10, 11, 13, 18, 19]. The lack of autonomous growth is surprising since mouse HBs are reported to be highly malignant [2, 4] and suggesting that the MHB-2 cell line may have originated from an early stage tumor. HGF is a pleiotropic cytokine which has been shown to be a growth factor as well as a morphogenic factor for a variety of epithelial cell lines [12]. Yaono et al. [24] previously reported that a single dose of recombinant human HGF increased the numbers and areas of glutathione S-transferase placental form positive foci, a putative preneoplastic lesion in a two-stage rat hepatocarcinogenesis model. Furthermore, Neaud et al. [14] demonstrated that myofibroblast-derived HGF could be involved in the development of hepatocellular carcinomas. Further studies are needed to elucidate the role of HGF in malignant progression of mouse HB.
In conclusion, the HGF-responsive MHB-2 cell line established from a DEN-PB-induced mouse HB would be useful for in vitro analysis for the biological characteristics of early stage mouse HB cells.

Fig. 4. Electron microphotograph of MHB-2 cells. Note large, irregularly shaped nuclei with prominent nucleoli. The cytoplasm contain small-sized mitochondria, abundant free ribosomes, and some myelinosomes (arrow heads). Microvillous cytoplasmic projections are apparent. × 7,000. Insert: Desmosome between contiguous cells. × 18,000.

Fig. 5. Response of MHB-2 cell line to growth factors in culture. Cells were cultured in control medium (cont) or medium supplemented with MITO, Insulin/transferrin (Ins/tr) or HGF. Growth of MHB-2 cell line was significantly accelerated by HGF but not by insulin/transferrin. *Significantly different from the control at P<0.01.

In conclusion, the HGF-responsive MHB-2 cell line established from a DEN-PB-induced mouse HB would be useful for in vitro analysis for the biological characteristics of early stage mouse HB cells.

Fig. 6. RT-PCR analysis for c-met. Expression of c-met, a receptor of HGF, was detected at the mRNA level. L, normal mouse liver; M, MHB-2 cells.

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REFERENCES


