Isolation of a Metastasizing Cancer Cell Line from an Aflatoxin B_1-Induced Rat Liver Tumor

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An attempt was made to isolate cancer cell lines from liver tumors that had been induced by aflatoxin B_1 (AFB_1) in rats. A clonal cell line named AFB-1 was isolated from a liver tumor that was histologically diagnosed as hepatocellular carcinoma. When AFB-1 cells were inoculated into the subcutaneous tissue at the dorsal region of syngenic animals, they metastasized from the site of inoculation into the abdominal cavity to form many tumor nodules throughout the serous membrane and metastatic foci in the kidney and pancreas. They also metastasized into the thoracic cavity to form metastatic foci in the lung. This is the first instance where a metastasizing AFB_1-induced cancer cell line has been isolated.

Keywords aflatoxin B_1; hepatocellular carcinoma; cancer cell line; metastasis

Introduction

Aflatoxins are produced by some toxigenic strains of *Aspergillus flavus* or *Aspergillus parasiticus*, occur in contaminated human foods and animal feeds, and have been inferred to be one of the etiological factors for human liver cancers.[1,2] Aflatoxin B_1 (AFB_1) is a potent hepatocarcinogen for many species of animals and has been extensively studied in the rat to elucidate the modes of toxic action and neoplastic induction.[1,3,4]

In view of our interest in the cell biology of AFB_1-induced liver cancer cells, we tried to isolate such cell lines in culture from liver tumors that had been induced in animals under a carcinogenic regimen. We report here that we have isolated a cancer cell line that has a potent metastatic activity.

Materials and Methods

Animals and Treatments To induce liver tumors, weanling male Fischer 344/JCI rats (CLEA Japan Inc., Tokyo) received daily intraperitoneal injections of 25 μg of AFB_1 (Makor Chemicals, Jerusalem, Israel) in 50 μl of dimethylsulfoxide (spectral grade, Wako Pure Chemicals Ind., Tokyo), 5 d/week for 8 weeks according to the method of McMahon *et al.*[5] The treatment of animals with injections started 4 weeks after birth and lasted for an 8 week period; thereafter animals were maintained on a basal diet CE-2 (CLEA Japan Inc.). The animals were housed in an air conditioned room kept at 23 °C and 60% relative humidity on a 12 h light/dark cycle. Moribund animals were killed under anaesthesia with pentobarbital sodium. Liver tumors for microscopic examination were fixed in 10% formalin solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Portions of liver tumors from moribund animals were used for the isolation of liver cancer cell lines.

Isolation of Liver Cancer Cell Line Liver tumors from moribund animals were freed from liver tissue and necrotic masses. Liver tumors were rinsed with Hanks' buffered salt solution (HBSS) and chopped finely with cross scalpels to about 1 mm^3. Pieces were transferred to a 100-ml Ehrenmeyer flask and suspended in 10 volumes of HBSS–0.01 M Hepes buffered solution (pH 7.5) containing 0.05% collagenase (type I, Sigma Chemical Co., St Louis, Mo.) and 0.01% trypsin inhibitor (type II-S, Sigma Chemical Co.). The suspension was incubated at 37 °C for 30 min under gentle shaking and dispersed with a rod. Pieces were allowed to settle for 1 min, and supernatant (the 1st supernatant) was filtered through 4 layers of gauze and placed in a 50-ml centrifuge tube. Settled pieces were again suspended in fresh collagenase plus trypsin inhibitor solution and incubated at 37 °C for 1.5 h. The 2nd supernatant was prepared and filtered as described above and placed in another centrifuge tube. Cells in the two supernatants were precipitated by centrifuging at $250 \times g$ for 10 min. Cell pellets were suspended in Williams' E culture medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% fetal bovine serum (Grand Island Biological Co.), 2 mM glutamine and gentamycin (50 μg/ml). The cell suspensions were fractionated into 2 fractions by stepwise centrifugations at $15 \times g$ for 1 min (fraction 1) and then at $250 \times g$ for 5 min (fraction 2). Cells of each fraction were suspended in the culture medium, seeded at a concentration of 1×10^6 or 4×10^6/ml in 100-mm (10 ml) polystyrene Petri dishes, and cultured at 37 °C under an atmosphere of 5% CO_2–95% air at 100% humidity. The medium was changed at 5 d after seeding and thereafter once a week. Several islands of epithelial cells were marked and maintained, while fibroblasts were removed by a rubber policeman or the round tip of a tapering glass rod. Approximately 3 months after seeding, two adjacent islands of epithelial cells began to grow rapidly in a dish that had been seeded with the cells of fraction 2 from the 2nd supernatant. The rapidly growing cells were passed 10 times by trypsinization and transfer to new dishes. The cloning of cell lines was performed by placing 8-mm stainless rings around the colonies to be isolated, trypsinizing the colonies within the rings, and transferring the dispersed cells into new dishes.[6] This step was repeated 4 times. In microscopic observation, almost all the clones showed a similar morphology in cell structure and colony formation.

JB1 Cells JB1 was another AFB_1-induced cancer cell line that had been established by Neal and his group.[7,8] JB1 cells were kindly provided by Dr. G. E. Neal, Medical Research Council Laboratories, United Kingdom and also studied in comparison with AFB-1 cells.

Electron Microscopy Cells were suspended in the culture medium at a concentration of 3×10^4 to 1×10^5/ml, placed in Lab-Tek culture chamber/slides (Nunc, Inc., Napelville, Ill.), and cultured for 3 d. The cells adherent to slides were fixed in 2.5% glutaraldehyde–0.1 M sodium phosphate solution (pH 7.4) for 6 h and post-fixed in 1% OsO_4 for 2 h at room temperature. After dehydration with a graded series of ethanol, the specimens were embedded in Epon 812. Ultrathin sections were cut with an ultramicrotome using a diamond knife, and stained with uranyl acetate and lead citrate. The stained sections were examined under a JEM-1200EX electron microscope (Japan Electron Optical Laboratory, Tokyo).

Results

Cloning of Liver Cancer Cell Line Attempts were made to isolate rapidly dividing epithelial cells in culture from the liver nodules of rats that were moribund in the carcinogenic regimen, and such cells were isolated from a rat that was killed at 565 d after the start of injections of AFB_1. The liver tumor was diagnosed as a hepatocellular carcinoma.[9] The cells were cloned as described in Materials and Methods, named AFB-1, and examined morphologically. AFB-1 cells adhered to polystyrene Petri dishes or glass plates of Lab-Tek culture chamber/slides and proliferated to form monolayers. The doubling time was estimated[6] to be approximately 16.6 h. Under electron microscope the cell nuclei were found to vary in size and exhibited irregular outlines with prominent development of nucleoli, 1 to 3 in number. The cytoplasmic portion was reduced and mitochondria were small and round. There

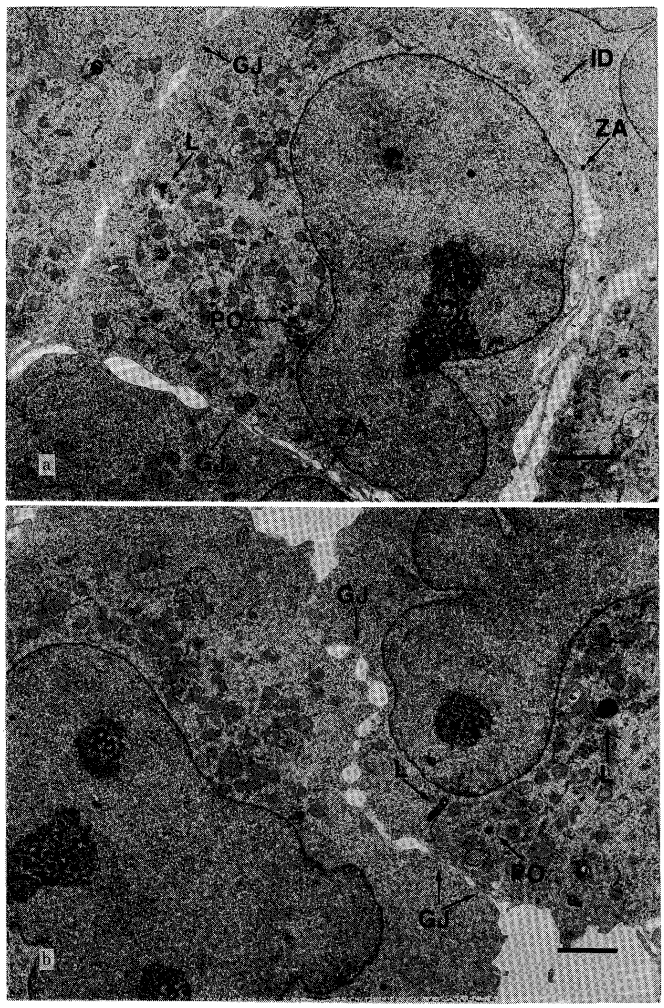

Fig. 1. Electron Micrographs of AFB-1 Cells in Culture

The cell nuclei show irregular outlines and the cytoplasmic portions are reduced. A small number of peroxisomes (PO) and lysosomes (L) are present. The intercellular attachment is so loose that many opening spaces are present between cells and interdigitations with microvilli (ID) poorly developed. Intercellular junctions are made mainly by gap junctions (GJ) and by zonula adherens (ZA) in parts. Bar = 2 μm. × 7000.

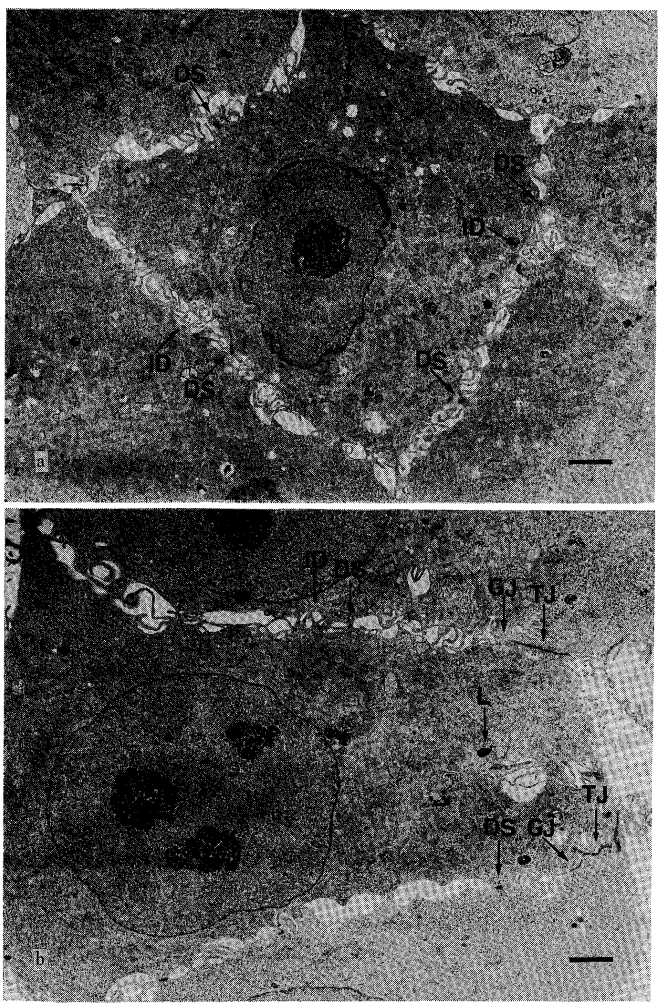

Fig. 2. Electron Micrographs of JB1 Cells in Culture

The cell nuclei showed round outlines and the cytoplasmic organella developed well. The intercellular attachment is made by both well-developed interdigitations with microvilli and desmosomes (DS) in the midst of the colony (a) and by a range of tight junction (TJ) and gap junction in the periphery (b). Bar = 2 μm. × 5000.

were well-developed rough surfaced endoplasmic reticulum and abundant polysomes. A small number of peroxisomes and lysosomes were noted. Intracellular attachments were so loose that many opening spaces were present between cells and interdigitations with microvilli poorly developed (Fig. 1a). Intercellular junctions were made mainly by gap junctions and zonula adherens in parts, but no strong attachment structures such as tight junctions or desmosomes were observed (Figs. 1a and 1b). These intercellular attachment structures of the cell line (AFB-1) were compared with those of JB1, another AFB_1-induced cancer cell line that had been established by Neal and his group.[7,8] The intercellular attachment structures of JB1 cells consisted of both well-developed interdigitations with microvilli and desmosomes in the midst of the colony (Fig. 2a) and a range of tight junction and gap junction in the periphery (Fig. 2b).

Transplantation of AFB-1 Cells A study was carried out to examine whether AFB-1 cells were transplantable in syngenic rats. Male Fischer rats, 4 weeks of age, were inoculated with 5×10^6 or 1×10^7 of AFB-1 cells into the subcutaneous tissue of the dorsal regions of the animals. In 40 to 60 d after the inoculation, all of the seven animals died or were moribund. The tumor nodules at the sites of inoculation were absent or small, if any, while the abdominal portions of the animals had swollen remarkably. In the abdominal cavity of the animals, numerous tumor nodules, small independent ones and large fused ones, were distributed throughout the serous membrane especially in the vicinity of stomach, spleen, diaphragm, pancreas, and kidneys. Under a microscope were seen many small well-demarcated colonies, indicating that AFB-1 cells grew and dispersed in small colonies throughout the serous membrane rather than growing to form a large nodule (Fig. 3a). A similar histological finding was noted in a tumor nodule at the site of inoculation. In addition, metastatic foci were found in the parenchymas of kidneys (Fig. 3b) and pancreas (Fig. 3c). Tumor nodules were also found in the thoracic cavity and metastatic foci were present in the lung parenchyma (Fig. 3d). A figure was obtained to show that a colony of AFB-1 cells infiltrated from the bronchial vein into lung parenchyma (Fig. 3e). For comparison, JB1 cells were also transplanted in the subcutaneous tissue of 5 animals. In contrast to AFB-1

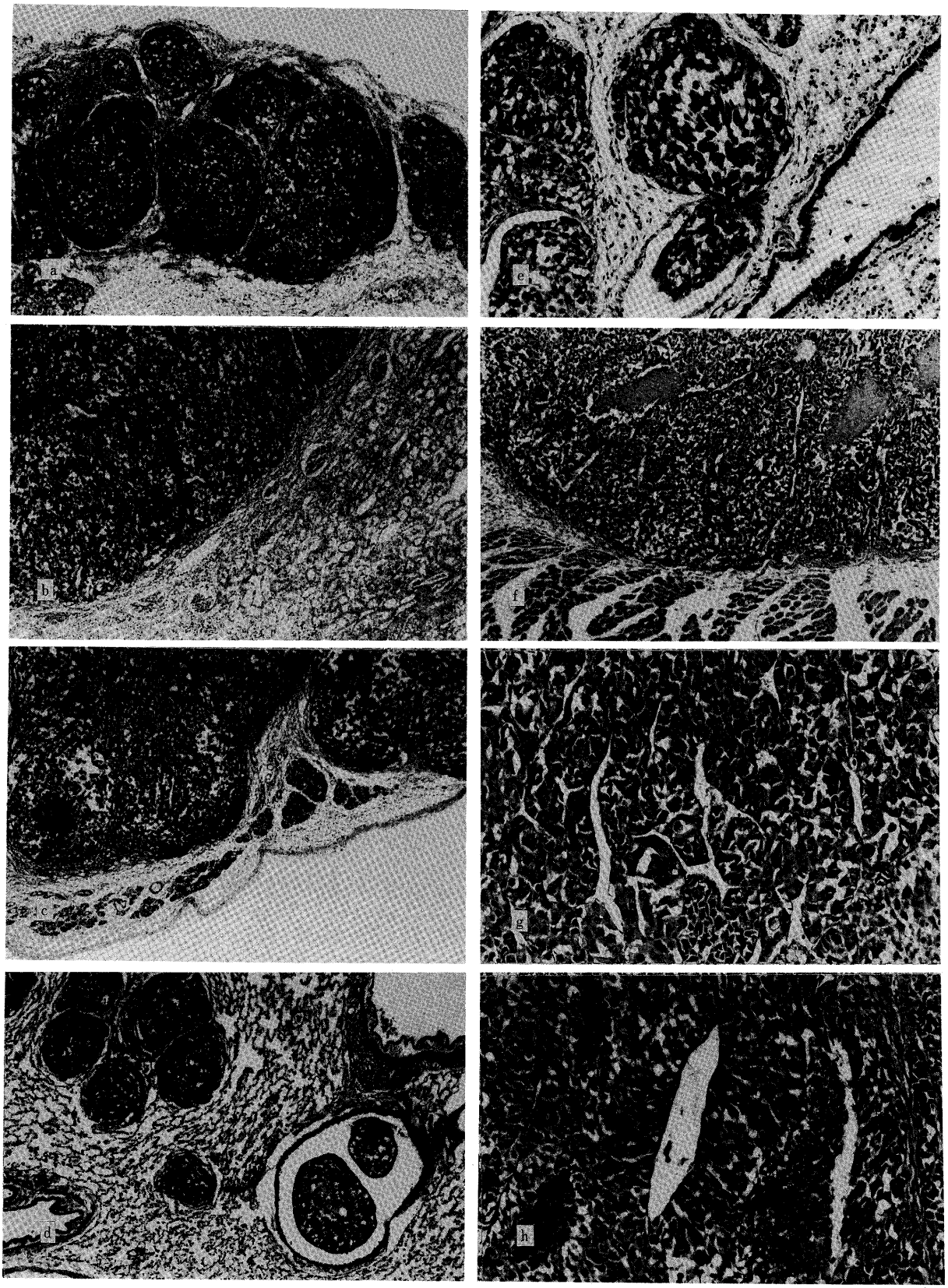

Fig. 3. Histology of Transplanted AFB-1 and JB1 Cells

a) Metastatic foci of AFB-1 cells in the abdominal serous membrane. They are an assembly of small colonies. ×60. b) Metastasis of AFB-1 cells in the parenchyma of kidney. ×60. c) Metastasis of AFB-1 cells in the parenchyma of pancreas. ×60. d) Metastasis of AFB-1 cells in the parenchyma of lung. ×60. e) Infiltration of AFB-1 cells from the bronchial vein into the parenchyma of lung. ×150. f) A nodule of JB1 cells at the site of inoculation in the subcutaneous tissue. JB1 cells form a large nodule, which is clearly demarcated from the surrounding subcutaneous tissue. ×60. g) A structure similar to sinusoids in the nodule of JB1 cells. ×150. h) A structure similar to the central vein in the nodule of JB1 cells. ×150.

cells, JB1 cells formed a large nodule only at the site of inoculation; at 40 d after the inoculation, the tumor nodules weighed between 8 and 15 g, while no metastatic foci were found in any tissues of the abdomen and chest. Histologically, the tumor nodule was clearly demarcated from the surrounding subcutaneous tissue and showed no invasive figures (Fig. 3f). The JB1 cells in the nodules arranged in cords and reticular structures and constituted duct structures which were similar to sinusoids or the central vein of the liver lobule (Figs. 3g and 3h).

Discussion

The purpose of this study was to establish an epithelial cell line from AFB_1-induced liver tumor nodule. We isolated and cloned rapidly growing epithelial cells from a tumor nodule which was histologically diagnosed as a hepatocellular carcinoma. The cloned cell line was named AFB-1. Then studies were carried out on the morphology and transplantability of AFB-1 cells in comparison with those of JB1, a cell line that had been established by Neal and his groups.[7,8] When AFB-1 cells were transplanted into the subcutaneous tissue of syngenic rats, they exhibited a marked potency of metastasis, *i.e.*, they metastasized from the site of inoculation, distributed throughout serous membrane and in some organs such as kidney, pancreas and lung, and finally killed the animals, while JB1 cells stayed and formed a large single nodule at the site of inoculation. Morphological findings in the cell structure and intercellular attachment structures indicated that AFB-1 was less differentiated than JB1; AFB-1 had a small size ratio of cytoplasm/nucleus, showed poor development of intercellular attachment structures, and formed a simple assembly of cells in a metastatic nodule. It is expected that these cell lines would be useful for further studies on the activated transforming genes[10,11] and the mechanism of metastasis in AFB_1-induced hepatic carcinomas.

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