Establishment and Characterization of Cell Lines (Kagura-1 and Kagura-2) from Aflatoxin B_1-Induced Rat Hepatoma

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Two hepatoma cell lines designated Kagura-1 and Kagura-2 were established from rat hepatocellular carcinomas induced by aflatoxin B_1, and have been propagated for over two years. Both cell lines grew as monolayered sheets with a population doubling time of about 20 h. Chromosome counts of Kagura-1 cells ranged from 34 to 45 with a modal number of 40, while that of Kagura-2 cells ranged widely from 40 to 130 with a modal number of 65. Subcutaneous inoculation of cultured cells of both these lines into nude mice resulted in tumor formation. The histopathological appearances of the induced tumors were similar to those of the original tumors. Kagura-1 and Kagura-2 cell lines express at least two tumor markers, glutathione-S-transferase P and γ-glutamyl transpeptidase; the level of c-*myc* messenger ribonucleic acid was also highly elevated.

Keywords rat hepatoma cell line; aflatoxin B_1; gene expression; tumor marker

Introduction

Aflatoxin B_1 (AFB_1), one of the dihydrobisfuranoids produced by *Aspergillus flavus*, is a potent hepatocarcinogen in experimental animals,[1] and several epidemiological surveys have demonstrated its association with the development of human liver cancer in South Africa,[2] China,[3] and the Philippines.[4]

We have demonstrated the overexpression of c-*myc* messenger ribonucleic acid (mRNA),[5] and McMahon *et al.*[6,7] observed the activation of c-Ki-*ras* gene in hepatocarcinomas induced by AFB_1 in rats.

In order to clarify the biochemical alterations of AFB_1-induced original tumors and established cell lines, we established novel hepatoma cell lines designated as "Kagura-1" and "Kagura-2" from AFB_1-induced rat hepatocellular carcinomas. In this paper, we report the chromosomal features, tumor markers, and the expression of c-*myc* mRNA in the established cell lines. Tyrosine aminotransferase (TAT) activities of these cells were also compared with those of Reuber hepatoma cell line, H4IIE, which is one of the minimal deviation hepatoma cell lines and maintains many biochemical and morphological properties of typical adult hepatocytes, including the ability to respond to glucocorticoid hormone.[8]

Materials and Methods

Induction of Rat Liver Tumors by AFB_1 Fourteen male Fischer 344 rats (Charles River Japan, Inc., Atsugi) were used in the experiments. A total dose of 1.5 mg/rat of AFB_1 dissolved in dimethyl sulfoxide was given to seven male F344 five-week-old rats by stomach tube in 40 equal doses over 8 weeks.[1] Fully developed liver tumors were observed in seven rats after 14—15 months.[5]

Primary Culture The rats were anesthetized with ether and were submerged in 1% phenol. Tumors were removed from the livers of two rats and washed with Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline PBS(−) containing penicillin (1000 units/ml) and streptomycin (1000 μg/ml) (GIBCO, Grand Island, NY).

The tumors were separately minced with sharp scissors into small pieces and treated with 0.1% collagenase (Sigma, St. Louis, MO) in PBS(−) containing 10 mM *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (HEPES) (pH 7.5) and 5 mM $CaCl_2$ for 15 min at 37 °C. Each cell suspension was passed through two layers of sterile gauze and centrifuged at 1000 rpm for 3 min. The cell pellets were suspended in 10 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and then centrifuged again in the same manner. After being washed 3 times with DMEM supplemented with 10% FCS, the cells were stained with trypan blue and counted in a hemocytometer.

Viable cells (1×10^6) were plated in 5 ml of DMEM supplemented with 10% FCS and 1×10^{-6} M each of insulin and dexamethasone in 60 mm culture dishes (Corning/Iwaki Glass, Tokyo) which had been coated with a solution consisting of 10 mg of bovine serum albumin and 3 mg of collagen (Collagen Co., Palo Alto, CA) in 100 ml of serum-free DMEM. The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Serial Subcultures The cell lines were passaged by trypsinization using 0.25% trypsin (DIFCO, Detroit, MI) and 0.02% ethylenediaminetetraacetic acid (EDTA) solution and maintained in DMEM supplemented with 10% FCS, 1×10^{-6} M insulin, 1×10^{-6} M dexamethasone, penicillin (100 unit/ml) and streptomycin (100 μg/ml). Subcultures were performed every 5 d. After passage 15, the culture medium was changed to DMEM supplemented with 10% FCS and antibiotics. During serial passages, cells of different passage numbers were frozen and stored in liquid nitrogen.

Growth Curves For the determination of the doubling time of Kagura-1 cells, Passage 60 (P-60) and Kagura-2 cells, Passage 43 (P-43), 5×10^4 cells were seeded in 35 mm culture dishes with 2 ml of growth medium, which was replaced every day. The number of viable cells was counted by the dye exclusion method after staining with 0.15% trypan blue solution at 24-h intervals.

Chromosomal Analysis Chromosomal analysis of Kagura-1 cells, Passage 80 (P-80) and Kagura-2 cells (P-59) was performed. Cells were treated with 0.2 μg/ml colcemid (GIBCO) for 2—3 h, then lysed with 0.075 M KCl for 15 min and fixed with methanol–acetic acid (3 : 1) solution. Giemsa staining was performed, and seventy metaphases were analyzed for chromosome number.

Transplantation of Kagura-1 and Kagura-2 Cells in Nude Mice Kagura-1 cells (P-50) or Kagura-2 cells (P-45) (2×10^6) were implanted subcutaneously into the flank of six-week-old athymic nude mice (BALB/c/nu/nu; CLEA Japan, Kumamoto). These animals were observed for at least 2 months for evidence of tumor formation. Mice with a tumor that grew to more than 0.4 cm in diameter at the site of inoculation were scored as positive. When necessary, the animals were killed and dissected. The tumor tissues were fixed in formalin and stained with hematoxylin and eosin for histological examination.

Preparation of Total RNA and Northern Blot Analysis Total RNA of tumors and cell lines was isolated by the guanidinium thiocyanate/CsCl method.[9] Total RNA (10 μg) was separated by electrophoresis in 1.2% agarose gel containing 2.2 M formaldehyde and then transferred to a nitrocellulose filter.[10] Hybridization and autoradiography were performed as described previously.[5]

Deoxyribonucleic Acid (DNA) Clones The following plasmid clones were used: pSmBH (rat c-*myc*),[11] pAF6 (mouse AFP),[12] prAlbI (rat albumin),[13] pGP5 (rat GST-P)[14] and pcTAT3 (rat TAT).[15] The insert fragments were eluted from the gel and labeled with [α-^{32}P]dCTP (3000 Ci/mmol) (ICN, Irvine, CA) by the random primer method.[16]

Assays for TAT and γ-Glutamyltranspeptidase (G-GT) Activity TAT activity of rat hepatoma cell lines was determined according to the method of Granner and Tomkins.[17] G-GT activity of these cell lines was

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determined according to the method of Orlowski and Meister[18] using γ-glutamyl-*p*-nitroanilide and glycylglycine as substrates. Protein concentration was determined by the method of Lowry *et al.*[19] with bovine serum albumin as a standard.

Results

Morphological Studies Figures 1 and 2 show phase-contrast photomicrographs of confluent cultures of Kagura-1 cells (P-80) and Kagura-2 cells (P-59), respectively. Most of the cells were polygonal in a cobblestone-like arrangement with prominent nucleoli, showing typical morphological characteristics of epithelial cells. When over-populated, many piled-up foci were observed.

Growth Curves In the exponential phase, the population doubling times of Kagura-1 cells (P-60) and Kagura-2 cells (P-43) were estimated as 20.2 and 20.4 h, respectively (Fig. 3).

Chromosomal Analysis Seventy metaphases were photographed and analyzed. The chromosome counts of Kagura-1 cells ranged from 34 to 45 with a model number of 40 at P-80, while that of Kagura-2 cells ranged widely from 40 to 130 with a modal number of 65 at P-59.

Histological Appearance of the Primary Tumors and the Transplanted Tumors The primary liver tumor (HCl) was well-differentiated hepato-cellular carcinoma mostly showing trabecular structures (Fig. 4).

When 2×10^6 Kagura-1 cells (P-50) were implanted subcutaneously into the flank of five nude mice, this cell line developed tumors in all mice. These tumors showed in general solid patterns but in parts had trabecular structures which consisted of hepatocyte-like cells (Fig. 5), and were similar to the primary tumor. They showed more malignant features, such as mitoses and nuclear atypia, than the primary tumor.

The histological findings of HC2 and xenografts of Kagura-2 cells in nude mice closely resembled those of HC1 (data not shown).

Northern Blot Analysis We examined the expression of various genes in the primary tumors induced by AFB_1 and in the new cell lines. Hybridization of the duplicate filters with various probes (c-*myc*, albumin, AFP, TAT, and GST-P) revealed no detectable expression (TAT), or changed expression characteristic for each gene (albumin, AFP,

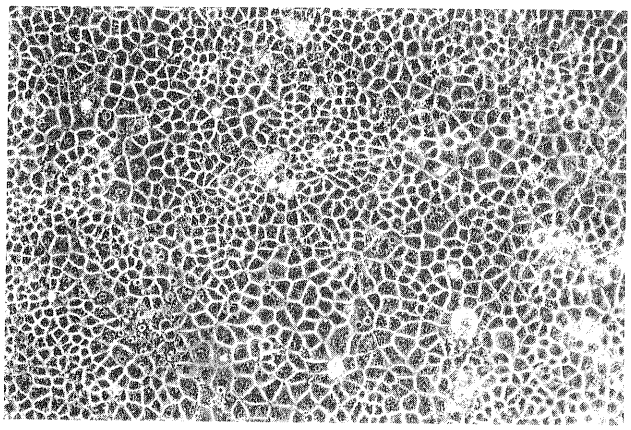

Fig. 1. Photomicrograph of Kagura-1 Cells (P-80)
Phase-contrast optics, × 100.

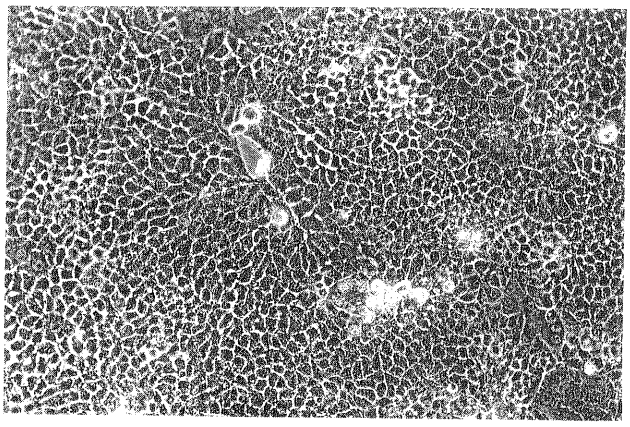

Fig. 2. Photomicrograph of Kagura-2 Cells (P-59)
Phase-contrast optics, × 100.

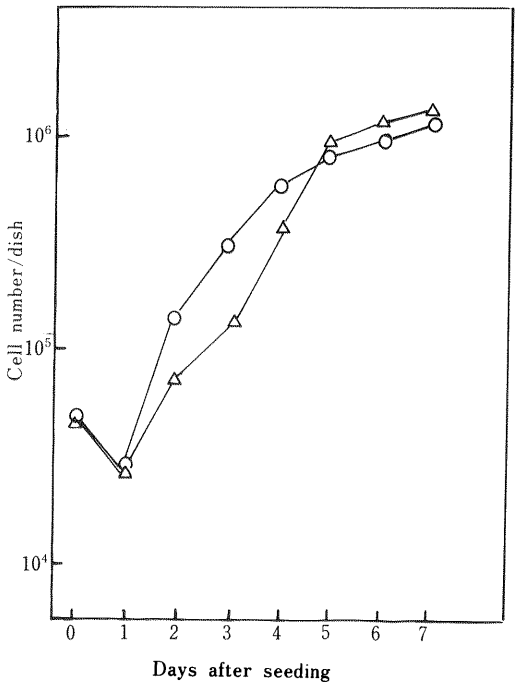

Fig. 3. Growth Curves of Kagura-1 and Kagura-2 Cell Lines
Viable cells (5×10^4) were seeded in 35 mm culture dishes and incubated at 37°C. Cells of each dish were harvested after the indicated time and counted using a hemocytometer. The doubling times of Kagura-1 (P-60) and Kagura-2 (P-43) were 20.2 and 20.4 h, respectively. ○—○, Kagura-1; △—△, Kagura-2.

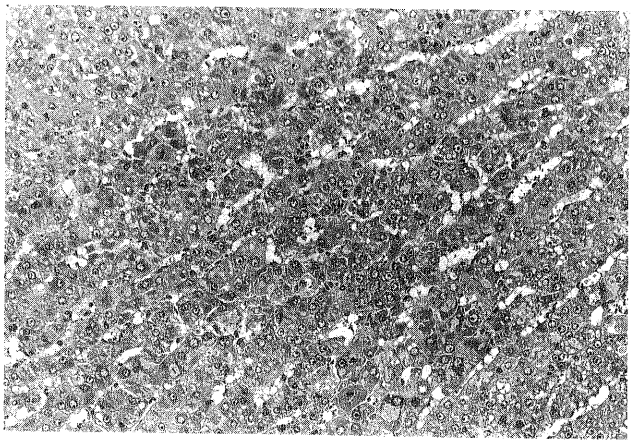

Fig. 4. Photomicrograph of the Primary Tumor (HC 1)
Hematoxylin and eosin, × 150.

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GST-P, and c-*myc*), in the primary tumors and cell lines (Fig. 6).

The level of 2.2 kb (kilobase) albumin mRNA, which was very high in normal liver, was decreased, but detectable, in the primary tumor. The 2.4 kb TAT mRNA was observed faintly only normal liver (Fig. 6A, 6B). We demonstrated a marked decrease in the levels of 2.2 kb albumin mRNA in both cell lines as compared to the levels found in normal liver and in the primary tumors. The 2.2 kb AFP mRNA was detected in only Kagura-1 cells (Fig. 6C).

The expressions of 0.75 kb GST-P mRNA and 2.4 kb c-*myc* mRNA were not observed in the normal liver, in agreement with the results of Sugioka *et al.*[14] and Hayashi *et al.*,[20] whereas they were markedly elevated in the primary tumors and in the cell lines derived from these tumors (Fig. 6D, 6E).

The extents of albumin mRNA decrease and c-*myc* mRNA increase approximately corresponded to each other during the establishment of cell lines from the primary tumors (Fig. 6B, 6D lanes 2 to 4 and lanes 3 to 5).

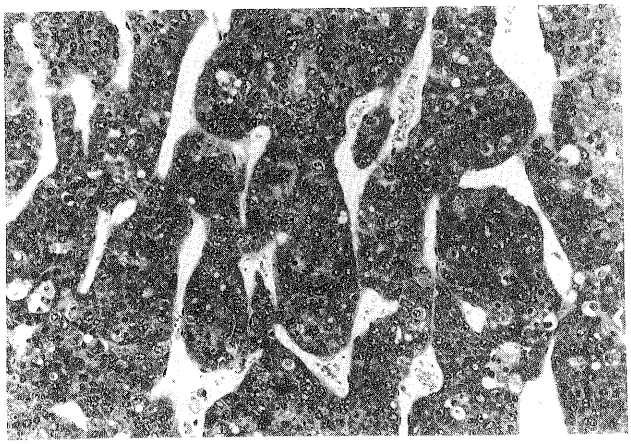

Fig. 5. Photomicrograph of Transplanted Kagura-1 Tumor in a Nude Mouse

Hematoxylin and eosin. × 150.

TABLE I. TAT and G-GT Activities in Rat Hepatoma Cell Lines

Cell lines	TAT[a,b] Dexamethasone Basal	TAT[a,b] Dexamethasone Induced[c]	G-GT[a,b]
	nmol product formed/min/mg protein		
Kagura-1	1.5±0.4	2.0±0.4	72 ±21
Kagura-2	1.9±0.5	1.9±0.4	72 ±18
H4IIE	13.9±3.5	45.4±9.6	0.4± 0.2

a) Assay methods of TAT and G-GT are given in Materials and Methods. *b*) Mean±S.D. from 4 experiments. *c*) Cells were pretreated for 6 h with 10^{-6} M dexamethasone.

TAT and G-GT Activity TAT activity of Kagura-1 and Kagura-2 cells was very low compared to that of H4IIE cells, and no marked increase was observed after dexamethasone treatment (Table I). However, G-GT activity of Kagura-1 and Kagura-2 cells was about 200 fold higher than that of the H4IIE cell line (Table I). The G-GT activity of H4IIE cells was comparable to that found in the primary culture of adult rat hepatocytes (data not shown).

Discussion

In the present study, we established two cell lines from hepatocellular carcinomas induced by AFB_1. Both cell lines have been continuously propagated by serial subculturing during the past 24 months.

The hepatocellular nature of Kagura-1 and Kagura-2 cell-lines was evident from the morphology of the tumors derived from the implanted cell lines. Moreover, in the case of Kagura-1 cells, the levels of AFP mRNA was very high.

It is well established that GST-P and G-GT are induced in the liver following administration of various carcinogens,[21,24] including AFB_1.[25,26] We investigated the levels of GST-P and G-GT in the established cell lines grown *in vitro*, and demonstrated that both cell lines expressed GST-P and G-GT to a high degree. The level of GST-P mRNA remained unchanged after the establishment of cell lines from the primary tumors.

The results indicated that GST-P and G-GT are useful tumor markers and retain stable biochemical characteristics during the establishment of cell lines from primary tumors.

Recent studies have suggested that the c-*myc* oncogene plays an important role in the initiation and/or progression of hepatocarcinogenesis. The steady-state level of c-*myc* gene transcripts was reported to be elevated in the primary

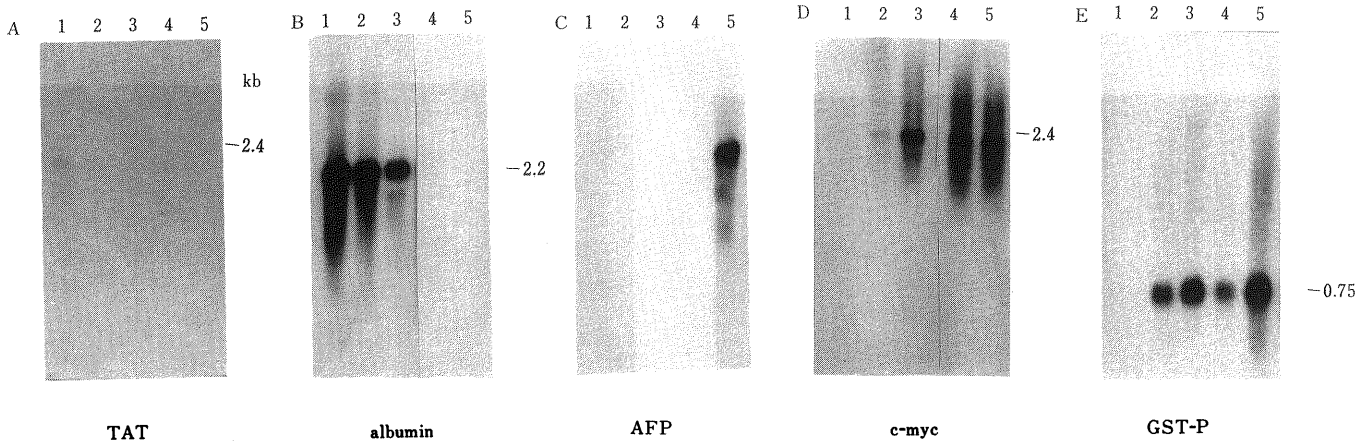

Fig. 6. Analysis of TAT (A), Albumin (B), AFP (C), c-*myc* (D), and GST-P (E) mRNAs in Primary Tumors and Cell Lines

Total RNAs were isolated from the normal F344 rat liver (lane 1), HC 2 primary tumor (lane 2), HC 1 primary tumor (lane 3), Kagura-2 cell line (P-62) derived from HC 2 (lane 4), Kagura-1 cell line (P-80) derived from HC 1 (lane 5). Each total RNA (10 μg) was fractionated in 1.2% agarose-formaldehyde gels, transferred to nitrocellulose filters, and hybridized to the appropriate ^{32}P-labeled probes. Specific transcripts were detected by hybridization and autoradiography as described in Materials and Methods. λDNA digested with Hind III was used as a size marker.

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liver tumors induced by ethionine[27] and 3′-MeDAB[28] as well as in Morris hepatomas,[20,29] and in hepatoma cell lines.[30,31] In a previous paper,[5] we also reported an overexpression of c-*myc* mRNA in AFB_1-induced hepatocellular carcinomas in rats.

Many studies have been done on the detection and comparison of GST-P and G-GT in primary tumors.[23,26] However, no reports have been found in which the amounts of c-*myc* mRNA in a primary tumor were compared to the amounts in cultured cell lines derived from the primary tumor.

It was, therefore, of interest to determine whether the alterations of gene expression were characteristic of the original primary tumors or phenotypes acquired during adaptation to *in vitro* culturing. We examined the levels of c-*myc* and albumin gene expression in primary tumors and in their cell lines. In normal livers and in primary tumors, as the levels of c-*myc* mRNA increased, those of albumin mRNA decreased. We found a reciprocal relationship between c-*myc* mRNA levels and albumin mRNA levels. Such a reciprocal relationship between c-*myc* mRNA levels and albumin mRNA levels was also evident in both cell lines.

The data presented in this paper on the levels of c-*myc* and albumin mRNAs confirm the generally accepted theory, that differentiation-specific genes (*e.g.* albumin and TAT) tend to be suppressed when the liver cells are growing *in vitro*.[29,32] This result (*i.e.* that a reciprocal relationship between c-*myc* mRNA and albumin mRNA holds not only in the primary tumors but also in cell lines derived from the primary tumors) may be important for the determination of the malignant potential of liver cells.

Further studies on the mechanisms involved in the regulation of various gene expressions in chemical hepatocarcinogenesis will be necessary, and the present novel cell lines are expected to contribute to such studies.

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