Properties of a Newly Established Adult Rat Liver Epithelial Cell Strain and its Application to Transformation Assay to Detect Epigenetic Carcinogens

Kazunori FURUKAWA, Toshikazu YOKOI, Toshinori KODAMA and Yohichi MOCHIZUKI

Department of Pathology, Cancer Research Institute, Sapporo Medical College South 1, West 17, Chuo-ku, Sapporo 060, Japan [*K. F., T. K., Y. M.*]

Department of Oral Surgery, Sapporo Medical College South 1, West 17, Chuo-ku, Sapporo 060, Japan [*T. Y.*]

SUMMARY

An adult rat liver epithelial (ARL) cell strain, ARLJ301-3, was established from a male F344/DuCrj rat, and its morphologies, growth properties, and availability for the transformation assay to detect genotoxic and epigenetic carcinogens were investigated. The ARL cells had a typical epithelial morphology and produced collagen. Immunocytochemical studies clarified that the intermediate filaments of the cells were positive in cytokeratins-PKK2 and vimentin. Since the cytokeratins-PKK2 antibodies exclusively react with biliary ducts and ductules but not with hepatocytes nor with sinusoidal endothelium, the ARL cells are likely to have originated from biliary ductal or ductular epithelial cells. The ARL cells displayed a diploid nuclear pattern with plating efficiency of $5.1\pm0.8\%$ and population doubling time (PDT) of 27 hr. The transformation study was carried out by a single exposure to 5×10^{-6}M N-methyl-N'-nitro-N-nitrosoguanidine. Growth in soft agar of the exposed cells was detected first in cultures of post-exposure passage (PEP) 16, and spontaneous transformation was definitely not manifested in the non-exposed cells. When the exposed and non-exposed cells were continually treated with 2×10^{-3}M clofibrate (CF) or phenobarbital (PB), the cell density of the cells at Day 7 of the PEP 2 and PEP 16 cultures and colony forming frequency at Day 11 of the PEP 5 cultures were reduced, irrespective of the exposure. Moreover, the

*Abbreviations:
ARL, adult rat liver epithelial cells; CF, clofibrate; PB, phenobarbital; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; WME, Williams' medium E; HIFBS, heat-inactivated fetal bovine serum; Dex, dexamethasone; HBSS, Hanks' balanced salt solution; AFP, α-fetoprotein; PEP, post-exposure passage; GGT, γ-glutamyl transpeptidase; PDT, population doubling time.

colony formation in soft agar of the exposed cells was also suppressed by the continual treatment with CF or PB. However, PDT was not affected by CF, but delayed by PB. On the other hand, cytochemical γ-glutamyl transpeptidase activity was detected in the exposed cells treated continually with CF and PB. The examination of the effect of the treatment for 10 days with CF or PB at 2×10^{-3}M or lower concentrations clarified that, at 1×10^{-4}M, both CF and PB enhanced the colony formation of the exposed and non-exposed cells. The suppressing or enhancing effect of these liver epigenetic carcinogens on the growth and induction of the anchorage independence are discussed.

Key words: Liver epithelial cells, Intermediate filaments, In vitro transformation, Anchorage independence, Clofibrate, Phenobarbital.

INTRODUCTION

In the studies on *in vitro* transformation of adult rat liver epithelial cell (ARL*) lines or strains(23, 24, 26), the most reliable marker of the transformation has been found to be anchorage independence which is determined by the growth in soft agar semisolid media. The anchorage independence has been demonstrated to be induced in the ARL cell strains by the exposure to different classes of genotoxic carcinogens(26), suggesting that the transformation assay using these ARL cell strains is useful for the detection of DNA-reactive genotoxic carcinogens.

Recently, we have developed a method for obtaining numerous clonogenic ARL cells and initiation of the cell strains(3, 6). However, phenotypic properties of the strains established by this method were not elucidated, except for the ultrastructural properties(6), and it was not determined whether the newly established ARL strains could be used for the transformation study without spontaneous transformation. In addition, the problem of origin of ARL cells has not yet been resolved. It was suggested in our previous fractionation studies(3, 6) that the epithelial cells were not derived from hepatocytes, but might have originated from bile ductular cells. The first purpose of the present study was to investigate the morphologic and growth properties of the cells of a newly established ARL cell strain, ARLJ301-3. The second was to examine availability of this clonal strain for the transformation study.

On the other hand, from the point of view of establishment of the transformation assay system using ARL cell strains, it is worthy of note to determine whether the transformation assay system can also be used for the detection of epigenetic carcinogens(32). The third purpose was to investigate the effects of well-known liver epigenetic carcinogens, clofibrate (CF) and phenobarbital (PB), on the

anchorage independence induced by a genotoxic carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). The experimental design for examination of the epigenetic effects is based upon the concepts of "initiation and promotion", that has been developed in the *in vivo* studies on hepatocarcinogenesis(19).

MATERIALS AND METHODS

Establishment of an Adult Rat Liver Epithelial Cell Strain and Culture Conditions.

The ARL cell strain used in the present study was derived from the liver of a 10-week-old male F344/DuCrj strain rat weighing 240 g. To obtain clonal growth of ARL cells in primary culture, hepatocytes and nonparenchymal cells were isolated from the liver by the cell isolation method II(3, 6) using 120 U/m*l* collagenase (Wako Pure Chemicals, Osaka) and 600 PU/m*l* dispase II (Godo Shusei, Tokyo) for enzymatic liver perfusion. The cells sedimented by the centrifugation at 50×g for 5 min were finally suspended in Williams' medium E (WME) (Flow Labs., Irving, UK) supplemented with 10% heat-inactivated calf serum (GIBCO, Grand Island, USA), 40 U/m*l* penicillin G and 40 μg/m*l* streptomycin sulfate and seeded in 60×15mm plastic tissue culture dishes (Corning; Iwaki Glass, Tokyo) at 3.0×10^5 hepatocytes and 7.1×10^4 nonparenchymal cells/dish. After a 1 hr attachment period, the cultures were washed three times with WME supplemented with 10% heat-inactivated fetal bovine serum (HIFBS), 10^{-6}M dexamethasone (Dex) and the antibiotics as mentioned above (WME : $HIFBS_{10}$: Dex), followed by maintaining the cells in this culture medium.

Colonies of ARL cells were observed at Day 10 of the primary culture at frequency of 1 to 7 colonies/dish. At Day 27, a dish containing one ARL cell colony/dish was selected, and the area of the colony was marked, The dish was washed three times with Ca^{++} and Mg^{++}-free Hanks' balanced salt solution (HBSS) containing 40 U/m*l* penicillin G and 40 μg/m*l* streptomycin sulfate, followed by incubation for 3 min at 37℃ with 750 PU/m*l* dispase II in Ca^{++} and Mg^{++}-free HBSS containing the antibiotics. After aspirating off the dispase solution, 3 m*l* of WME : $HIFBS_{10}$: Dex were added, and gentle pipetting was done on the marked area. The dissociated cell suspension was then diluted 20 times with the same medium, followed by seeding of the cells at 1.5 m*l* of the diluted cell suspension/35×10 mm tissue culture dish (Corning). At Day 5 of the first passage (passage 1) culture, 6 dishes containing one colony/dish were selected, and the cells in each dish were harvested at Day 21 using the dispase II solution as described above and seeded at 1.0×10^4 cells/60-mm dish. At Day 4 of the passage 2 culture, the culture medium was changed to WME containing 10% HIFBS but not supplemented with Dex (WME : $HIFBS_{10}$), followed by maintaining the cells in this culture medium. For the passage 3 culture, each clonal cells was dissociated with

0.01% trypsin (2914 NF units/mg; GIBCO) in Ca^{++} and Mg^{++}-free HBSS containing 0.01% EDTA-Na_2 and the antibiotics. The washing solution was Ca^{++} and Mg^{++}-free HBSS containing 0.005% EDTA-Na_2. The cells were then routinely subcultured by the trypsinization at intervals of once per 2 weeks with the seeding density of 1.0×10^4 cells/60-mm dish. For storage of the cells in liquid nitrogen, the cells seeded at 7.5×10^5 cells/T-75 flask (Sumitomo Bakelite, Tokyo) and harvested at Day 7. Thus, the six ARL cell strains designated as ARLJ301-1 to -6 were established, and ARLJ301-3 strain frozen at passage 9 was used in this study. There were no mycoplasmal infections in the passage 10 cultures of ARLJ301-3 cells, that was examined with Hoechst Stain Kit (Flow).

Morphological Examinations.

Ultrastructural properties of ARLJ301-3 cells were examined by the previously described method(4). Immunocytochemistries of albumin, α-fetoprotein (AFP), factor VIII-related antigen or the intermediate filament proteins, vimentin, desmin, cytokeratins-PKK1 and cytokeratins-PKK2 were carried out by the immunoperoxidase staining method using avidin-biotin-peroxidase complex or FITC-conjugated avidin. All antibodies and chemicals for the immunocytochemistries were purchased from the commercial sources as follows: anti-rat albumin IgG fraction (sheep) from Cappel Labs. (West Chester, USA); anti-rat AFP IgG fraction (sheep) from Nandi Immunological Labs. (Tilburg, Netherlands); mouse monoclonal IgG to human factor VIII-related antigen, vimentin and desmin from DAKOPATTS a/s (Glostrup, Denmark); mouse monoclonal IgG to cytokeratins-PKK1 or cytokeratins-PKK2 from Labsystems Oy (Helsinki, Finland); Vectastain ABC (avidin-biotin complex) sheep and mouse IgG Kits, Blocking Kits and FITC-conjugated avidin from Vector Labs. (Burlingame, USA).

Measurement of Nuclear DNA Contents.

In order to examine ploidy of ARLJ301-3 cells, the cells were seeded on a HIFBS-coated glass cover slip in a 35-mm dish. The cells in an exponential growth phase were fixed with buffered formalin, and nuclear DNA was stained by Feulgen reaction. DNA contents in interphase nuclei were measured at 550 nm with a microspectrophotometer (MMSP; Olympus, Tokyo).

Cytotoxicity Test of MNNG.

The purpose of the cytotoxicity test is to determine the dose of MNNG for obtaining a fraction of the cells that resist acute toxicity of MNNG and therefore could be transformed by its genotoxic carcinogenic effect. The cells were seeded at 3.0×10^4 cells/60-mm dish in 3 m*l* of WME: $HIFBS_{10}$ and fed the fresh medium at

Day 1. At Day 7, when the cells were in an exponential growth phase, the cells were washed with three changes of WME containing antibiotics (incomplete WME) and fed 5 m*l*/dish of incomplete WME. To this, 5 μl of a MNNG (Sigma Chemical Co., St. Louis, USA) stock solution (×1000) prepared in dimethyl sulfoxide was added. After the exposure to MNNG for 1 hr, the media were removed, and the cells were washed three times with incomplete WME, followed by feeding fresh WME : $HIFBS_{10}$. At Day 14, the cells were dissociated by trypsinization, and the mean number of viable cells/dish was determined by the trypan blue dye exclusion method. The cytotoxicity was expressed as the number in the exposed cultures relative to that of the solvent control. The dose that killed the exposed cells and resulted in reduction of the number of viable cells/dish to around 75% was used for the transformation assay.

Eyposure and Treatment Conditions for Transformation Assay.

The ARLJ301-3 cells in passage 10 cultures were dissociated and seeded at 7.5×10^5 cells in 10 m*l* of WME : $HIFBS_{10}$/T-75 flask. At Day 7, the cells were exposed to the 75% cell-growing dose of MNNG in incomplete WME for 1 hr. Seven days after the removal of MNNG, the cells were dissociated by trypsinization and subcultured to pose-exposure passage (PEP) 1, meaning one passage after the exposure of cultures. The unexposed control cells were also seeded at 4.0×10^5 cells/T-75 flask and subcultured. The seeding density of PEP 1 cultures was 1.0×10^4 cells/60-mm dish. The treatment with CF or PB was started at Day 4 of PEP 1. The exposed and unexposed cell cultures were divided into 3 groups, one of which was fed fresh WME : $HIFBS_{10}$ containing 2×10^{-3}M CF (sodium salt ; Ayerst Labs., New York, USA), another was fed the culture medium containing 2×10^{-3}M PB (Wako) and the third was fed the medium without CF or PB, followed by feeding the respective media twice a week. The cells of each group were subcultured to PEP 2 at the seeding density of 1.0×10^4 cells/60-mm dish using WME : $HIFBS_{10}$ without CF or PB. At Day 4, each treatment was started again. Further passages and treatments were performed in the same manner as those for PEP 2.

Growth in Soft Agar.

Induction of the anchorage independence was examined by the soft agar method of Shimada *et al.*(26) using WME deprived of Ca^{++} and Mg^{++} (Flow) and HIFBS at 10%. Agar noble and bacto-peptone were obtained from Difco Labs. (Detroit, USA). The cells of each group were subcultured in a T-75 flask at 7.5×10^5. At Day 7, the cells were dissociated and seeded into the upper layer of the soft agar at 5.0×10^4 cells/60-mm dish. Colony number/dish was determined at Day 21 of the soft agar culture.

Cytochemistry of γ-Glutamyl Transpeptidase (GGT).

Since GGT activity has been expressed by the ARL Cells exposed to MNNG (26), the cytochemical examination of GGT activity was carried out at every two passages by the method of Rutenburg *et al.*(21). The cells at Day 14 of the passage cultures were used.

RESULTS

Some Properties of ARLJ301-3 Cell Strain.

As shown in Fig. 1A, the ARL cells displayed a typical epithelial morphology, and production of collagen fibers was observed in the confluent cultures maintained up to 14 days, as shown in Fig. 1B. The collagen fibers were detected also by ultrastructural examinations (not shown). Immunocytochemically, albumin, AFP and factor VIII-related antigen were not found in the cells. Vimentin and cytokeratins-PKK2 were detected in the cells, as shown in Figs. 1C and 1D, while cytokeratins-PKK1 and desmin were not detected. Cytokeratins-PKK2 was also identified in the cells by the avidin-biotin-FITC complex method (Fig. 1E).

Fine structures of the ARL cells are shown in Fig. 2. Their nuclei were oval in shape and contained one to three nucleoli. Abundant polysomes and rough endoplasmic reticulum were observed in the cytoplasms (Fig. 2A). A few microperoxisomes without crystalloid nucleoids were observed. On the plasma membranes facing the dish surface, a marked pinocytosis was observed (Fig. 2B), while the membranes on the upper surface of the cells were not infolding, and sieve plates were not found. No formation of sieve plates was detected also by scanning electron microscopy (not shown). The cells formed gap junctions and desmosomes (Figs. 2C and 2D).

The ploidy pattern of ARLJ301-3 cells at passage 12 is illustrated in Fig. 3. They displayed a typical diploid pattern. The plating efficiency of the cells was $5.1 \pm 0.8\%$, which was determined at Day 11 with the seeding density of 500 cells per 60-mm dish, and the population doubling time was 27 hr.

Determination of MNNG dose.

As shown in Table 1, the dose of MNNG exhibiting the cytotoxicity of 75% was calculated to be 5.12×10^{-6}M. Therefore, the dose used for the transformation studies was determined to be 5×10^{-6}M.

Effects of CF and PB on the growth of the cells exposed or not exposed to MNNG.

The cell density at Day 7 of the exposed and non-exposed cells in the PEP 2 and PEP 16 cultures were reduced by the continual treatments with CF and PB (Table 2). The effect of CF was greater than that of PB, and the efficacy of each treat-

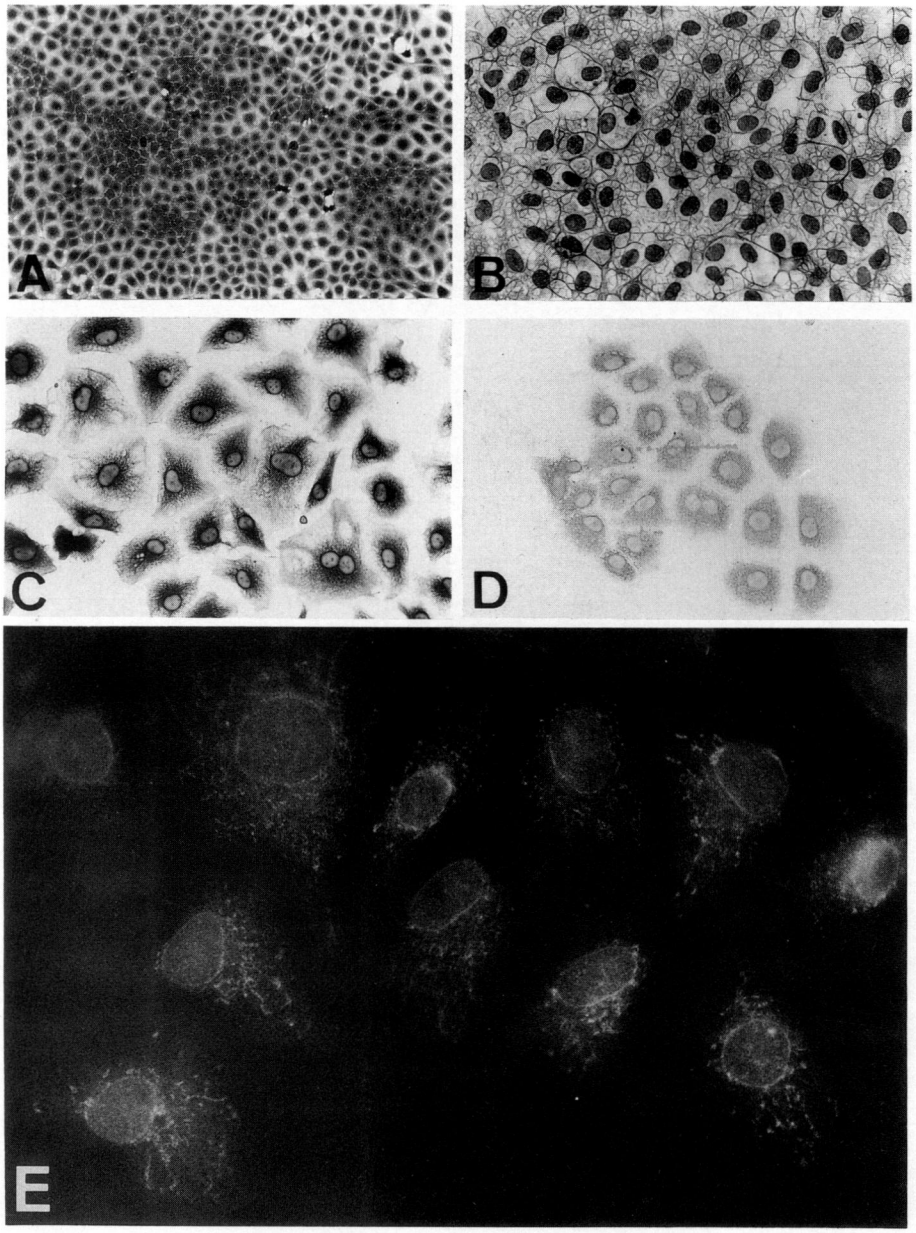

Fig. 1 **A.** Epithelial morphology of APLJ301-3 cells. Coomassie brilliant blue staining, ×50. **B.** Collagen production of the ARLJ301-3 cells in the aged culture at Day 21. Watanabe's silver impregnation method, ×100. **C** and **D.** Immunoperoxidase staining of ARLJ301-3 cells using avidin-biotin complex, ×200. **C.** Vimentin; **D.** cytokeratins-PKK2. **E.** Immunofluorescence staining of ARLJ301-3 cells with the cytokeratins-PKK2 antibody, ×634.

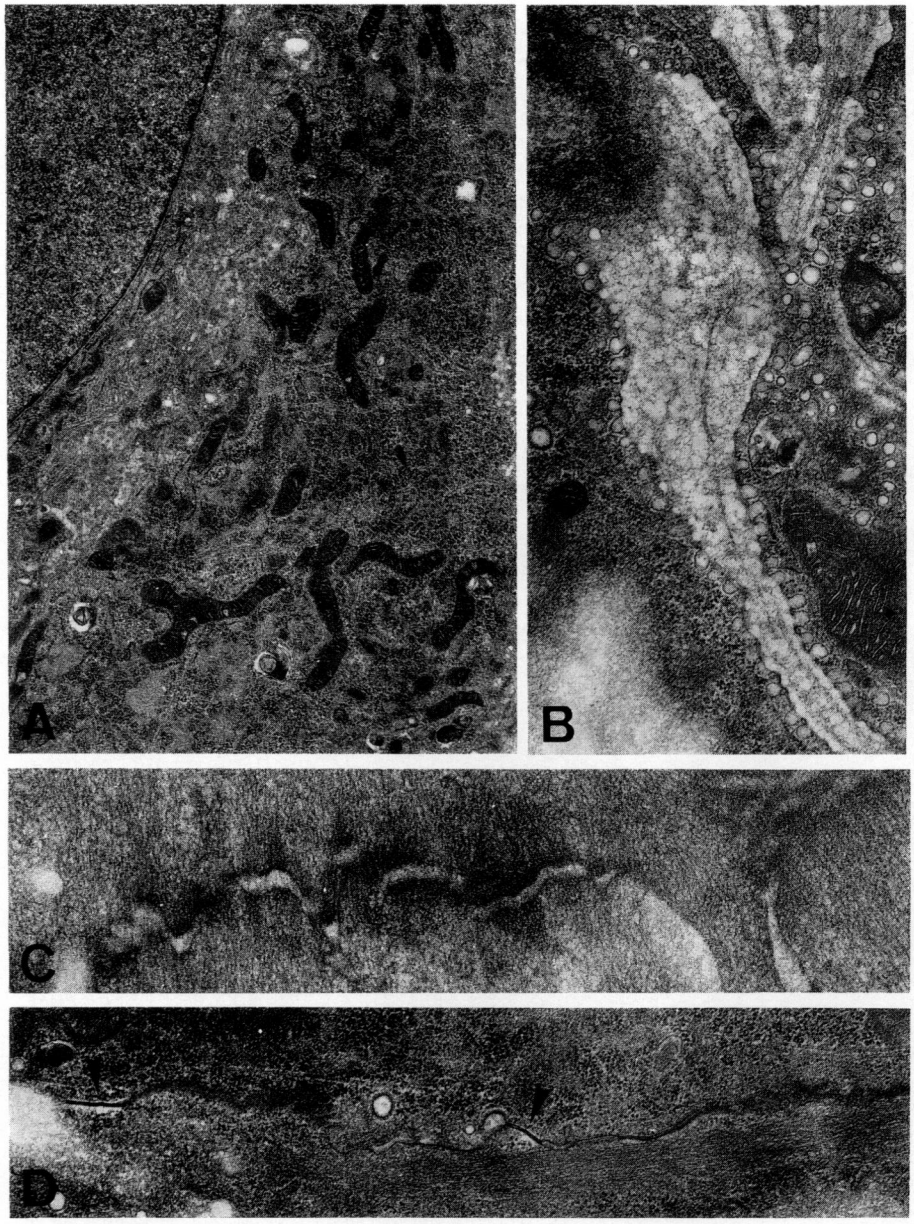

Fig. 2 Transmission electron microphotographs of ARLJ301-3 cells. **A.** ×8,000. **B.** A marked pinocytosis observed on the cell membranes faced with the dish surface, ×20,000. **C.** Desmosomes, ×40,000. **D.** Gap junctions (*arrow heads*), ×20,000.

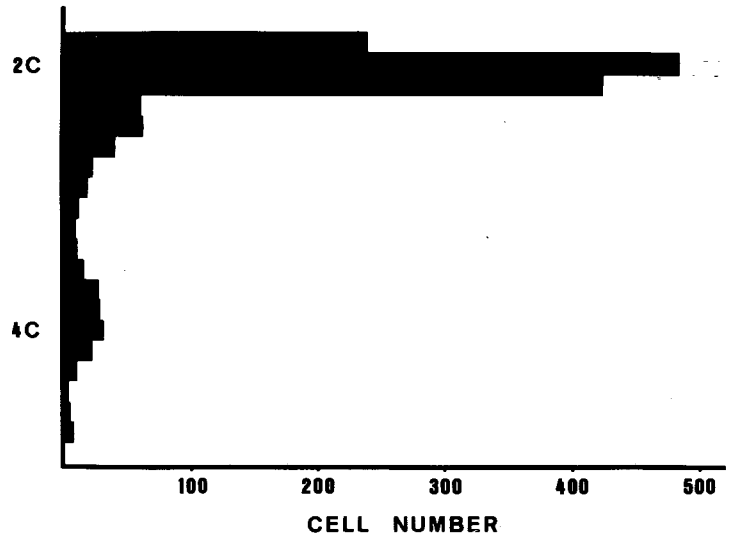

Fig. 3 Histogram of nuclear DNA contents of ARLJ301-3 cells.

Table 1 *Cytotoxicity test of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG).*

Concentration of MNNG [X]	Viable cell number/60-mm dish relative to that of non-exposed control (%) [Y]
0 (DMSO control)	100
5×10^{-6} M	80
1×10^{-5} M	48
2×10^{-5} M	40
5×10^{-5} M	13.5

Hemilogarithmic linear regression equation:

$$Y=-257.01-62.6X \quad (r=0.977\,;\ p<0.05)$$

$25\%=3.16\times10^{-5}$ M, $50\%=1.26\times10^{-5}$ M, $75\%=5.12\times10^{-6}$ M

ment showed no difference between the exposed and non-exposed cells or between the cells at PEP 2 and PEP 16. The population doubling time (PDT) of the exposed cells at PEP 5 was slightly longer than that of the non-exposed controls, as shown in Table 3. PDT of the exposed cells with the CF treatment was somewhat shorter than that of the untreated counterparts, while PDT of the non-exposed cells was not affected by the CF treatment. On the other hand, the PB treatment delayed PDT of the exposed cells as well as that of the non-exposed cells.

Table 2 *Effect of the treatment with clofibrate or phenobarbital on growth of the adult rat liver eqithelial cells exposed or not exposed to MNNG[a]: Cell density at Day 7[b] of post-exposure passage (PEP) 2 and 16.*

	Cell number/T-75 flask relative to that of non-exposed, untreated control (%)	
	PEP 2	PEP 16
Non-exposed control:		
Untreated	100	100
Clofibrate at 2×10^{-3} M	33	32
Phenobarbital at 2×10^{-3} M	62	60
Exposed to 5×10^{-6} M MNNG:		
Untreated	99	93
Clofibrate at 2×10^{-3} M	22	26
Phenobarbital at 2×10^{-3} M	42	66

[a] MNNG, N-methyl-N'-nitro-N-nitrosoguanidine.
[b] The seeding density was 7.5×10^{5} cells/flask.

Table 3 *Effect of the treatment with clofibrate or phenobarbital on growth of the adult rat liver epithelial cells exposed or not exposed to MNNG[a]: Population doubling time[b] at post-exposure passage (PEP) 5.*

	Population doubling time (hr)
Non-exposed control:	
Untreated	26.4
Clofibrate at 2×10^{-3} M	25.9
Phenobarbital at 2×10^{-3} M	59.3
Exposed to 5×10^{-6} M MNNG:	
Untreated	33.6
Clofibrate at 2×10^{-3} M	27.1
Phenobarbital at 2×10^{-3} M	45.1

[a] MNNG, N-methyl-N'-nitro-N-nitrosoguanidine.
[b] The seeding density was 1.0×10^{4} cells/60-mm dish. Viable cell number/dish was determined at Days 7 and 11 of PEP 5.

Effects of CF and PB on anchorage independence.

Throughout 15 passages after the exposure, growth in soft agar was not detected. The colony formation by the exposed cells was detected first in PEP 16 cultures, as shown in Table 4. The colony forming frequency in soft agar of the exposed cells with the CF and PB treatments was lower than the untreated counterparts, and the frequency of the cells treated with PB was smaller than that of the

Table 4 *Effect of the treatment with clofibrate or phenobarbital on the colony formation in soft agar culture[a] of the adult rat liver epithelial cells exposed or not exposed to MNNG[b] at post-exposure passage 16.*

	Number of colonies/60-mm dish
Non-exposed control:	
Untreated	0
Clofibrate at 2×10^{-3} M	0
Phenobarbital at 2×10^{-3} M	0
Exposed to 5×10^{-6} M MNNG:	
Untreated	43 ± 4[c]
Clofibrate at 2×10^{-3} M	11 ± 3*
Phenobarbital at 2×10^{-3} M	3 ± 2*,**

[a] The seeding density was 5.0×10^4 cells/60-mm dish. The medium was Ca^{++} and Mg^{++}-free WME containing 10% HIFBS. The number of colonies/dish was determined at Day 21.

[b] MNNG, N-methyl-N'-nitro-N-nitrosoguanidine.

[c] Values are means±SD and derived from 6 dishes.

* The significance level as compared with values for the exposed control cells; $p<0.001$.

** The significance level as compared with values for the exposed cells treated with clofibrate; $p<0.001$.

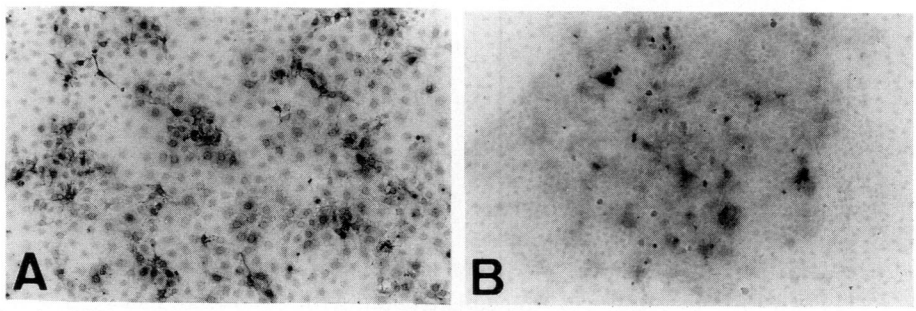

Fig. 4 GGT cytochemistry of the ARLJ301-3 cells exposed to MNNG and treated with CF and PB, ×50. They are counterstained with hematoxylin. **A.** The exposed cells in the PEP 15 culture with CF-treatment. **B.** The exposed cells in the PEP 15 culture with the PB-treatment.

cells treated with CF. The non-exposed cells did not form colonies in the soft agar cultures throughout 16 passages of the transformation study, irrespective of the CF and PB treatments.

Effects of CF and PB on GGT activity.

GGT activity was not detected cytochemically in the unexposed cells throughout

Table 5 *Effect of the short-term treatment[a] with clofibrate or phenobarbital on growth of the adult rat liver epithelial cells exposed or not exposed to MNNG[b] : Colony formation at Day 11 of post-exposure passage 5.*

	Number of colonies/60-mm dish	
	Non-exposed	Exposed
Untreated	30±3[c]	22±3*
Clofibrate :		
1×10^{-4} M	30±4	30±3**
5×10^{-4} M	18±4**	24±4
2×10^{-3} M	3±2**	3±1**
Phenobarbital :		
1×10^{-4} M	30±8	32±7***
5×10^{-4} M	29±5	25±6
2×10^{-3} M	13±5**	22±3

[a] The seeding density was 1.0×10^3 cells/60-mm dish. At Day 1, the medium was changed to WME : $HIFBS_{10}$ containing each concentration of clofibrate or phenobarbital, and the cells were maintained for 10 days.

[b] MNNG, N-methyl-N'-nitro-N-nitrosoguanidine. The cells were exposed for 1 hr to 5×10^{-6} M MNNG at Day 7 of post-exposure passage 0.

[c] Values are means±SD and derived from 6 dishes.

* The significance level as compared with values for the non-exposed, untreated cells; $p<0.001$.

** The significance level as compared with values for the non-exposed or exposed control cells; $p<0.001$.

*** The significance level as compared with values for the non-exposed or exposed control cells; $p<0.01$.

the 16 passages of the transformation study, irrespective of the treatments with CF and PB. The activity was also not detected in the exposed, untreated cells, while the PEP 11 to PEP 16 cultures of the cells treated with CF or PB displayed a focal GGT activity, as shown in Figs. 4A and 4B. In addition, the intensity of the cytochemical staining was higher in the CF-treated cells than in the PB-treated cells.

Effect of the short-term treatment with CF or PB on the growth of the cells exposed or not exposed to MNNG.

To re-evaluate the effects of CF and PB on the growth of the exposed and non-exposed cells, the cells in PEP 5 cultures with a seeding density of 1.0×10^3 cells per 60-mm dish were treated for 10 days with CF or PB at concentrations not exceeding 2×10^{-3}M. As shown in Table 5, the colony forming frequency of the exposed cells

without the treatments was lower than that of the non-exposed counterparts, while the fregquency of the exposed cells was enhanced by the short-term treatments with 1×10^{-4}M CF and PB. At 2×10^{-3}M, both CF and PB were effective for markedly reducing the colony forming frequency of the exposed and non-exposed cells.

DISCUSSION

The newly established adult rat liver epithelial cell strain, ARLJ301-3, was demonstrated to be a diploid cell strain with the fine structures characteristic of epithelial cells that were similar to those of the previously established ARL cell strains(6). Moreover, the cells produced collagens in aged cultures, which is also the phenotypic property common to the liver epithelial cell line(1, 22, 28). The cells of the present strain were not transformed spontaneously throughout 26 passages after the initiation of the culture. It is suggested that the ARL cell strain can be used for the transformation studies as reported previously(26).

The origin of the ARL cells has not yet been clarified. The chemically or spontaneously transformed tumorigenic ARL cells have been demonstrated to form well-differentiated adenocarcinomas when transplanted into syngeneic newborn rats (16, 33). In our previous studies to develop a highly efficient method for obtaining clonogenic ARL cells(3, 6), it was suggested that the ARL cells might be derived from bile ductular cells. Grisham(8) proposed that the terminal bile ductules were the hepatocytic stem cells which could proliferate in culture as the ARL cells with phenotypic properties of "oval cells"(28), while Sell and Salman(25) reported that the "non-descriptive cells" located around the terminal bile ductules could be the origin of "oval cells".

In the present study, the ARLJ301-3 cells were found to have cytokeratins-PKK2 and vimentin, while the cells were negative in cytokeratins-PKK1 and desmin. The previous studies(2, 15, 27, 31) have demonstrated that hepatocytes, biliary duct or ductular epithelial cells, sinusoidal endothelial cells and Ito cells have the intermediate filament proteins which react exclusively with the antibodies against cytokeratins-PKK1, cytokeratins-PKK2, vimentin and desmin, respectively. Although cultured sinusoidal endothelial cells were vimentin-positive(31), adult rat hepatocytes and rat hepatoma cells also had vimentin as well as cytokeratins-PKK1 in culture (7, and unpublished observation). Therefore, in culture, vimentin is a intermediate filament protein not specific to the endothelial cells. In contrast, cytokeratins are definitely the intermediate filament proteins of epithelial cell type even in culture(9, 15, 27). In addition, sieve plates were not formed in the ARLJ301-3 cells, and the cells did not have factor VIII-related antigen, albumin and AFP. It is very likely that the ARL cells are derived from bile duct or ductular epithelial cells.

The sensitivity of ARLJ301-3 cells to the cytotoxic effect of MNNG was similar to that of ARL15 clone 1 cells used in the previous transformation study(26). The anchorage independence of the ARLJ301-3 cells exposed to MNNG was detected first in the PEP 16 cultures without apparent changes in growth properties.

CF at 2×10^{-3}M has been found to be effective for induction of peroxisomal proliferation in adult rat hepatocytes in primary culture(4), and hepatocarcinogenicity of peroxisome proliferators are considered to be induced by the long-term treatment at the high dose sufficient to induce peroxisomal proliferation(20). On the other hand, PB has been effective at 2×10^{-3}M to induce the clonal growth in primary culture of hepatocytes of the rats treated with genotoxic carcinogens (10, 11). In contrast, in the present study, it was shown that continual treatment with 2×10^{-3}M CF or PB reduced the cell density of both the exposed cells and the non-exposed cells. Moreover, colony forming frequency in the soft agar culture at PEP 16 was lowered by continual treatments, although the emergence of the anchorage independence was not delayed by these treatments. It is suggested that the ARL cells are different from hepatocytes in the responsiveness to the CF and PB treatments, and it is likely that the suppression of growth of the exposed cells for a long time is associated with low level of growth in soft agar. Additionally, the inhibitory effect of PB at 2×10^{-3}M or higher concentrations on growth of the established rat liver diploid epithelial cell line(28) has been reported by Tsao *et al.* (29, 30).

The difference between the effects of CF and PB on growth of the exposed and unexposed cells was manifested by the examination of PDT. The continual treatment with PB delayed PDT, while the treatment with CF did not. It is likely that PB is effective for delaying the intervals of the growth cycle. We have demonstrated previously that CF at 2×10^{-3}M or higher concentrations is cytotoxic to adult rat hepatocytes in primary culture(4). CF is likely not to delay the proliferation rate of the ARL cells, but to reduce the cell density of the cells in culture by its cell-killing effect.

GGT activity of periportal hepatocytes has been enhanced in the rats fed PB, but inhibited in the rats fed the peroxisome proliferators(5, 13, 18, 19). Moreover, in the preneoplastic stages of hepatocarcinogenesis, many of the altered hepatocyte foci in the PB-fed rats were GGT-positive while many or all of the foci were GGT-negative in the liver of rats fed peroxisome proliferators(18, 19, 20). However, GGT activity of intrahepatic biliary ducts and ductules was not suppressed by the peroxisome proliferator(5). In the cultures of the exposed cells, cytochemical GGT activity was induced not only by the continual treatment with PB, but also by the CF treatment. This effect of CF has not been reported. With respect to GGT expression, the ARL cells as well as bile ductal and ductular epithelial cells may be

different from hepatocytes. Although the emergence of GGT-positive cells is likely to be associated with genetic deviations induced by the exposure to MNNG, the cytochemical GGT activity is not correlated with the transforming ability of the exposed cells because of the suppression of anchorage independence by the continual treatments with CF and PB.

We have demonstrated previously(14) that low doses of CF promoted and the highest dose inhibited rat hepatocarcinogenesis. Moreover, tumor-promoting activity of CF has manifested itself at a very low concentration in the cells of fibroblastic cell line transformed by methylcholanthrene(12). The present short-term treatments with CF and PB at 1×10^{-4}M enhanced the colony forming frequency of the exposed cells. It is possible that the continual treatments with CF and PB at this low concentration could promote the anchorage independence. The transformation study using 1×10^{-4}M CF and PB is now ongoing.

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