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Simian virus 40 large tumor antigen-immortalized normal human liver epithelial cells express hepatocyte characteristics and metabolize chemical carcinogens

(serum-free medium/aflatoxin B_1/benzo[*a*]pyrene/*N*-nitrosodimethylamine)

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ABSTRACT **Normal human liver tissue and cultured human hepatocytes are valuable models to study xenobiotic metabolism and toxicity, but they only have a limited *in vitro* life-span and are not readily available. This report describes the establishment of replicative cultures of human adult liver epithelial cells in serum-free medium. The longevity of three of these cultures, derived from different donors, was extended by introduction of the simian virus 40 large T antigen gene. Two cell lines, THLE-2 and -3, established with a recombinant simian virus 40 large T antigen virus have undergone >100 population doublings, are nontumorigenic when injected into athymic nude mice, have near-diploid karyotypes, and do not express α-fetoprotein. The cells express cytokeratin 18 and albumin in early passage, whereas higher-passage cells in logarithmic-phase growth also express cytokeratin 19. THLE-2 and -3 cells metabolize benzo[*a*]pyrene, *N*-nitrosodimethylamine, and aflatoxin B_1 to their ultimate carcinogenic metabolites that adduct DNA, which indicates functional cytochrome P450 pathways. Other enzymes involved in metabolism of chemical carcinogens, such as epoxide hydrolase, NADPH cytochrome P450 reductase, superoxide dismutase, catalase, glutathione *S*-transferases, and glutathione peroxidase are also retained by THLE cells. Thus, these immortalized human liver cells constitute an *in vitro* model for pharmacotoxicological studies and for the investigation of etiology and pathogenesis of human hepatocellular carcinoma.**

Establishing conditions to support long-term cell cultures of normal adult human liver has proven difficult. Kaighn and Prince (1) established clonally derived cultures from fetal, infant, and adult donors and showed evidence that a small subpopulation of liver epithelial cells could complete several population doublings (PDs). Further evidence for the existence of a "stem" cell population in the adult human liver was provided by the observation that replicative cultures of normal human liver epithelial cells grow in serum-free medium (LCM) for a limited period (2, 3). The life-span of cultured human epithelial cells can be significantly increased or made indefinite by transformation with the simian virus 40 large T antigen (SV40 T antigen) (4). For rat hepatocytes transformed with SV40 T antigen, several differentiated features of normal hepatocytes were retained, including the expression of albumin, transferrin, hemopexin, and glucose-6-phosphatase (5, 6). These cells, however, did not produce detectable α-fetoprotein, a characteristic of fetal or abnormal liver cells. Similar results were obtained when rat hepatocytes were transformed with adenovirus or adenovirus DNA, whereas transformation of rat fetal hepatocytes with 3′-methyl-4-dimethylaminoazobenzene led to cell lines that lack expression of albumin and β_2-macroglobulin (7, 8).

Metabolic activation of environmental carcinogens from several chemical classes have been studied in human liver tissue explants or microsomes and isolated human hepatocytes (4, 9–19). Furthermore, observed animal species-specific differences in aflatoxin B_1 (AFB_1) and 2-acetylaminofluorene metabolism indicate the need for studying human liver or hepatocytes (17, 18, 20). However, because tissue availability is limited, individuals vary in their propensity for xenobiotic metabolism, and standardized *in vitro* conditions are difficult to establish, a reproducible system with human liver cells for pharmacotoxicological studies has not been established.

In this report, the immortalization of adult human liver epithelial cells from two different nondiseased donors with the SV40 T antigen is described. These cell lines are shown to be nontumorigenic, express hepatocyte differentiation markers, and possess enzymatic pathways responsible for xenobiotic metabolism. For example, AFB_1, a known risk factor for human hepatocellular carcinoma (21), requires metabolic activation to exert its carcinogenic effects and is associated with a mutational hotspot in the p53 tumor-suppressor gene at the codon for Ser-249 (22, 23). Thus, THLE-2 and -3 liver cell lines will be beneficial for multiple applications: standardized toxicological *in vitro* tests; investigations of species-specific mechanisms of carcinogens and anticarcinogens; assessment of the mode of action of biologically active compounds; *in vitro* studies to assay factors involved in liver cancer, such as hepatitis infection; and protooncogene activation or tumor-suppressor gene inactivation.

MATERIALS AND METHODS

Cell Isolation and Culture. Normal human liver epithelial cells were obtained by a collagenase/dispase perfusion of the

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Abbreviations: SV40 T antigen, simian virus 40 large tumor antigen; AFB_1, aflatoxin B_1; AFB_1-FAPyr, 8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl-formamido)-9-hydroxyaflatoxin B_1; DMN, *N*-nitrosodimethylamine; B[*a*]P, benzo[*a*]pyrene; BPDE, (±)-*r*-7,*t*-8-dihydroxy-*c*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; γ-GT, γ-glutamyl transpeptidase; GST, glutathione *S*-transferase; GPX, glutathione peroxidase; SOD, superoxide dismutase; PD, population doubling; CYP, cytochrome P450; GADPH, glyceraldehyde-3-phosphate dehydrogenase.

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left lobe from adult autopsy donors with no clinical evidence of cancer at "immediate autopsy" (24) and cultured into flasks precoated with collagen I (Vitrogen, Celtrix Laboratories, Palo Alto, CA) at a cell density of 6000 cells per cm^2. After incubation overnight in Waymouth's medium (GIBCO)/10% fetal bovine serum (BRFF, Ijamsville, MD), the culture medium was changed to LCM containing HepG2-conditioned medium (33%) (3). Following the observation that medium conditioned 72 hr by confluent cultures of THLE-2 cells had a higher growth-stimulatory effect (D.T.S., unpublished observation), LCM medium supplemented with THLE-2-conditioned medium (33%, T2-CM) was used to culture the THLE-2 and THLE-3 cells.

For metabolism studies and Southern and RNA blot analyses, high-cell-density cultures were initiated in roller bottles (800 ml) at an initial cell density of 1×10^4 cells per cm^2 and harvested when the cell density reached 4×10^4 cells per cm^2.

Immortalization of Human Liver Cells. The THLE-1 cell strain was developed by transfecting plasmid pRSV-T (from B. Howard, National Cancer Institute) into a primary culture of human liver epithelial cells by strontium phosphate coprecipitation (25). Cell lines THLE-2 and THLE-3 were established by infection of primary cultures with a high titer of viruses collected in serum-free PC-1 medium (Ventrex Laboratories, Portland, ME) in the presence of Polybrene (8 μg/ml). The virus was generated by introducing a retroviral vector containing the *Bgl* I–*Hpa* I fragment of SV40 T antigen into the amphotropic packaging cell line PA317 (26, 27).

Karyotyping, Immuno-, and Enzyme-Cytochemical Analyses. Karyotyping was done by W. Peterson (Children's Hospital, Detroit). For immunocytochemistry, cells were fixed for 30 min in a phosphate-buffered saline containing paraformaldehyde (albumin) or 100% methanol at 4°C (cytokeratins, T antigen, α_1-antitrypsin, and α_2-macroglobulin).

For factor VIII analysis, cells were fixed in ice-cold acetone (2 min) and incubated with a mouse monoclonal antibody to human factor VIII (45 min; Zymed Laboratories) at room temperature. Primary cultures of human umbilical cord endothelial cells were used as a positive control.

Cells for γ-glutamyl transpeptidase (γ-GT) were histochemically analyzed after fixation in ice-cold acetone (28). The HepG2 cell line was used as a positive control, and the hamster embryonic cell line 3T6 was used as a negative control.

Southern and RNA Blot Analysis. Southern blot analysis of cellular DNA digested with *Bam*HI or *Cla* I was done as described (29). Integration of the SV40 T antigen gene was evaluated by hybridizing with a 1.17-kb *Hin*dIII fragment of the large T antigen gene labeled with ^{32}P by nick translation (30).

RNA extraction and blot analysis was done as described (31). Poly(A)$^+$ mRNA was isolated from cells by hybridization to biotinylated-oligo(dT) probes according to the manufacturer's protocol (Promega). The presence of epoxide hydrolase was determined by using a human *Sma* I–*Xho* I (0.4-kb) or a *Nco* I–*Nsp* I (0.9-kb) fragment derived from plasmid R60 (Oxford Biochemicals, Oxford, MI). The 2.4-kb *Eco*RI probe for NADPH cytochrome P450 reductase was isolated from plasmid hP450 (from F. Gonzales, National Cancer Institute). Superoxide dismutase (SOD) expression was determined by hybridization to a 0.45-kb human *Eco*RI–*Hin*dIII fragment from plasmid Sp65/SOD containing a cDNA fragment for human SOD (32). Glutathione peroxidase (GPX) was analyzed with a 0.8-kb *Eco*RI human cDNA derived from plasmid pSPT19/6PX (33). Expression of glutathione *S*-transferase (GST) π, α, and μ was tested by hybridization to a 0.7-kb *Eco*RI fragment of plasmid pGEM4/GST π (from J. A. Moscow, National Cancer Institute), a 0.7-kb *Eco*RI fragment of plasmid pGST2-PvuII (34), and 0.67-kb *Pst* I–*Eco*RI cDNA isolated from plasmid pGST-T-NCO (from P. G. Board, The Australian National University), respectively. The probe for albumin was isolated from plasmid B44 (35). For catalase detection, a nick-translated probe was prepared by using a 1.25-kb fragment removed by *Hin*dIII and *Eco*RI digestion of a SP64 construct of the human catalase gene (36). Cytochrome P450 (CYP) mRNA was detected by using probes corresponding to the following recombinant DNA fragments: 1.0-kb 3′ *Eco*RI CYPIA2, 1.0-kb 3′ *Eco*RI CYPIA1, 1.3-kb 3′ *Bam*HI–*Eco*RI CYPIIA3, 1.6-kb 3′ *Bam*HI–*Eco*RI CYPIIE1, 1.1-kb 3′ *Eco*RI CYPIIIA4, and 0.8-kb 5′ *Eco*RI IIB1 cDNA fragment or the entire 1.6-kb CYPIID6 cDNA isolated by *Eco*RI digestion. The CYP cDNAs were prepared from Puc18 (IA1 and IIA3), Puc9 (IA2, IIIA4, IIE1, and IIB1), or p91023 (IID6) plasmids (from F. Gonzales, National Cancer Institute). The quantity of RNA in each lane was normalized for differences in loading by reprobing the blots with glyceraldehyde-3-phosphate dehydrogenase (pRGAPDH-1, GAPDH) (37). Autoradiograms were analyzed by densitometry.

Immunoblot Analysis of Secreted Albumin. Cell supernatants (10 ml from 72-hr cultures of $\approx 0.6 \times 10^6$ cells per ml in flasks or 1.2×10^6 cells per ml in roller bottles; the cells were switched to MLCM without conditioned medium 24 hr before the assay) were adjusted to the salt and detergent concentration of RIPA (1× RIPA is 0.15 mM NaCl/0.05 mM Tris·HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% $NaDodSO_4$) (38), and the albumin was immunoprecipitated (4°C; 1 hr) with goat anti-human albumin (DAKO, Carpinteria, CA), and subjected to immunoblot analysis and quantitative densitometry (Fig. 2).

DNA Adduct Determination. DNA adduct formation was tested in cells incubated (24 hr) with ^3H-labeled benzo[*a*]pyrene (B[*a*]P, 1.5 μM, 11 Ci/mmol; Amersham; 1 Ci = 37 GBq), ^3H-labeled AFB_1 (32 μM, 19 Ci/mM; Moravek Biochemicals, Brea, CA), or *N*-nitrosodimethylamine (DMN) (50 μM). Cells were pretreated with Aroclor 1254 (10 μg/ml; National Cancer Institute Chemical Repository, Kansas City, MO), where indicated, 24 hr before carcinogen incubation. Adducts from DNA treated with B[*a*]P, AFB_1, and DMN were determined for each carcinogen in two separate experiments (39–41).

RESULTS

Immortalization of Normal Liver Epithelial Cells. Primary cell culture of normal liver epithelial cells replicated within 2–4 days after cell isolation. These cultures usually developed into a confluent monolayer after 10–14 days of incubation. Subcultures could then be obtained with a collagenase/dispase solution. Cells from all donors averaged 12 divisions before senescence. The first donor's cells were transfected with the SV40 early-region gene plasmid. Colonies having an altered morphology and high mitotic index arose 6–8 weeks later at a frequency of 2×10^{-4}. The generation time of THLE-1 cells was 48 hr until the 12th passage (40 PDs), after which time no mitoses were seen for 180 days. At the third passage, ≈30% of the THLE-1 cells expressed SV40 T antigen within their nuclei by indirect immunofluorescent staining; virtually 100% of the cells were positive five PDs later.

Primary cultures of the second and third donors were transformed by retroviral infection. In contrast to the focal growth pattern upon transfection with the SV40 T antigen gene plasmid, most virus-exposed cells rapidly divided and did not enter a cell crisis. The two cell lines that developed (THLE-2, second donor; and THLE-3, third donor) were maintained as mass cultures (Fig. 1*A*). All third- and fifth-passage cells showed T antigen in their nuclei (Fig. 1*B*). Further, Southern blot analyses showed that THLE-2 cells contained approximately one copy of the SV40 T antigen gene (data not shown). Growth decreased markedly after ≈25 PDs during the first 6 weeks after infection. At this time early-passage-cryopreserved THLE-2 cells were used to determine the growth responses to LCM medium supple-

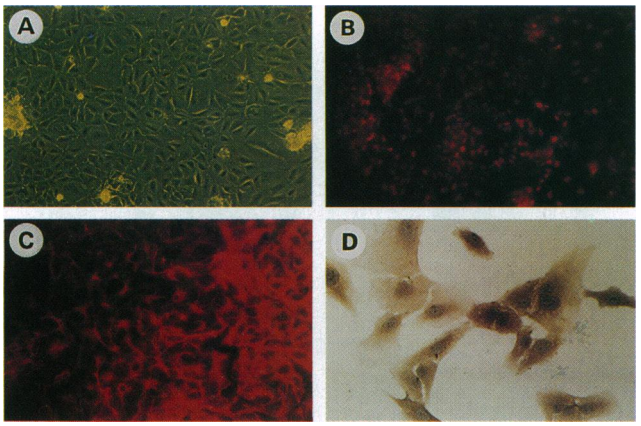

FIG. 1. Morphology and expression of cellular markers in THLE-2 cells. Phase-contrast micrograph showing the epithelial appearance of THLE-2 cells (*A*). Indirect immunofluorescence staining for SV40 T antigen (*B*) and cytokeratin 18 (*C*) showing both proteins in nearly 100% of passage-5 cells. Immunoperoxidase staining showed clonal expression of albumin in THLE-2 cells of passage 5 (*D*). For immunofluorescence analyses, nonspecific binding was blocked with the appropriate blocking serum (1:100 dilution, 20 min). Subsequently, antibodies (IgG) specific for albumin (1:20; American Qualox, La Mirada, CA), general cytokeratins (1:15; ICN), cytokeratin 18 and cytokeratin 19 (1:20 each; ICN), α-fetoprotein (1:50; Zymed Laboratories), α_1-antitrypsin, α_2-macroglobulin (1:50; Chemicon), and SV40 T antigen (1:5; Oncogene Sciences, Mineola, NY) were incubated at room temperature (30 min–1 hr) and developed with fluorescent secondary antibodies diluted 1:32 at room temperature (1 hr). Immunocytochemical staining for albumin was done by incubation with a secondary (rabbit anti-mouse; DAKOCorp, Santa Barbara, CA) and tertiary (swine anti-rabbit; DAKOCorp) antibody and development with horseradish peroxidase at room temperature (30 min each incubation).

ments. The clonal growth rate could be optimized by omitting lipid (ExCyte V) and cholera toxin supplements, replacing ornithine with arginine, and replacing HepG2-conditioned medium with T2-CM (D.T.S., unpublished observation). With this modified growth medium (MLCM), THLE-2 cells have undergone >130 PDs with no evidence of senescence. Their apparent maximal PD time is 24 hr, and their colony-forming efficiency is ≈15%. THLE-3 cells have been cultured in MLCM since early passage and, thus, have never gone into quiescence. More than 100 PDs have been completed. Their growth rate is 0.7 PDs per day, and their colony-forming efficiency is 15%. In contrast, THLE-1 cells cryopreserved at early passage still ceased dividing after ≈40 PDs, even when cultured in MLCM.

Expression of Hepatocyte Phenotypic Traits. Cytokeratin 18, but not cytokeratin 19, was uniformly expressed in early-passage cells of THLE-2 and -3, whereas at passage 10–12 all cells also expressed cytokeratin 19 (Fig. 1*C*). α-Fetoprotein or factor VIII expression was not detected at early- or late-cell passages, whereas α_1-antitrypsin and α_2-macroglobulin were present (data not shown). Albumin was readily detected in the cytoplasm of early-passage THLE-1, THLE-2, and THLE-3 cells by immunocytochemistry (Fig. 1*D* and data not shown). Islands of albumin-positive cells were surrounded by clusters of less intensely staining cells, indicating different cell clones or types. Immunoblot analyses showed that late-passage THLE-2 and THLE-3 cells can secrete albumin (Fig. 2). The albumin secretion by THLE-2 cells in roller bottles or THLE-2 and THLE-3 cells in flasks was ≈300 pg/ml, 70 pg/ml, and 14.5 ng/ml, respectively. γ-GT was weakly positive by cytochemistry in some colonies of THLE-2 or -3, as well as in the primary cultures before introduction of SV40 T antigen (data not shown). In the same test 3T6 cells were negative, whereas HepG2 cells exhibited high enzyme activity.

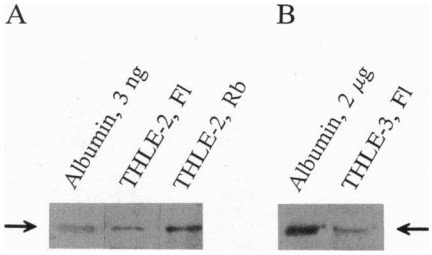

FIG. 2. Albumin secretion of THLE-2 (*A*) and THLE-3 (*B*). Albumin was immunoprecipitated from 10 ml of supernatant isolated from cells grown in serum-free medium for 24 hr in roller bottles (Rb) or flasks (Fl) (0.6×10^6 or 1.2×10^6 cells per ml, respectively; 72 hr) with 10 μl of goat anti-human albumin and analyzed by immunoblot (42) with rabbit anti-human albumin antibody (DAKO; 1:800 diluted). The membrane was developed with streptavidin alkaline phosphatase in the presence of chromogen (ABComplex, DAKO). When normalized by densitometry to the simultaneously immunoprecipitated albumin standard of 3 ng in *A* and 2 μg in *B* albumin at ≈300 pg/ml, 70 pg/ml, and 14.5 ng/ml was secreted in 24 hr by THLE-2 (Rb), THLE-2 (Fl), and THLE-3 (Fl), respectively.

Karyotype and Tumorigenicity Analysis. Karyotype analysis showed that THLE-2 and -3 are hypodiploid with most karyotypes being near-diploid (data not shown). The karyotype for each cell line was found distinctive. Monosomy of chromosomes 2 and 10, a break of chromosome 1, and 22q+ translocation leading to the marker chromosome M_{1A} characterize the near-diploid metaphase of THLE-2 at passage 18. Typical SV40 T antigen effects were also detected in THLE-3 cells at passage 22—i.e., monosomy of chromosomes 13 and deletions of chromosomes 2 and 8. When the cell lines were tested for tumor formation by s.c. injection of 10^6 cells per athymic nude mouse (20 animals), no tumors were found after 12 mo of observation.

Metabolic Studies. The metabolism, cytotoxicity, and DNA adduct formation of three different chemical classes of carcinogens were investigated in THLE-2 and -3 cells. AFB_1, B[*a*]P, or DMN caused dose-dependent cytotoxicity of THLE-2 cells (data not shown), suggesting metabolic activation of these promutagens to genotoxic metabolites. AFB_1, DMN, or B[*a*]P formed 3.5 ± 0.9, 30.4 ± 3.9, and 1.5 ± 0.1 fmol of adduct per μg of DNA, respectively, in THLE-2 or -3 cells grown in roller bottles (see Fig. 4). The major adduct found in cells treated with ^{3}H-labeled B[*a*]P was chromatographically indistinguishable from the major product formed when (±)-*r*-7, *t*-8-dihydroxy-*c*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE) was allowed to react with DNA (Fig. 3*A*). ^{32}P-postlabeling analysis revealed the N^7-methyldeoxyguanosine adduct in THLE cells incubated with DMN (Fig. 3 *C* and *D*). The major adduct in AFB_1-exposed THLE cells was 8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl-formamido)-9-hydroxyaflatoxin B_1 (AFB_1-FAPyr), whereas AFB_1-diol and 8,9-dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin B_1 were minor adducts (Fig. 3*E*). Preincubation of cells with Aroclor 1254, an inducer of CYPIA1/1A2, enhanced formation of B[*a*]P-related adducts 3-fold to 4.9 ± 2.7 fmol/μg of DNA, decreased DMN-related adducts to 3.4 ± 0.1 fmol/μg of DNA, and did not affect AFB_1–DNA adduct formation (1.6 ± 0.4 fmol/μg of DNA). Pretreatment with β-naphthoflavone abolished the ability of THLE cells to activate B[*a*]P. Similarly, ethanol treatment of the THLE cells decreased metabolic activation of DMN (data not shown).

Expression of Phase I and II Enzymes. RNA analyses of CYPIA1 mRNA steady-state levels were consistent with the results from DNA-adduct analyses. CYPIA1 mRNA was undetectable in control cells grown as roller bottle cultures. Aroclor 1254 or B[*a*]P exposure increased steady-state levels of CYPIA1 mRNA. When cells were treated with both

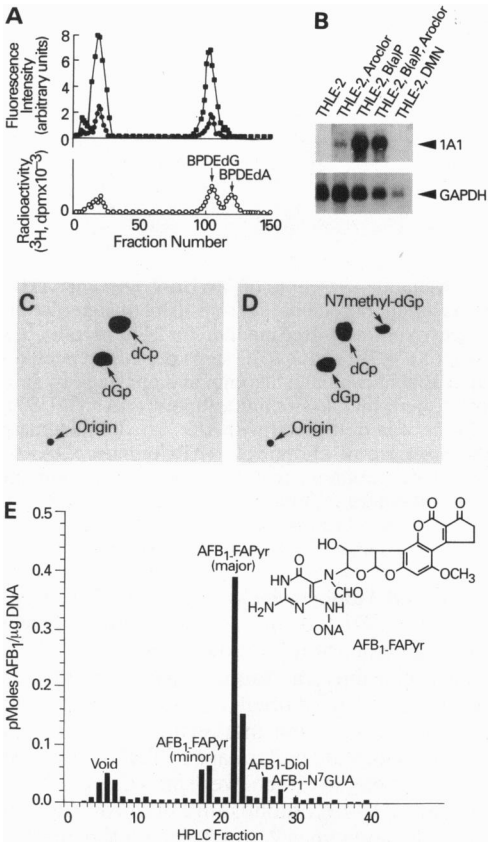

FIG. 3. Metabolic activation of carcinogens. THLE-2 cells were incubated (24 hr) with 1.5 µM ^{3}H-labeled B[*a*]P, 32 µM ^{3}H-labeled AFB_1 or 50 µM DMN, respectively. Carcinogen-modified DNA was isolated from cells (43) and BPDE–DNA (-dG or -dA) adducts were identified as described (39): arochlor induced (□); uninduced (●). RNA blot analysis (*B*) of poly(A)$^+$-selected mRNA showed an induction of CYPIA1 (1A1) normalized to GAPDH, where Aroclor < B[*a*]P < Aroclor + B[*a*]P. The relative CYPIA1/GAPDH ratios are 0.73, 10.0, and 13.1, respectively. Alkyl-DNA-adduct analysis was done by HPLC and detected by ^{32}P-postlabeling (41). Only cells exposed to DMN (*D*) had detectable levels of N^7-methyldeoxyguanosine (N7methyl-dGp). DMN adducts were absent in untreated cells (*C*). The level of adduct was determined by using scintillation counting and calibration curves for known vs. detected molar ratios of adduct to unmodified deoxyguanosine phosphate (dGp). AFB_1-FAPyr and less prominent AFB_1 adducts were identified by coelution with authentic HPLC standards (*E*). AFB_1-N^7GUA, 8,9-dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin B_1.

agents, the CYPIA1-inducing effects with both components appeared additive (Fig. 3*B*). In contrast, neither DMN (Fig. 3*B*) nor AFB_1 induced expression of CYPIA1 mRNA in roller bottle cultures of THLE-2. Other CYPs (CYPIA2, CYPIIA3, CYPIIE1, CYPIID6, and CYPIIIA4) were not detectable by RNA blot analysis.

The THLE-2 and -3 cells express the same amount of epoxide hydrolase mRNA but less NADPH CYP reductase mRNA (Fig. 4*A*). Detoxifying enzymes such as SOD, catalase, and GPX are expressed in THLE-2 and -3 cells at mRNA steady-state levels similar to the amounts found in human liver tissue (Fig. 4*B*). GST π mRNA was not found in the donor's liver tissue but were expressed by THLE cells. In contrast, GST-α mRNA was only detected in the original human tissue (data not shown).

DISCUSSION

The immortalization of normal adult human liver epithelial cells using SV40 T antigen is reported herein. In contrast to reports of the transformation of adult rat hepatocytes (5, 6),

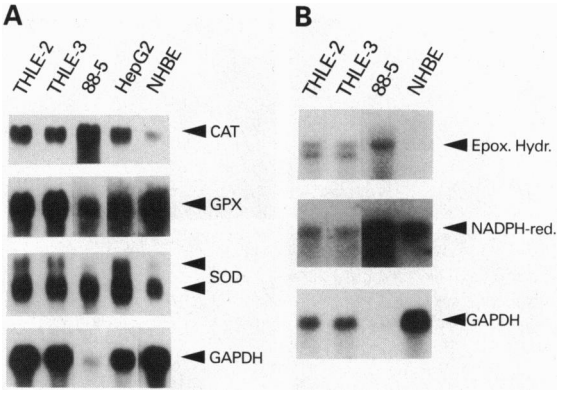

FIG. 4. RNA blot analysis of phase II enzymes. Total RNA was isolated from THLE cells, normal human liver tissue, case 88-5 (which led to THLE-2 establishment), the hepatoblastoma cell line HepG2, and normal human bronchial epithelial cells (NHBE). Quantification was based on densitometric analysis of ethidium bromide staining because GAPDH underestimated the RNA amount loaded from liver tissue. Similar expression of epoxide hydrolase (Epox. Hydr.) (*B*), GPX, SOD, and catalase (CAT) (*A*) was found in THLE cells and human liver, whereas expression of NADPH CYP reductase (NADPH-red.) (*B*) was reduced.

immortalized cultures of human liver epithelial cells could only be reproducibly established by retroviral infection. Infection with SV40 T antigen 2–3 days after isolation of the primary hepatocytes led to proliferating cells that never entered a crisis. This result was due either to a higher efficiency of gene transfer by retroviruses, as suggested by gene transfer studies in rat hepatocytes (44, 45), or to introduction of the infected genes to transcriptionally active regions of the genome. The former is more likely because only 30% of THLE-1 cells but 100% of THLE-2 and THLE-3 cells stained positive for SV40 T antigen at passage 3.

Early-passage THLE-1, -2, and -3 cells formed colonies with mixed ability to secrete albumin. We hypothesize that these cells constitute dedifferentiated hepatocytes that have various abilities to express albumin or arose from liver stem cells differentiated to cells with hepatocyte characteristics. In rats treated with hepatic carcinogens or toxic compounds, oval cells, which are much smaller than parenchymal hepatocytes or nodular cells, can differentiate to liver parenchymal cells under particular conditions *in vivo* (46–50) and may be stem cells with the potential of being neoplastically transformed to cholangiocellular, as well as hepatocellular, carcinomas (48). Rat oval cells are characterized by the expression of phenotypic markers such as albumin, cytokeratin 18 and 19, γ-GT, α-fetoprotein, and GST π, whereas glucose-6-phosphatase activity is only weakly positive (47, 49). THLE cells have an epithelial morphology; early-passage cells secreted albumin, expressed cytokeratin 18, transferrin, α_1-antitrypsin, α_2-macroglobulin, GST π (Figs. 1 and 2), and very low γ-GT levels. They were uniformly negative for α-fetoprotein and factor VIII. Therefore, THLE cells represent a population with a differentiation grade between oval cells and hepatocytes and may be derived from hepatocyte precursors, such as oval cells. However, that cytokeratin 18 is expressed and α-fetoprotein is absent in a very early stage of their establishment indicates a derivation from differentiated hepatocytes and dedifferentiation of the cells in culture shown by the appearance of cytokeratin 19 and decreased albumin secretion.

A second consequence of dedifferentiation of hepatocytes is the loss of drug-metabolizing enzymes, including CYP and associated mixed-function oxidases (51). Culture conditions such as extracellular matrices (52–54), coculture systems (55, 56), and hormone supplementation (6, 57, 58) have been

reported to positively influence differentiated functions, including phase I and II enzyme activities of primary hepatocytes (53, 56). Although SV40-immortalized rat liver cell lines have not been extensively characterized for their metabolic potential, maintenance, and/or inducibility of CYPIIB and CYPIA, NADPH CYP reductase, GSTs, and UDP-glucuronyltransferases with levels higher than in human or rat hepatoma cell lines have been reported (51). THLE cells expressed mRNAs of phase II enzymes such as epoxide hydrolase, catalase, GPX, SOD, and GSTs at levels comparable to human liver. GST π and α mRNAs are the dominant forms seen in THLE cells or human liver, respectively. NADPH CYP reductase was maintained but at a lower steady-state mRNA level than in human liver.

The cells metabolized carcinogens AFB_1, DMN, and B[*a*]P, which represent three different chemical classes. In each case, highly reactive electrophilic metabolites were formed that resulted in the same DNA adducts as formed *in vivo* (40, 41, 59). These data suggest the presence and function of CYPIA2/1A1, CYPIIE1, and CYPIIIA4 in these cells. The B[*a*]P and Aroclor 1254 treatment induced CYPIA1 mRNA analogous to findings in rat hepatocytes and the hepatoma cell line H4-II-E (60, 61). In addition, B[*a*]P metabolic activation was inhibited by α-naphthoflavone, indicating inhibition of CYPIA. However, CYPIA2, the predominant isoform in human liver, as well as CYPIIE1, CYPIIIA4, CYPIIA3, or CYPIID6, were not detected by RNA blot analysis. The AFB_1–DNA adducts, therefore, might have been formed through a mechanism involving CYPIA1 (12, 13). There is no reported evidence for metabolic activation of DMN by cytochromes other than CYPIIE1. Therefore, the cells probably express low CYPIIE1 amounts, which is consistent with an inhibition of adduct formation by ethanol pretreatment strongly blocking CYPIIE1 after an initial induction phase (62). The decrease in DMN–adduct formation after treatment with Aroclor 1254 may indicate enhanced detoxification of critical metabolites.

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