Growth in Culture and Tumorigenicity after Transfection with the ras Oncogene of Liver Epithelial Cells from Carcinogen-treated Rats

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ABSTRACT

Two epithelial cell lines designated LE/2 and LE/6 were established from cells isolated by centrifugal elutriation from the livers of carcinogen-treated rats. Both cell lines exhibit some characteristics of fetal liver cells, such as the expression of the 2.3-kilobase α-fetoprotein mRNA, aldolase A, and lactate dehydrogenases 4 and 5. Primary cultures contain γ-glutamyl transferase-positive cells which do not proliferate in vitro. After the first passage, the LE/2 and LE/6 cell lines are uniformly γ-glutamyl transferase-negative. Neither cell line is transformed as assayed by morphology, anchorage-independent growth, or tumor formation in nude mice. By the 50th passage, LE/6 cells form numerous colonies in soft agar in the presence of epidermal growth factor, while no colonies grow in medium lacking this growth factor. Clonal cell populations derived from five epidermal growth factor-induced soft agar colonies were not tumorigenic in nude mice. This indicates that, although epidermal growth factor-responsive late passage cells had acquired some of the phenotypic properties commonly associated with tumor cells, these cells were not fully transformed. Transformation of LE/6 cells was accomplished by transfection of the ras oncogene (EJ). Subcutaneous inoculation of ras(EJ)-transfected LE/6 cells produced tumors at the site of injection with histological features of moderate to well-differentiated trabecular hepatocellular carcinomas. Tumor cell lines derived from the nude mouse tumors are γ-glutamyl transferase positive and express α-fetoprotein mRNA. One clonal cell line expresses both α-fetoprotein and albumin mRNA. These results show that nonparenchymal liver epithelial cells transfected with an activated oncogene can give rise to differentiated hepatocellular tumors similar to those induced in livers of rats fed a carcinogenic diet.

INTRODUCTION

Experimental rat liver carcinogenesis is a common model for the study of the development of cancer in animals. However, studies on the cellular origin of experimentally induced rat hepatocellular carcinomas are complicated by the likelihood that there may be multiple pathways involving different target cells and/or different progression steps in hepatocarcinogenesis. In general it is assumed that hepatocytes are the target cells for most chemical carcinogens and as such represent the cellular precursor for hepatocellular carcinomas (1). The normal liver is, however, a histologically complex tissue and, although hepatocytes account for approximately 90% of the volume of the liver, there is evidence to suggest that other liver cell types, including Kupffer cells and bile ductular cells, may contribute to the regulation of normal and abnormal liver growth (2).

A striking feature of the early stages of hepatocarcinogenesis induced by some chemical carcinogens is the proliferation of small nonparenchymal epithelial cells which are morphologically and biochemically distinct from mature hepatocytes. The initial appearance of these cells, termed oval cells, in the portal areas of the liver and their formation of duct-like structures have led to suggestions that oval cells derive from cells of the terminal bile ducts (3–5). However, the role these cells play in the neoplastic process is uncertain (6). Based on the demonstration of cells morphologically intermediate between oval cells and hepatocytes, several investigators have proposed that oval cells give rise to hepatocytes during azo-dye carcinogenesis (3, 7, 8). In other experimental systems, the evolution of oval cells into hepatocytes has been difficult to establish (9), and clearly more experimental data are required to determine the significance of oval cells in the development of liver tumors (10).

Propagable lines of small, relatively undifferentiated epithelial cells which vary express hepatocytic properties have been established in continuous culture (11). The in vivo origin of these cells was not known, and most investigators in the past concluded that they derived from the dedifferentiation of mature hepatocytes in culture. Grisham (11), however, showed by cloning of cells from collagenase-digested livers that mature hepatocytes are not clonogenic under usual culture conditions. Given the close embryological relationship between hepatocytes and terminal bile ductular cells, he suggested that clonogenic hepatic epithelial cells originate from cells in the terminal bile ducts and that these cells may function as facultative liver stem cells, especially under conditions where hepatocytes are subjected to prolonged damage. This finding raises the question of whether oval cells and propagable hepatic epithelial cells share a common cellular origin.

A more detailed analysis of the biological properties of oval cells has been made possible by the development of methods to purify oval cells and hepatocytes from the livers of carcinogen-treated (12–17). Extensive biochemical characterization of oval cells and hepatocytes purified from the livers of rats fed a CDE diet has shown that many of the changes such as the expression of oncodevelopmental proteins and elevated levels of the c-myc and c-ras-protooncogenes seen in whole livers and in the resulting hepatomas occur in the oval cell population but not in the liver parenchymal cells, at least at the earliest stages of the process (18–20).

In order to further investigate the origin and fate of the oval cell population in liver carcinogenesis, we have established cell lines using oval cells isolated from rats after different periods on the CDE diet. In this paper, we report (a) a detailed characterization of two cell lines, LE/2 and LE/6, isolated from the livers of rats fed a CDE diet for 2 or 6 wk, respectively; (b) the transformation of LE/6 cells by transfection with the activated c-ras (EJ) gene; and (c) the tumorigenicity of these cells in nude mice. The availability of untransformed and transformed oval cell lines enabled us to dissect some of the sequential steps in the neoplastic conversion of nonhepatocyte liver epithelial cells and to evaluate the capacity of these cells to generate hepatocytic tumors.

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*The abbreviations used are: CDE diet, choline-deficient diet containing 0.1% ethionine; LDH, lactate dehydrogenase; GGT, γ-glutamyl transferase; ATP, α-fetoprotein; ABC, avidin-biotin complex; LE, liver epithelial; FBS, fetal bovine serum; PBS, phosphate-buffered saline; cDNA, complementary DNA.

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MATERIALS AND METHODS

Isolation and Culture of Oval Cells. Oval cells from the livers of 6-8 wk old male rats (Fischer for LE/6 cells or Sprague-Dawley, Holtzman strain for LE/6 cells) aged 6 to 8 wk fed a choline-deficient diet for 2 or 6 wk were isolated as previously described by Yaswen et al. (12). The livers were first perfused with 0.1% collagenase and finely minced. The minced tissue was then incubated with 0.1% collagenase:0.004% DNase:0.1% Pronase to dissociate the cells and selectively digest parenchymal cells. The oval cell-enriched cell suspension was subjected to centrifugal elutriation, and a total of 6 fractions were collected. On the basis of size and histochemical staining for GGT, the cells in Fraction 5 (12, 18) were used for in vitro culture. Oval cells were plated at a density of 1 x 10^6 cells/60-mm Petri dish on a 0.1% confluent irradiated feeder layer of mouse 3T3 fibroblasts (4000 rads, X-ray source) in Dulbecco’s minimal essential medium:Ham’s F-10 (1:1) (Gibco Laboratories) supplemented with 10% FBS, 1 μg/ml insulin (Sigma Chemical Co.), 0.5 μg/ml hydrocortisone (Sigma Chemical Co.), and 25 μg/ml gentamicin sulfate. Parallel cultures were established using no feeder layer or using collagen-coated Petri dishes.

To establish primary cultures, cells were allowed to attach for 24 h, at which time unattached cells were removed and fresh medium was added. Medium was changed every 3 to 4 days, and when cultures were nearly confluent (10 days), contaminating fibroblasts were selectively removed from the Petri dish by pipetting with a 0.02% EDTA solution (21). Essentially pure cultures of epithelial cells were obtained in this way. To establish cell lines, cultures were passaged once a week on plastic at a 1:5 split ratio.

Generation times and saturation densities were determined by counting the cells in a hemocytometer at regular intervals after plating 1 x 10^6 cells/cm².

Histochemistry. Cells from primary cultures were grown on cover-slips, air dried, and stained for GGT for 30 min on Days 1, 3, and 7 at passages 5, 10, 15, and 50 (22). Histochemical staining for primary cultures for peroxidase was performed on Days 3 and 7 (23, 24).

Isozyme Assays. Cells grown in Petri dishes were washed 2 times with PBS and scraped from the dishes with a rubber policeman. Pelleted cells were homogenized in 10 mm Tris:1 mM EDTA, pH 7.4, and centrifuged at 100,000 x g for 30 min at 4°C. Extracts were either frozen or used immediately. Electrophoretic separation of extracts and enzyme staining for aldolase and LDH isoforms were performed as previously described (18).

Immunohistochemical Localization of Keratin, AFP, and Albumin. Indirect immunofluorescent staining for keratin was performed as described by Riggs (25). Cells grown on glass cover slips were fixed in cold acetone for 10 min, air dried, and tested immediately or stored at −70°C. Cover slips were first incubated with anti-bovine prekeratin diluted 1:20 (Miles Scientific), washed 3 times in PBS, and then treated with fluorescein-conjugated secondary antibody (Miles Scientific). After further washes in PBS, cover slips were mounted on glass slides with glycerol and examined with a Zeiss microscope.

Immunoperoxidase staining for AFP and albumin was performed by the ABC method of Hsu (26) using ABC vectastain kits (Vector Laboratories). Cells grown on coverslips were fixed in acid alcohol (99% ethanol:1% acetic acid) for 30 min at 4°C. The rabbit anti-mouse AFP (Miles Scientific) was diluted 1:20, and rabbit anti-rat albumin (Cooper Biomedical) was diluted 1:100. All other reagents were supplied with the ABC kits. Normal rabbit serum was used as a control for both antibodies.

RNA Analysis. Total cellular RNA was isolated from cell cultures by the guanidine thiocyanate method of Chirgwin et al. (27). Cell pellets were homogenized in a Dounce homogenizer in guanidine thiocyanate buffer, passed 3 times through a 21-gauge needle, and layered onto a 3.5-ml cushion of 5.7 M cesium chloride in 25 mM sodium acetate (pH 5). The homogenate was centrifuged for 20 h at 28,000 rpm in a Beckman SW40 Ti rotor. The buffer was removed, and the pellet containing the RNA was dissolved in 0.2 ml of water. The RNA was then precipitated with ethanol and redissolved in water.

Twenty μg of each total cellular RNA were fractionated on 1.1% agarose:formaldehyde gels and transformed to nitrocellulose filters (28). 32P end-labeled HindIII digests of phage λ-DNA were used as size markers. Filters were prehybridized and hybridized with 32P-labeled AFP (cDNA clones pRAF87 and pRAF65 provided by Dr. T. Sargent) (29) as previously described (30), or hybridized with a 32P-labeled albumin probe (pM.L2). Filters were then washed and examined by autoradiography.

For cell cycle studies, cells were grown to confluence and placed in 0.1% FBS for 72 h. Quiescent cultures were then stimulated to divide by the addition of 10% FBS, and RNA was extracted at various times after serum stimulation.

Soft Agar Assay. The soft agar assay was performed essentially as previously described (31). Cells (10⁶ and 10⁹) in 1 ml of complete medium with 0.3% agar (Bacto-Difco) were gently overlaid on a 2-ml basal layer of 0.6% agar in 35-mm Petri dishes. Five replicate cultures were prepared for each cell line. Epidermal growth factor was added at a concentration of 10 ng/ml. Cultures were fed every 5 days with a small amount of complete medium. Colony formation was determined at 21 days, at which time a large variation in colony size was observed. True colonies were defined as clones with a diameter greater than 0.1 mm. Microcolonies were defined as clones with a diameter of less than 0.1 mm. Only true colonies continued to grow and could be subsequently propagated in monolayer culture. SV40-transformed SVT2 cells grown in an identical manner served as positive controls.

Transfection of LE/6 Cells. LE/6 cells (passage 25) were seeded at a density of 3 x 10⁶ cells/10-cm tissue culture dish. The plasmid DNAs used for transfection were pUC/EJ and pGV-16. pUC/EJ is a PBR322 derivative which contains the 6.6-kilobase BamHI fragment of the activated ras gene isolated from the human EJ bladder carcinoma cell line (32). pGV-16 is a plasmid containing the neo gene which confers resistance to the antibiotic G418 (Sigma Chemical Co.) (33). Twenty-four h after plating, cells were fed fresh medium, and 4 h later, they were transfected by the calcium phosphate precipitation method (34) using 3 μg of pGV-16, 15 μg of pUC/EJ DNA, and 12 μg of rat liver DNA (30 μg of DNA, total). Control plates were transfected with 30 μg of normal rat liver DNA with or without 3 μg of pGV-16. Eighteen h after transfection the precipitate was removed, and cells were fed fresh medium. A day later cells were placed in selective medium containing 150 μg/ml G418. After 2 wk in selective medium, G418-resistant colonies were scored, pooled, and expanded for further analysis.

Tumorigenicity in Nude Mice. Nude mice were inoculated s.c. on the back with 2.5 x 10⁶ neo-transfected cells. Control animals received 5 x 10⁶ neo-transfected cells or 5 x 10⁶ untransfected LE/6 passage 36 cells. The mice were examined twice weekly for tumor formation. Animals bearing tumors were killed, and a portion of each tumor was fixed in formalin for routine histology. The remaining tumor tissue was either frozen for DNA and RNA analysis or finely minced, trypsinized, and placed in Petri dishes to establish cell lines.

RESULTS

Isolation, Morphology, and Growth Properties of Liver Epithelial Cells. Two hepatic epithelial cell lines, LE/2 and LE/6, isolated from the livers of rats fed a CDE diet for 2 and 6 wk, respectively, have been successfully established in continuous culture. Since it is thought that Pronase may decrease the ability of cells to form colonies in culture, we initially omitted the Pronase digestion step from the isolation procedure of Yaswen et al. (12). However, Pronase was used in later experiments and had no effect on the morphological and biochemical properties of the cells grown in culture. Plating efficiency was approximately 20% for both cell lines on untreated Petri dishes, although not all cells which attached formed colonies.

The isolated cells were examined for GGT staining immediately after isolation, 24 h after placing them in culture, and at frequent intervals thereafter. For both cell lines the percentage of cells which stained for GGT at 24 h after plating corresponded to the percentage of positive cells in the isolated fraction (60 to 90%). However, by Day 3 the number of GGT-
positive epithelial cells had declined to about 20 to 30%, and after the first passage virtually no GGT-positive epithelial cells could be detected in our cultures regardless of the experimental conditions or the origin of the cell lines.

Primary cultures of LE/2 cells contained a mixture of spindle-shaped fibroblastic cells and large polygonal epithelial cells. Fibroblasts continued to proliferate until the fifth passage, at which time the cultures underwent a crisis and most cells died. This resulted in pure cultures of GGT-negative epithelial cells (Fig. 1a). LE/2 cells have been maintained continuously in culture for over 1 yr and are now in the 30th passage. The generation time of these cells is approximately 24 h, and they reach a saturation density of 1.7 x 10^5 cells/cm^2 in 7 to 9 days.

Four morphologically distinct cell types could be distinguished in cultures of primary LE/6 cells: (a) large stellate cells with irregular outlines, which were morphologically distinct from typical fibroblasts; (b) small epithelial-like cells which formed densely packed colonies of 10 to 12 cells; (c) fibroblasts; and (d) large epithelial cells. LE/6 cells never entered a crisis regardless of the experimental conditions, and after the first few passages only large epithelial cells survived. These four cell types were also apparent when hydrocortisone was not added to the culture medium. The large epithelial cells replicated to form uniform monolayers with a cobblestone appearance. The morphology of LE/6 cells is shown in Fig. 1. b and c. A consistent morphological feature of cultured LE/6 cells is the presence of islands of multinucleated giant cells seen at both early and late passages. The significance of these cells is unclear, but such cells have been reported previously by Heine et al. (35) in rat liver cells transformed in culture by DL-ethionine. The epithelial origin of our cell lines has been confirmed by immunofluorescent staining of the cells for keratin using antiserum against bovine prkeratin (data not shown).

LE/6 cells have been maintained in culture for over 100 passages, making it possible to characterize the alterations in the growth patterns of this line with passage in culture. Late passage (greater than 50) LE/6 cells reach a saturation density of 1.2 x 10^5 cells/cm^2, whereas early passage (less than 20) LE/6 cells grow to a saturation density of only 6.0 x 10^4 cells/cm^2. Morphologically, early and late passage LE/6 cells are similar until about passage 60, at which time the cells appear smaller, have a higher nucleocytoplasmic ratio, and tend to pile up.

The ability of LE/6 cells to grow in reduced serum (0.2% FBS without supplementary growth factors) differed markedly according to passage level. At passage 15, LE/6 cells grew poorly with a saturation density of only 1.0 x 10^5 cells/cm^2, whereas at passage 50 LE/6 cells grew to a saturation density of 3.9 x 10^6 cells/cm^2, approximately 38 times higher than early passage cells. Late passage cells can be subcultured for a period of time in medium containing 0.2% FBS. Apparently, this does not select for a more transformed phenotype, since the cells remain morphologically benign and senesce after approximately 10 passages in reduced serum. Late passage cells can, however, be maintained continuously in 10% FBS.

Expression of Aldolase and LDH Isozymes in Cultured LE/2 and LE/6 Cells. In order to further characterize the liver epithelial cell lines and to compare them to purified cell populations isolated from normal and carcinogen-treated livers, we examined the electrophoretic patterns of two enzymes, LDH and aldolase, which have been previously shown to change during hepatocarcinogenesis (18). A comparison of the LDH profile of cultured LE/2 and LE/6 cells to that of in vivo liver cells reveals some interesting results (Fig. 2a). Approximately equimolar amounts of LDH-4 and -5 are present in LE/6 cells, a pattern of expression which closely resembles that found in fetal livers at 15 days of gestation, whereas the LDH profile of LE/2 cells is very similar to that found in extracts of fetal livers at 21 days of gestation. By this point in fetal liver development, a shift towards LDH-5, the predominant form detected in adult liver or isolated parenchymal cells, has occurred.

We also compared the expression of aldolase isozymes among cultured oval cells, normal adult and fetal livers, normal parenchymal cells, and isolated oval cells from CDE livers. As shown in Fig. 2b normal adult liver and parenchymal cells purified from adult livers contain mostly aldolase B. Fetal livers at 15 days of gestation express aldolase A, and a small amount of aldolase B, whereas by 21 days of gestation a marked shift toward the expression of aldolase B is seen. In LE/2 cells the predominant isozyme is aldolase A, but aldolase B is also present. In contrast, LE/6 cells contain exclusively aldolase A. Again, as noted with LDH, the aldolase profile in LE/2 and LE/6 cells resembles that seen in fetal livers.

Expression of AFP and Albumin mRNAs. To further assess the functional characteristics of LE/2 and LE/6 cells, the expression of AFP, albumin, and their corresponding RNAs was studied in both cell lines. We could not detect AFP or albumin by immunoperoxidase staining of the cultured cells.
We analyzed the abundance of AFP transcripts in LE/2 passage 10 cells and in LE/6 cells at the tenth and 53rd passages using the Northern blot technique (Fig. 3). The major transcript detected in both cell lines is a 2.3-kilobase mRNA which corresponds in size to the full-length AFP mRNA found in large amounts in fetal liver, isolated oval cells, and primary hepatocellular carcinomas and at low levels in nonparenchymal cells from normal adult rat liver (30). The overall abundance of AFP mRNA did not decrease with passage in culture of LE/6 cells and was slightly higher in late passage cells. In both cell lines regardless of passage level, AFP mRNA levels were low in quiescent cells and increased in abundance as the cells progressed through the cell cycle. These results are consistent with the work of others (36-38) in which the synthesis of AFP in hepatocytes and epithelial cells was found to vary during the cell cycle. In addition to the 2.3-kilobase AFP mRNA, LE/2 and LE/6 cells contain a 1.7-kilobase RNA which hybridizes with the AFP cDNA probe. This RNA has been detected in hepatocytes from normal livers, but its biological significance is unknown at this time (30).

Low levels of albumin mRNA were detected in LE/2 cells at the third passage but not in LE/2 cells at later passages. Albumin mRNA was only infrequently found in LE/6 cells (data not shown).

Transformation Potential of LE/2 and LE/6 Cells. Colony formation in soft agar is usually associated with tumorigenicity in animals. Recent studies in a number of cell lines indicate that soft agar growth can be induced by a number of growth factors, including transforming growth factors, multiplication-stimulation activity, fibroblast growth factor, and epidermal growth factor (39). Therefore, we decided to examine the ability of LE/2 and LE/6 cells to form colonies in soft agar in the presence or absence of epidermal growth factor. The results of these studies are shown in Table 1. As indicated in “Materials and Methods,” true colonies were defined as clones with a diameter of greater than 0.1 mm on Day 21 and were scored independently from clones with a diameter of less than 0.1 mm.

Early passage LE/2 and LE/6 cells do not grow in soft agar, and only a few small but no true colonies formed in agar medium containing epidermal growth factor. Although late passage LE/6 cells do not grow in soft agar under the usual assay conditions, a striking epidermal growth factor-mediated induction of anchorage-independent growth occurred at the 50th passage (Table 1). In order to determine if LE/6 cells, which were induced to grow in soft agar by epidermal growth factor, were transformed, we isolated 5 soft agar clones, expanded them in monolayer culture in the presence of epidermal growth factor, and injected 5 x 10<sup>6</sup> cells from each clone into 5 nude mice. No tumors from any of the clones were observed after 4 mo. These results suggest that, whereas the growth properties of late passage LE/6 cells have been altered as indicated by their enhanced sensitivity to epidermal growth factor, these cells are not tumorigenic.
ONCOGENE TRANSFECTION OF LIVER EPITHELIAL CELLS

Fig. 4. Southern blot analysis of LE/6 cells transfected with the ras™ (EJ) oncogene. Genomic DNA (10 μg) from untransfected cells (1), pGV-16 transfected cells (2), pGV-16 and pUC/EJ cotransfected cells (3), and cell line from nude mouse tumor obtained by inoculation of pGV-16 and pUC/EJ cotransfected cells (4) was digested with HindIII, separated on 0.8% agarose gels, transferred to nitrocellulose filters, and hybridized with a v-ras™-specific probe (BS-9) under conditions previously described (19). Arrows on the right denote the additional bands corresponding to the presence of the ras™ (EJ) oncogene in the ras-transfected cells. HindIII-digested phage λ DNA served as size markers, left, in kilobases. Filters were autoradiographed at -70°C for 3 days.

showed some cellular heterogeneity, but the predominant cell type was a fairly uniform cuboidal cell which formed flat colonies (Fig. 5a). These cultures grew at a higher density than untransformed cell cultures but had no other obvious alterations which might be indicative of transformation. To test the tumorigenicity of the EJ-transfected liver epithelial cells, the uncloned cells were maintained for 6 to 11 passages and inoculated into 8 nude mice (2.5 x 10⁶ cells/animal). Visible nodules formed at the site of inoculation in all animals with a latency of 4 to 5 wk. The tumors reached an average diameter of 2 cm in 6 to 7 wk at which time the mice were killed. In contrast, only one of 5 mice given injections of neo-transfected cells developed a tumor, which appeared after a period of 4 mo. No tumors developed in mice given injections of untransfected passage 36 LE/6 cells.

Tumor Histology. All 8 tumors examined contained histological features of moderate-to well-differentiated hepatocellular carcinoma (Fig. 6, a to c). Differentiated hepatic cells predominated in 4 of the tumors. The other 4 tumors were more mixed, consisting of some areas which clearly resembled typical hepatocellular carcinoma and other areas which were less differentiated. The differentiated tumors contained cells with large, regular, round nuclei with a single, central nucleolus as well as binuclear cells. These features are very similar to those observed in liver tumors seen in rats fed the CDE diet (Fig. 6d). The differentiated hepatocellular tumors induced in nude mice were cellular with little connective tissue; hepatic cells were often arranged in organized rows or trabeculae. The less-differentiated tumors were pleomorphic, containing large cells with deep staining, large nuclei, and scant cytoplasm. In 2 of these tumors there were small areas in which abnormal, elongated cells resembling fibroblasts proliferated, and in one tumor an area of papillary cholangiocarcinoma was present.

Cells obtained from one of the differentiated tumors have been placed in culture. These cultures are morphologically heterogenous containing large cells with irregular outlines which form islands surrounded by smaller, more uniform cells (Fig. 5b). In contrast to what we observed in the untransfected LE/6 cells, the tumor cell line was GGT positive with the intensity of staining roughly corresponding to differences in cell morphology. In general, the large cells were faintly GGT positive, whereas the smaller cells stained intensely for GGT. The tumor cell line and 8 clones derived from this cell line...
express AFP mRNA, and one of the clones expresses albumin mRNA (Fig. 7).

DISCUSSION

In this paper we describe the in vitro culture and biochemical characterization of 2 liver epithelial cell lines isolated from livers of rats fed a CDE diet for different periods of time. We transfected the EJ oncogene into cells of the LE/6 line and showed that the transfected cells can form moderate- to well-differentiated trabecular hepatocellular carcinomas when injected into nude mice.

A summary of the phenotypic properties of both cell lines is presented in Table 2. Although similar in morphology, the LE/2 and LE/6 cell lines can be most easily distinguished by their isozyme profiles. Both cell lines express low levels of AFP RNA transcripts and fetal liver patterns of LDH and aldolase isozymes. LE/2 cells isolated after 2 wk on the diet also express albumin transcripts and aldolase B, the isozyme synthesized by adult hepatocytes. On the other hand, LE/6 cells consistently express only aldolase A and AFP transcripts. Aldolase B had not previously been demonstrated in cultured liver epithelial cells. Since early passages of both cell lines have proven difficult
ONCOGENE TRANSFECTION OF LIVER EPITHELIAL CELLS

![Image](https://cancerres.aacrjournals.org/)

**Table 2** Summary of phenotypic properties of LE/2 and LE/6 cells

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<th>Passage</th>
<th>LE/2</th>
<th>LE/6</th>
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<td>A</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Albumin</td>
<td>Transient</td>
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<td>Transformation potential</td>
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<td>1.2 x 10^4</td>
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</tr>
<tr>
<td>+EGF</td>
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<td>Tumors in nude mice</td>
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<td>ND</td>
<td>0/5</td>
</tr>
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</table>

* Values expressed as the average number of cells/cm² in triplicate cultures.

ND, not determined.

Fig. 7. Expression of AFP mRNA (top) and albumin mRNA (bottom) in untransfected LE/6 cells (Lane 1), EJ and neo-transfected LE/6 cells before injection into nude mice (Lane 2), the tumor cell line derived from a nude mouse given injections of EJ and neo-transfected LE/6 cells (Lane 3), and representative clones derived from the tumor cell line (Lanes 4 to 6). Total RNAs (20 μg) were fractionated on an agarose gel, transferred to nitrocellulose filters, and hybridized with the appropriate probe. Filters were exposed to Kodak XAR-2 film for 3 days with the use of intensifying screens (DuPont).

cells in vivo but differ from our cell lines in that they express both aldolase A and C, LDH 2-5, and albumin and are weakly positive for GGT. Although it appears that LE/2 and LE/6 are different cell types from WB-F344, we cannot exclude the possibility that all of these lines originate from a single cell type and that some of the dissimilarities in morphology and patterns of gene expression between the LE and WB-F344 lines are consequences of culture conditions. The cultured oval cell lines described by Yoshimura et al. (41) differ from our lines in that they are uniformly GGT positive and spontaneously transform between the 20th and 30th passages. Evidence for the biochemical heterogeneity of oval cells has been presented by several investigators. Using a double immunofluorescent labeling technique with antibodies against AFP and a specific cytoketatin which recognizes oval cells and bile ductular cells but not hepatocytes, Germain et al. (43) distinguished 3 oval cell populations in 3-methyl-4-dimethyl aminoazobenzene-treated rat liver. Likewise Hixson and Allison (44) concluded that oval cells which proliferate in the livers of rats fed a choline-deficient diet containing ethionine or 2-acetylaminofluorene are composed of 3 distinct subpopulations based on immunofluorescence studies with a panel of monoclonal antibodies. In summary, these results suggest that liver epithelial cell lines established from normal and carcinogen-treated rat livers are phenotypically heterogenous with diverse biological potentials.

The lack of histochemical staining for GGT in both our cell lines after the first passage in culture is somewhat surprising given the large proportion of oval cells which stain for this enzyme in vivo. Our culture conditions do not inhibit GGT activity; the large stellate cells in the primary cultures stain for GGT, and hepatocytes tend to become GGT positive with time in culture. In addition, the tumor cell line derived from the nude mouse tumors stains intensely for GGT. Although the failure to detect GGT activity in our cells could be an adaptive response of the cells to the in vitro environment, it is more likely that we have selected a subpopulation of oval cells which are negative for this marker. The absence of GGT staining in LE/2 and LE/6 cells may, therefore, reflect their undifferentiated nature.

Since the LE/2 and LE/6 cell lines are derived from a population of cells which proliferate during in vivo exposure to a carcinogenic diet, the transformation or tumorigenic potential of these cell lines is of particular interest. Based on the most stringent criteria for transformation—anchorage-independent growth and tumorigenicity in animals—early passage LE/2 and LE/6 cell lines are not transformed. However, by the 50th passage the LE/6 cells have acquired an altered sensitivity to growth factors such that they can form colonies in soft agar in the presence of epidermal growth factor. Cell lines derived from the soft agar colonies, however, are not tumorigenic in nude mice. Similar observations were made by Tsao et al. (45) in their liver epithelial cell lines transformed by repeated treatments with N-methyl-N-nitro-N-nitrosoguanidine. Our work suggests that a still undefined pretransformation event has taken place in late passage LE/6 cells. Whether in vivo exposure to ethionine or passage in culture (46) has primed the oval cells for this event is not known. We are examining the regulation of expression of certain protooncogenes, especially that of c-myc (47), and of genes encoding growth factors in early and late passage cells to help define the nature of the pretransformation event and the mechanism by which epidermal growth factor induces anchorage-independent growth in LE/6 cells.

The developmental fate and tumorigenic potential of liver epithelial cells that proliferate during hepatocarcinogenesis are...
subjects of considerable debate. One way to approach these questions is to isolate and maintain these cells in culture and analyze the spectrum of tumors induced in suitable recipients after transfection of the cells with known cellular oncogenes. Although a detailed analysis of the expression of various markers in the tumors is needed, it is clear that passage 25 LE/6 cells transfected with the EJ oncogene are capable of forming moderately to well-differentiated hepatocellular carcinomas of the trabecular type when injected into nude mice. These tumors have a similar morphology to those induced in vivo in livers of rats fed the CDE diet for 50 wk or more. Although spontaneously transformed or transfected liver epithelial cells have been reported to produce undifferentiated tumors when inoculated into syngeneic animals or nude mice (41, 48), the formation of hepatocytic tumors from liver epithelial cells which have been transformed in culture by chemical carcinogens has been described by 2 different laboratories (49, 50). Thus, it does appear that liver epithelial cells have the potential of evolving into transformed hepatocytes. Whether or not this developmental path occurs in vivo may depend on the specific conditions of hepatocarcinogenesis or liver damage. It has, however, been clearly demonstrated that liver epithelial cells can give rise to hepatocytes during azo-dye carcinogenesis. The results presented in this paper strengthen the view that there are facultative stem cells in the mammalian liver which can follow different developmental pathways including that of hepatocytic differentiation, depending on the nature of the induction process.

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ONCOGENE TRANSFECTION OF LIVER EPITHELIAL CELLS


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