Phenotypic Expression in Spontaneously Transformed Cultured Rat Liver Epithelial Cells

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ABSTRACT

Five clonal cell strains of an early-passage normal rat liver epithelial cell line were transformed spontaneously using the protocol of "selective culture" condition. Twelve cell lines were established from the tumors produced after injecting these transformed cells into 1-day-old syngeneic rats. The phenotypic expressions of these spontaneously transformed tumor cell lines were studied and compared to those of cell lines obtained from tumors produced by rat liver epithelial cells transformed by N-methyl-N'-nitro-N-nitrosoguanidine. Like the chemically induced tumor cells, spontaneously transformed tumor cells exhibited phenotypic heterogeneity in the expression of isoenzymes, proto-oncogenes, growth factors and their receptors, and cellular responses to the effect of growth factors. However, unlike the chemically induced tumor cells, these spontaneously induced tumor cells did not express the "resistant phenotypes" characteristic of chemically induced or promoted tumors. Although all the spontaneously induced tumor cell lines expressed variable amounts of transforming growth factor-α mRNA, it was not functionally coordinated with the expression of its receptor, the epidermal growth factor receptor. Thus, spontaneously transformed rat liver epithelial cells demonstrated both similarity and diversity in their phenotypic expression when compared to their chemically induced counterpart. This model of spontaneous transformation of cultured rat liver epithelial cells may be useful for the mechanistic study of non-chemically induced carcinogenesis.

INTRODUCTION

We have previously reported that, during malignant transformation of cultured rat liver epithelial cells induced by multiple treatments with MNNG, heterogeneous biological and biochemical changes occur that mimic those occurring during in vivo rat chemical hepatocarcinogenesis (1, 2). The tumors produced by these chemically transformed rat liver epithelial cells are morphologically diverse but closely resemble hepatocellular carcinoma, cholangiocarcinoma, or hepatoblastoma (3). More importantly, these chemically induced tumorigenic cultured rat liver epithelial cells demonstrate the "resistant" phenotypes typical of chemically induced rat preneoplastic/neoplastic hepatocytes, including elevated cellular glutathione content and activities of GGT and GST (2-5). TGF-α expression and secretion is common in these chemically transformed rat liver epithelial cells (6) and its level of expression appears to correlate well with the tumorigenicity of these cells. This is especially so when there is an overexpression of the proto-oncogene c-myc, suggesting an important mechanistic role for this growth factor in the neoplastic transformation of these cells (7).

During our previous studies using the carcinogen-treated clones of cultured rat liver epithelial cells on the co-segregation of paratumorigenic phenotypes and tumorigenicity (7, 8), we were concerned with the possibility that repeated chemical carcinogen treatments would induce an "abundance" of genetic lesions and aberrant phenotypic expressions that were epiphenomena to the critical mechanism(s) of neoplastic transformation. However, these changes would greatly complicate the mechanistic studies of transformation. The fact that repeated treatments with MNNG introduced progressively greater heterogeneities in karyotypic changes and phenotypic expressions (1), most of which except anchorage-independent growth capacity and TGF-α expression were poorly correlated with tumorigenicity, supports this hypothesis. We have subsequently found that, using a "selective" culture condition, normal cultured rat liver epithelial cells can be consistently induced to undergo neoplastic transformation spontaneously or after only a single treatment with MNNG (9). With the hypothesis that spontaneous transformation would greatly reduce the number of undesired epiphenomena of neoplastic transformation induced by repeated carcinogen treatments, we have undertaken further studies into the phenotypic expression of spontaneously transformed clones of cultured rat liver epithelial cells. The results indicate that phenotypic heterogeneity and diversification is a phenomenon inherent to both spontaneous and chemically induced neoplastic transformation; however, consistent differences in some of their phenotypes may be representative of the difference(s) in the molecular pathogenesis of the two types of transformation system.

MATERIALS AND METHODS

Cell Strains/Cell Lines. Four cell strains (Ei1, Ei6, Er1, Er4) cloned from an early-passage normal rat liver epithelial cell line (10) were transformed spontaneously using the protocol of "selective" culture condition (9). A fifth cell strain, Ei7, transformed spontaneously when it was not routinely subcultured before reaching confluence. Spontaneously transformed tumor cell lines were derived from tumors formed in syngeneic animals after injection of these cell strains, when they demonstrated anchorage-independent growth capacity in soft agar, into 1-day-old Fischer 344 rats. Chemically transformed tumor cell lines were derived from tumors formed by clones of WB-F344 normal cultured rat liver epithelial cells that have been subjected to 11 episodic treatments with MNNG. The transformation protocol and phenotypes of these MNNG-induced tumor cell lines have been reported previously (1, 2, 4-6).

Although derived from two different animals, RL-F344-1 (10) and WB-F344 (11) cell strains share many common features and are considered identical phenotypically. They were both derived from the livers of adult (6- to 8-week old) male Fischer 344 rats using the trypsin digestion method of Williams et al. (12). Both cell lines express identical lactate dehydrogenase and aldolase isoenzymes, have low levels of α-fetoprotein and albumin mRNA and low GGT activity, and appear to be identical ultrastructurally.

Cells were routinely cultured in Richter's IMEM-ZO medium without insulin (Irvine Scientific, Santa Ana, CA) but supplemented with 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 2.6 mM sodium bicarbonate, 40 μg/ml gentamycin, and 10% fetal bovine serum (GIBCO Laboratories, Grand Island, NY).

Selective Culture for Induction of Spontaneous Transformation. The methodology has been previously reported in detail (9). Briefly, each cycle of selective culture consisted of seeding 2 × 10^6 cells in 100-mm tissue culture plates (Corning) in 10 ml culture medium. The cells were
allowed to grow to confluence in 7–10 days, after which the medium was replaced with fresh medium every week for the next 4 weeks. The cells were then dissociated with Ca²⁺-Mg²⁺-free Hanks' balanced salt solution containing 0.1% trypsin and 1 mM EDTA, and a new cycle of selective culture was initiated. At the end of each cycle, cells were also tested for anchorage-independent growth in 0.3% soft agar medium, at a density of 5 × 10⁴ cells/60-mm plate. One day later, each group of triplicate plates was supplemented with medium containing serum only or with medium containing serum plus EGF at a final concentration of 10 ng/ml. Colonies were counted as described previously (13).

Previous studies (9) have indicated that, when cells start to show colony-forming ability in agar containing EGF, imminent tumorigenic transformation usually occurs. When this was observed, the cells of the following cycle were tested for tumorigenicity by injection of 2 million cells into the s.c. tissue of each 1-day-old syngeneic Fischer 344 rat. Animals were sacrificed when tumors reached 1–2 cm in diameter or at the end of 8 months. Parts of the tumors were either frozen at −80°C, fixed in 2% glutaraldehyde for electron microscopic studies, or fixed in 10% buffered formalin for routine hematoxylin-eosin sections. Parts of some tumors were also explanted following collagenase digestion to obtain tumor cell lines.

Enzyme Assays. Assays for the activities of GGT and GST were performed as previously described (2, 5).

MNNG Toxicity. Two hundred cells in 10 ml medium were seeded into 100-mm tissue culture plates. Two days later, MNNG dissolved in dimethyl sulfoxide was added to a final concentration of either 1 or 5 µg/ml. The medium was replaced 24 h later with fresh medium and the cells were allowed to form colonies during the next 10–14 days. Colonies were stained with 4% giemsa and counted. Colonizing cells that survived the MNNG treatment were expressed as the percentage of colonies that formed in control plates, which had been treated with solvent only. Triplicate plates were used for each treatment group.

RNA Isolation and Northern Blot Hybridization Studies. These studies were performed according to the methods previously described in detail (5). Total cellular RNA was extracted from two plates of confluent culture of each cell line, as described by Chirgwin et al. (14). Thirty µg of each RNA sample were resolved in a 1% formaldehyde-agarose gel, transferred to a Hybond-N nylon membrane (Amersham Canada, Oakville, Ontario), and hybridized to the appropriate ³²P-labeled cDNA probes using the procedures of Church and Gilbert (15).

cDNA Probes. The following plasmids or inserts were used as cDNA probes: Saffi-EcoRI insert of pGP5 for the Yp subunit of rat GST (16); PstI insert of pGTA/c44 for the Yb subunit of rat GST (17); 2.3-kilobase EcoRI insert of prTGFr2 for rat TGF-α (18); 2.4-kilobase ClaI insert of pE7 for the EGF receptor (19); 752-base pair PstI-Xmal fragment of v-Ha-ras insert in HB-11 plasmid for Ha-ras (20); 600-base pair SstI-PstI fragment of v-Ki-ras insert in KBE-2 plasmid for Ki-ras (20); and chicken actin cDNA (Oncor, Gaithersburg, MD) for actin. The cDNA probes were labeled with [³²P]βCTP (ICN Canada, St. Laurent, Quebec) to high specific activities, using the Oligolabelling kit of Pharmacia (Dorval, Quebec).

Growth Responses to EGF/TGF-α and TGF-β. Tumor cells (10⁵ or 5 × 10⁴) were seeded in 0.3% soft agar medium, and 24 h later, fresh medium containing serum alone or with EGF, TGF-α, or TGF-β at final concentrations of 10, 10, and 1 ng/ml, respectively, were added. Colonies were allowed to form during the next 4 weeks and were then stained and counted as described previously (6).

Statistical Analysis. All enzymatic assays and soft agar colony-forming efficiency experiments were performed using triplicate samples or plates and the statistical significance was evaluated using Student's t test.

RESULTS

When the four clones of normal rat liver epithelial cells were subjected to the selective culture condition, spontaneous neoplastic transformation invariably occurred (Table 1). Two of the cell strains (Er1 and Er4), whose growth in serum-containing medium is stimulated by EGF (10), transformed after only the third cycle of prolonged maintenance in confluent culture. The other two cell strains (Ei1 and Ei6), whose growth in serum-containing medium is initially inhibited by EGF, trans-
The collection of spontaneously transformed tumor cell lines derived from clones of RL-F344-1 and chemically transformed tumor cell lines derived from clones of MNNG-treated WB-F344 were used to compare the phenotypic expression of these two types of neoplastic cells.

We first compared the expression of the "resistant phenotypes" in these cells. In contrast to the chemically induced tumor cell lines, which expressed high activities of GGT, spontaneously induced tumor cell lines expressed low activities of GGT (Table 3). Likewise, the specific activities of GST of spontaneously transformed tumor cells were not elevated, as compared to their normal parental cells. In contrast, chemically induced tumor cells expressed significantly elevated GST activity when compared to their normal parental cells (Table 3). The differences in these biochemical findings were also reflected in the susceptibility of these cells to the toxicity of MNNG. Treatment with 1 µg/ml MNNG resulted in a significantly greater loss of colony-forming cells in spontaneously transformed than in chemically induced tumor cells (Fig. 1).

We studied further the mRNA expressions of the GST isoenzyme subunits Yb and Yp, c-Ha-ras, c-Ki-ras, EGF receptor, and TGF-α in these tumor cells during confluent culture. Among several tumor cell lines derived from a single clonal normal cell strain (E11 and Er1), heterogeneity in the expression of these phenotypes was evident (Figs. 2 and 3). Heterogeneity in the expression of growth factors and their receptors was also reflected in the heterogeneous responses of tumor cells to the proliferative effects of EGF/TGF-α and TGF-β. In general, EGF/TGF-α stimulated the colony-forming efficiency in soft agar (10 of 12 or 83%), while TGF-β suppressed it (8 of 12 or 67%) (Table 4). EGF and TGF-α at equiconcentration yielded similar levels of colony growth stimulation, in terms of either colony number or size (data not shown). None of the tumor
Table 4  Effects of EGF and TGF-β on the growth of soft agar colonies of spontaneously induced rat liver epithelial tumor cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>CFE*</th>
<th>Increased by EGF (10 ng/ml)</th>
<th>Decreased by TGF-β (1 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ei1C8TA</td>
<td>3.94</td>
<td>+ (2.39)</td>
<td>− (1.06)</td>
</tr>
<tr>
<td>Ei1C8TC</td>
<td>4.20</td>
<td>+ (1.79)</td>
<td>+ (0.69)</td>
</tr>
<tr>
<td>Ei1C8TD</td>
<td>0.29</td>
<td>+ (11.10)</td>
<td>+ (0.27)</td>
</tr>
<tr>
<td>Ei1C8TF</td>
<td>0.22</td>
<td>+ (2.18)</td>
<td>+ (0.05)</td>
</tr>
<tr>
<td>Er6C5TA</td>
<td>1.17</td>
<td>+ (2.83)</td>
<td>+ (0.63)</td>
</tr>
<tr>
<td>Er1C3TD</td>
<td>1.86</td>
<td>+ (3.20)</td>
<td>+ (0.42)</td>
</tr>
<tr>
<td>Er1C3YH</td>
<td>0.58</td>
<td>− (0.98)</td>
<td>− (0.89)</td>
</tr>
<tr>
<td>Er1C5YH</td>
<td>1.60</td>
<td>+ (3.95)</td>
<td>− (0.88)</td>
</tr>
<tr>
<td>Er1C5TA</td>
<td>0.91</td>
<td>+ (4.55)</td>
<td>+ (0.67)</td>
</tr>
<tr>
<td>Er1C6TB</td>
<td>2.15</td>
<td>+ (1.24)</td>
<td>− (0.77)</td>
</tr>
<tr>
<td>Er4C4TA</td>
<td>4.10</td>
<td>− (1.12)</td>
<td>+ (0.65)</td>
</tr>
<tr>
<td>Er7TA</td>
<td>0.34</td>
<td>+ (2.06)</td>
<td>+ (0.67)</td>
</tr>
</tbody>
</table>

* The plating density was 10^4 cells/60-mm plate and assays were always performed using triplicate plates. The colony-forming efficiency (CFE) indicates the number of colonies as a percentage of the number of cells seeded. +, significant increase or decrease in colony-forming efficiency occurs; −, no significant response. The values in parentheses indicate the ratio of the colony-forming efficiencies between the growth factor-treated plates and the control plates.

Fig. 3. Heterogeneity in the phenotypic expressions among cell lines derived from four different tumors produced by transformed Ei cells after eight cycles of selective culture. Yb and Yp represent subunits of the GST enzyme. Thirty μg of total cellular RNA are used in each lane.

Fig. 4. The expression of the EGF receptor (EGFR) and TGF-α gene in the parental Er4 cell strain and a cell line derived from one of the tumors produced by the transformed Er4 cells after the fourth cycle of selective culture. This tumor cell line expresses abundant TGF-α mRNA but very little message for EGF receptor. Its colony-forming efficiency in soft agar is also nonresponsive to the presence of exogenously added EGF or TGF-α.

Er4C4 cells also showed focal areas of intestinal glandular cell differentiation (Fig. 5d). Ultrastructurally, cells in poorly differentiated areas contained moderately abundant dilated rough endoplasmic reticulum and mitochondria and formed numerous bile canalicule-like intercellular lumens (Fig. 6a), suggesting their hepatocytic differentiation, while columnar cells that

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Unpublished data.

* Unpublished data.
Fig. 5. The representative histological appearances of tumors produced by spontaneously transformed rat liver epithelial cells. a. Most of the tumors consist of anaplastic carcinoma showing sheets of epithelioid cells. b. Focal areas of tumors show glands formation which indicate that they are poorly-differentiated adenocarcinoma. c. Some tumors, especially those produced by the E11 and E16 cell strains, show significant host fibroblastic reaction and stromal fibrosis (desmoplasia). d. Tumors derived from the spontaneously transformed E14 cell strain contain areas of infiltrating glands lined by columnar cells with periodic acid-Schiff-stained apical brush border, which suggests intestinal epithelial cell differentiation. a. b. and c. H & E stain; d. PAS stain. × 500.

DISCUSSION

In this report we have confirmed previous findings (9, 21) that subjecting cells to repeated cycles of prolonged confluent culture (selective) periods may consistently induce spontaneous transformation. We have previously reported (9) that spontaneous transformation occurs more rapidly in WB-F344 normal rat liver epithelial cells that were grown in selective conditions than cells that were always subcultured soon after they became confluent (nonselective condition). A single treatment with the carcinogen MNNG further reduced the number of cycles required before the emergence of transformed cells. Using clonally derived parental normal cells, we have also demonstrated that cells which are stimulated in their proliferation by EGF transformed earlier than cells whose proliferation is suppressed by EGF. It is interesting to note that the EGF-suppressed early-passage normal cultured rat liver epithelial cells may lose their susceptibility to the EGF-induced growth suppression after being maintained in a prolonged confluent culture period (10), thus suggesting a possible similarity in the mechanisms that facilitate spontaneous transformation and that modify cellular responsiveness to the EGF growth effect during such culture conditions. Results of preliminary studies indicate that a prolonged confluency period both facilitates the selective/preferential accumulation of “transforming” cells and induces the progressive expression of the “transformed” phenotypes.

Our studies of the phenotypic expression by both the spontaneously and chemically induced malignant rat liver epithelial tumor cell lines have demonstrated both commonality and diversity. Tumor cells derived using both methods of transformation show extensive phenotypic heterogeneity in the expressions of isoenzymes, proto-oncogenes, and growth factors and their receptors and in cellular responses to the effect of growth factors. These results should not be surprising, since numerous studies (reviewed in Refs. 22 and 23) have indicated that heterogeneity in phenotypic expressions is common in both experimentally induced animal tumors and naturally occurring human neoplasms and that the generation of phenotypic and karyotypic instability and diversification is inherent to and probably essential in the process of carcinogenesis and neoplastic progression.

Although spontaneous transformation does not appear to simplify the problem of phenotypic heterogeneity in the mechanistic studies of neoplastic transformation of these rat liver epithelial cells, the results of this study suggest that at least two differences may distinguish the phenotypic expressions in spontaneous versus chemically induced neoplastic rat liver epithelial cells. The lack of induction of the resistant phenotypes characteristic of chemically induced or promoted neoplasms in these
spontaneously transformed cells suggests that the expression of such phenotypes is not universal in all neoplasms and is highly dependent on the method used to induce the neoplasms. There exist at least two in vivo models of carcinogenesis which corroborate our in vitro findings. In rat hepatocarcinogenesis studies using the nonmutagenic peroxisome proliferators, the pre-neoplastic and neoplastic lesions that result also lack the expression of the resistant phenotypes (24, 25). In the mouse mammary carcinogenesis model, the preneoplastic hyperplastic alveolar nodules and carcinomas induced by a single treatment with 7,12-dimethylbenzanthracene and promoted by growth factors released by pituitary isograft also fail to express the resistant phenotypes (26). Further studies using experimentally induced tumors or human cancers of various organs are required to confirm our hypothesis that the expression of the resistant phenotypes or the lack of it in naturally occurring human tumors may provide clues as to their etiology and pathogenesis.

The apparent dissociation between the functional expression of TGF-α and EGF receptor expression in these spontaneously transformed rat liver epithelial tumor cells superficially does not support an "autocrine" role for TGF-α in the growth of these cells. However, the possibility that the expression of in vitro phenotypes may be modulated when the cells are growing in vivo requires that in situ localization studies for the expression of these growth factor/receptors on the tumor tissues be carried out before a definitive conclusion can be reached. In
previous studies (6, 7), we have reported close correlation between the expression of TGF-α and tumorigenicity in clones of MNNG-transformed rat liver epithelial cells, suggesting an autocrine role of TGF-α in the tumorigenesis of these chemically transformed cells. This relationship is especially strong when c-myc is also overexpressed. Although all of the spontaneously transformed tumor cell lines reported here express TGF-α to various degrees, none of them were found to overexpress the c-myc proto-oncogene (data not shown).

The pattern of growth responses to EGF/TGF-α and TGF-β, when growing in soft agar medium, by spontaneously or chemically induced malignant rat liver epithelial tumor cell lines appears similar. Most of these tumor cells are stimulated by EGF/TGF-α in their soft agar colony-forming efficiency. In contrast, TGF-β may suppress or have no effect on their growth and none of the spontaneously induced tumor cells are stimulated by TGF-β.

The cellular, biochemical, or molecular basis for the model of rat liver epithelial carcinogenesis that we describe is completely unknown but deserves further study. In the classical concept of multistage carcinogenesis, the unknown “initiation” step may possibly be represented by a rare spontaneous genetic change, such as mutation in a proto-oncogene or suppressor “anti-oncogene” or a critical karyotypic alteration, which creates “promotability” and response modification in these initiated cells (27). Both the prolonged period of confluency and the periodic bursts of cell proliferation after each subculture that characterize this transformation protocol may play important roles in the promotion of these altered cells. Prolonged suppression of cellular proliferation in normally highly proliferative cultured cells may induce alteration in the expression of growth factors, in the paracrine responses to these growth factors, or in junctional cell to cell communications. Characterization of the phenotype and genotype of the early transforming cells as represented by soft agar-colonizing cells may further elucidate the mechanism of this spontaneous transformation. The model may also be used to study the effect and role of response modifiers such as hormones or growth factors in the promotion of neoplastic transformation of cultured rat liver epithelial cells.

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REFERENCES

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