ras Transformation of Simian Virus 40-immortalized Rat Hepatocytes: An in Vitro Model of Hepatocarcinogenesis

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ABSTRACT

Primary rat hepatocytes were transfected with simian virus 40 DNA and cultured in a chemically defined medium. Proliferating colonies developed after 2–3 weeks. Three cell lines were established by cloning albumin-secreting colonies, as identified by an immunooverlay assay. Two of the cell lines, ALB-6 and ALB-8, expressed all five liver-specific mRNAs studied, albumin, α-1-antitrypsin, fibrinogen, α-1-acid glycoprotein, and histidase. ALB-6 cells were non-tumorigenic in nude mice while ALB-8 cells were weakly tumorigenic with only one of four injected nude mice developing a slowly growing tumor. Further transfection of ALB-6 and ALB-8 cells with an activated c-Ha-ras or N-ras oncogene resulted in strongly tumorigenic cells. The tumors induced by ras-transformed ALB-6 cells were moderately differentiated hepatocellular carcinomas. The tumors derived from ras-transformed ALB-8 cells were poorly differentiated, while the slowly growing tumors induced by untransformed or control DNA-transfected ALB-8 cells were well-differentiated trabecular hepatocellular carcinomas, suggesting histological dedifferentiation of cells following ras transformation. However, the synthetic capabilities of the tumors of the ras-transfected cultures and the tumors induced by ras-transformed cells retained the ability to synthesize the five liver-specific mRNAs. Thus we have developed an in vitro model of carcinogenesis in which, by sequential exposure to SV40 DNA and a ras oncogene, primary rat hepatocytes are transformed.

INTRODUCTION

Activated protooncogenes have been identified in some human and rodent liver malignancies. Members of the ras family are the most frequently detected (1, 2). The presence of altered ras genes in tumor cells, however, does not prove a causal relationship. One approach to define the role of mutated ras oncogenes in carcinogenesis has been to study the oncogenic effect of transfected ras genes on cultured cells. Neoplastic transformation of established rat liver epithelial cell lines with activated ras oncogenes has been reported (3, 4). These rat liver epithelial cells are poorly differentiated cell lines, derived from long-term liver cell cultures (5, 6). Since their in vivo counterparts have not been clearly established, the relevance of these cells to the process of hepatocarcinogenesis is uncertain. Some rat liver epithelial cell lines are thought to be derived from “oval” cells (7, 8), proposed hepatic stem cells. Braun et al. (9) recently reported transformation of two oval cell lines by an activated c-Ha-ras oncogene into malignant cells which formed differentiated HCC in nude mice.

Although hepatocytes are thought to be the target cells for most chemical carcinogens and as such may also be precursors for HCC (10), the limited capacity of differentiated hepatocytes to proliferate in culture has precluded their use in assessment of transforming effect of protooncogenes. Recently it has become possible to establish rat or mouse cell lines which resemble the phenotype of differentiated hepatocytes in continuous culture by using viral oncogenes from SV40 (11–13) or adenovirus (14). Although they eventually become highly malignant after repeated passaging, these SV40-immortalized hepatocyte cell lines are nontumorigenic or only weakly tumorigenic at early passage (12, 13). Similarly, transgenic mice harboring SV40 transforming sequences did not develop tumors rapidly but rather showed a stepwise neoplastic progression in vivo, from normal liver to HCC (15–17), analogous to the process of chemical hepatic carcinogenesis. These observations suggest that, in addition to the SV40 T-antigen, other cellular event(s) occurring during in vitro/in vivo passages are involved in the complete transformation of hepatocytes. Therefore, early passages of SV40-immortalized hepatocyte cell lines are potentially useful in the study of mechanisms behind the hepatic malignant progression and to assess the transforming effect of protooncogenes on differentiated hepatocytes.

In this report, we have evaluated the ability of an activated ras oncogene to transform SV40-immortalized, differentiated rat hepatocytes and the effect of ras transformation on the differentiation of these hepatic cells. Hepatocyte cell lines were established by transfection of primary rat liver cells with SV40 DNA. These cell lines were further transformed into a frankly malignant phenotype using an activated c-Ha-ras or N-ras oncogene. We have thus established an in vitro model of carcinogenesis in differentiated rat hepatocytes.

MATERIALS AND METHODS

DNA. pGEM4-SV40 was constructed by ligating EcoRI-digested SV40 genomic DNA (American Type Culture Collection, Bethesda, MD) into the unique EcoRI site of pGEM4 (Promega Biotech, Madison, WI). The plasmids pEJ6.6, pSV2neo, and pSVneo-N-ras were kindly provided by Dr. A. Pawson (University of Toronto, Toronto, Ontario, Canada). pEJ6.6 is a pBR322 derivative which contains an activated c-Ha-ras 6.6-kilobase oncogene from the human EJ bladder carcinoma cell line (18). pSV2neo is a modified pBR322 vector containing the neo gene which confers resistance to the antibiotic G418 (19). pSVneo-N-ras is a pSV2neo derivative which carries the activated N-ras oncogene isolated from the human SK-N-SH neuroblastoma cells (20).

The DNA probes used to analyze cellular mRNA included complementary DNA of five liver proteins: rat albumin (21) (a gift of Dr. M. Zern, Brown University, Providence, RI); mouse AAT (22) (a gift of Dr. D. Wilson-Cox, University of Toronto); mouse fibrinogen (22); rat AAGP (23) (gifts of Dr. D. Shafritz, Albert Einstein College of Medicine, New York, NY); and rat histidase (24) (a gift of Dr. R. R. McInnes, University of Toronto); and the complementary DNA of chicken β-actin (25) (a gift of Dr. D. Drucker, University of Toronto). The structures of the above plasmids were verified by digestion with appropriate restriction enzymes. The inserts were isolated from agarose gels by Gene Clean (Bio/Can Scientific, Inc., Mississauga, Ontario, Canada) and labeled with [32P]dATP or [32P]dCTP by random primer extension.
Primary Rat Hepatocytes. Hepatocytes were isolated from adult male Fischer 344 rats (180–220 g) by in situ collagenase perfusion essentially as described by Berry and Friend (26). Following perfusion, cells were suspended in a defined William's E medium (27) supplemented with 10% FCS. Only cell preparations of greater than 85% viability, as determined by trypan blue exclusion, were used for subsequent transfection experiments and for RNA isolation.

Transfection. DNA was introduced into cells by electroporation (27). Plasmids pGEM4, pGEM4-SV40, pSV2neo, pEJ6.6, and pSVneo-N-ras were linearized with appropriate restriction enzymes. Approximately 1 x 10^6 cells, suspended in 0.6 ml phosphate-buffered saline, were electroporated with 40 µg DNA. Since pEJ6.6 did not contain the neo gene as a selective marker, cells were cotransfected with 60 µg pEJ6.6 and 10 µg pSV2neo (molar ratio, 3:1). This molar ratio was chosen to ensure that the majority of resultant G418-resistant colonies carried both the neo and the c-Ha-ras genes (28).

Transfected cells were plated in RPMI 1640 supplemented with 5% FCS at 1 x 10^4 cells/60-mm dish. Culture dishes were precoated with collagen (Collagen Corp., Palo Alto, CA). At 12 h, the medium was changed to a CDM (11) supplemented with 20 ng/ml EGF (Boehringer Mannheim, Laval, Quebec, Canada). Two days after transfection with plasmids carrying the neo gene, G418 (300 µg/ml; GIBCO, Burlington, Ontario, Canada) was added to cultures for selective growth of transformants.

Immunofluorescence assay. Immunofluorescence analysis of albumin secretion by SV40-immortalized colonies was carried out as described by Sammons et al. (29). Goat anti-rabbit albumin antibody and rabbit anti-goat IgG (Cappel Laboratories, Inc., Cochraneville, PA) were both used at a 1:64 dilution to distinguish albumin-producing colonies from non-albumin-producing colonies.

Establishment and Culture of Cell Lines. To establish SV40-immortalized, differentiated hepatocyte cell lines, discrete colonies of albumin-producing cells, identified by the immunofluorescence assay, were isolated by ring subcloning. Cloned cells were expanded on collagen-coated plates in CDM plus EGF to the desired numbers of passages. Cells were always plated initially in RPMI 1640 with 5% FCS to improve efficiency of attachment. After 4–6 h, the medium was changed to CDM plus EGF.

As controls for Northern blot analyses of liver-specific mRNAs, the Morris rat hepatoma cell line, McA-RH7777 (American Type Culture Collection) (30), and a rat glomerular mesangial cell line, kindly provided by Dr. K. Skorecki (University of Toronto) (31), were cultured under recommended conditions.

Immunostaining for SV40 T-Antigen. Indirect immunofluorescence staining for the SV40 T-antigen was performed according to the method of Banerji et al. (32). A monoclonal antibody raised against the SV40 T-antigen was selectively reactive with SV40-transformed cells. The monoclonal antibody was raised against a CDM plus EGF-stained cell line derived from the 14–15th passage were assessed for tumorigenicity in athymic nude mice (Table 1). ALB-6 cells were nontumorigenic in nude mice. ALB-8 was weakly tumorigenic in that one of four injected mice developed a tumor after a latent period of 12 weeks. The tumor grew slowly and had not reached 1 cm in diameter by 18 weeks after injection.

Transformation by an Activated ras Oncogene (c-Ha-ras and N-ras). Having established that ALB-6 and ALB-8 were well-differentiated lines which were either weakly tumorigenic or nontumorigenic in nude mice, we further transfected these two cell lines with an activated c-Ha-ras or N-ras oncogene to evaluate the oncogenic effect of an activated ras oncogene on these two lines. ALB-6 and ALB-8 cells at the 11th–12th passage were transfected with pSVneo-N-ras or cotransfected with pEJ6.6 and pSV2neo. Control cultures were transfected with pSV2neo alone. G418-resistant colonies were visible 3–4 weeks posttransfection. There was no morphological difference observed between resistant colonies derived from pSV2neo-transfected and from ras-transfected cultures (data not shown).
ras TRANSFORMATION OF SV40-IMMORTALIZED RAT HEPATOCYTES

transfected ALB-6 cells induced tumors in 3 of 4 mice and pSVneo-N-ras-transfected ALB-6 cells formed tumors in all 4 animals. Like untransfected ALB-6 cells, pSV2neo-transfected ALB-6 cells were nontumorigenic 20 weeks after injection. After ras transformation, ALB-8 cells became strongly tumorigenic. All mice grew tumors 2–3 weeks after inoculation of either pEJ6.6- or pSVneo-N-ras-transfected ALB-8 cells. These tumors reached 1 cm in diameter within 4–6 weeks. pSV2neo-transfected ALB-8 cells showed an oncogenic potential similar to that of the untransfected ALB-8 cells at the 14th-15th passage. Small tumors developed in 2 of 7 injected animals after a latent period of 11–13 weeks.

Expression of Introduced ras Oncogenes in the ras-transformed Cells/Tumors. Northern blot analysis of RNA isolated from passaged cultures of pooled G418-resistant colonies developing

Pooled G418-resistant colonies were expanded for 3 more passages and then used for the tumorigenicity assay and for RNA analyses. Woodworth et al. (12) have previously shown that high passages of SV40-immortalized hepatocyte cell lines were strongly tumorigenic. To avoid the influence of passaging on the tumorigenicity of cells, we chose to use pooled G418-resistant colonies, instead of individual colonies, to limit the number of passages required to obtain sufficient cells for further experiments.

As outlined in Table 1, both cell lines were efficiently transformed by the activated c-Ha-ras or N-ras oncogene. pEJ6.6-

### Table 1 Tumorigenicity of SV40-immortalized, well-differentiated rat hepatocyte cell lines in nude mice before and after transformation by activated ras oncogenes

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Plasmids</th>
<th>Tumor frequency</th>
<th>Latency</th>
<th>Growth rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALB-6</td>
<td>Untransfected</td>
<td>0/4</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>pSV2neo</td>
<td>0/4</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>pSV2neo + pEJ6.6</td>
<td>3/4</td>
<td>6–9</td>
<td>8–13</td>
<td></td>
</tr>
<tr>
<td>pSVneo-N-ras</td>
<td>4/4</td>
<td>4–5</td>
<td>6–8</td>
<td></td>
</tr>
<tr>
<td>ALB-8</td>
<td>Untransfected</td>
<td>1/4</td>
<td>12</td>
<td>&gt;18</td>
</tr>
<tr>
<td>pSV2neo</td>
<td>2/7</td>
<td>11–13</td>
<td>&gt;18</td>
<td></td>
</tr>
<tr>
<td>pSV2neo + pEJ6.6</td>
<td>4/4</td>
<td>2–3</td>
<td>4–6</td>
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<tr>
<td>pSVneo-N-ras</td>
<td>4/4</td>
<td>2–3</td>
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* Interval (weeks) between injection of cells into nude mice and the first appearance of a discrete nodule.

* Time (weeks) required for a tumor to reach 1 cm in diameter.

* N/A, not applicable.
after transfection with the control plasmid pSV2neo and with ras plasmids, and from tumors derived from ras-transformed cells, showed moderate expression of c-Ha-ras or N-ras in the ras-transformed cells and higher expression in the corresponding tumors (Fig. 3). The apparent difference between the cells and the tumors in the expression level may reflect up-regulation of ras genes in vivo or a selective growth of cells overexpressing ras genes in tumors. Weak expression of the endogenous c-Ha-ras was observed in pSV2neo-transfected ALB-8 but not in ALB-6 cells. Transcripts of the endogenous N-ras oncogene were not detected in either cell line after transfection with pSV2neo. The differential expression of c-Ha-ras and N-ras in the pSV2neo-transfected cells compared to the ras-transformed cells/tumors indicated that the foreign ras oncogenes were being expressed and were responsible for the acquisition of tumorigenicity.

Tumor Histology. The tumors induced by ras-transformed ALB-6 cells were moderately differentiated HCCs with some areas forming trabecular patterns and others composed of less differentiated epithelial cells (Fig. 4, A and B). As shown in Table 1, untransfected and pSV2neo-transfected ALB-8 cells were weakly tumorigenic. The resultant tumors showed the histological features of a well-differentiated trabecular HCC. The individual cells throughout the tumors resembled normal hepatocytes in that they were cuboidal, with large, rounded nuclei and abundant cytoplasm. The cells were organized into mono- or multilayered cords which were lined by endothelium (Fig. 4C). After ras transformation, however, ALB-8 cells produced rapidly growing tumors. These tumors were consistently poorly differentiated HCCs composed of sheets of epithelial cells with few architectural features (Fig. 4, D and E), suggesting that histological dedifferentiation had occurred following ras transformation.

Expression of the Liver-specific Markers in the ras-transformed Cells/Tumors. To investigate whether ras expression or ras-mediated transformation extinguished the expression of liver-specific proteins in cells, we compared the expression of the five liver-specific markers between pSV2neo- and ras-transformed cultures by Northern blot analysis of RNA isolated from passaged cultures of the pooled G418-resistant colonies developing after transfection with pSV2neo [ALB-6 (Lane 1) and ALB-8 (Lane 4)] and with pSV2neo+peJ6.6 (ALB-6 (Lane 2) and ALB-8 (Lane 3)], and from tumors induced by pEJ6.6-transformed cells [ALB-6 (Lane 3) and ALB-8 (Lane 6)]. The c-Ha-ras-specific probe was the 2.9-kilobase (kb) SacI-Sal fragment of pEJ6.6 (18). At right, the order of RNA samples is the same as at left except that RNA was extracted from pSV2neo-N-ras-transformed cells/tumors instead of pEJ6.6-transformed cells/tumors. The N-ras-specific probe was a PnuI-PvuII fragment (p52c-5) (49) isolated from pSVneo-N-ras. The sizes of the two major transcripts (c-Ha-ras, 1.4 and 4.4 kilobases; N-ras, 2.2 and 5.2 kilobases) were measured by comparison with the locations of 28S and 18S RNA (indicated at the right of each panel) and were labeled at the left of the panels.

DISCUSSION

In this study, we have established an in vitro model of carcinogenesis in which primary rat hepatocytes were first immortalized by SV40 DNA and then fully transformed either by an activated c-Ha-ras or by N-ras oncogene. The target cells retained the ability to express the liver-specific genes albumin, A1AT, fibrinogen, A1AGP, and histidase, during immortalization and the subsequent transformation. The present work is consistent with a previous abstract by Meng et al. (34) about transformation of SV40-immortalized hepatocytes with c-Ha-ras oncogene. In their study, only a single cell line and a single liver specific gene, albumin, were evaluated.

An advantage of the model system using SV40-immortalized cells over previous in vivo/in vitro models is that the target cells are clearly derived from hepatocytes. In our study, albumin mRNA levels in ALB-6 and ALB-8 cells are comparable to that in primary hepatocytes. Although a high percentage of oval cells have been shown to express albumin (35), only hepatocytes in the liver are known to produce large quantities of albumin. Furthermore, ALB-6 and ALB-8 express other liver-specific proteins evaluated although at lower levels compared to primary hepatocytes. It is clear that, like the SV40-immortalized cell lines described by Woodworth et al. (11, 12), ALB-6 and ALB-8 resemble primary hepatocytes in biochemistry more closely than any other previously established rat liver cell lines.

The limited capacity of primary hepatocytes to divide and to maintain their differentiated state has precluded the direct evaluation of the effects of oncogenic stimuli on hepatocytes. We know of no report of successful transformation of differentiated hepatocytes by direct exposure to a single oncogene. In our model, SV40 DNA was used to induce primary hepatocytes to divide continuously, thus conferring the potential to be transformed by an activated ras oncogene. Demonstration of the oncogenic effect of an activated ras oncogene on immortalized, well-differentiated hepatocytes raises the possibility that mutational activation of ras oncogenes in vivo could be a critical signal to actively proliferating hepatocytes.
The cooperative interaction between SV40 T-antigen and activated c-ras or v-ras oncogenes has been previously demonstrated to cause transformation of other cell types (36–39). For example, transformation of nonestablished rat embryo fibroblasts by SV40 DNA was greatly accelerated by cotransfection with Ha-ras (36). In another study by Hirakawa and Ruley (37), transformation of established rat embryo fibroblasts REF 52 by ras was dependent on a second oncogene, SV40 T-antigen or adenovirus E1a oncogene. Epithelial cells are in general more difficult to transform than fibroblasts. However, in response to the combined actions of SV40 T- and ras oncogenes, neoplastic transformation of human amniocytes (38) and uro-
epithelial cells (39) has been described.

It has been documented that conversion of SV40-immortalized rat or mouse hepatocytes into frankly malignant cells requires other undefined event(s) during in vitro/in vivo passaging (12, 13, 15–17). Woodworth et al. (12) reported that the malignant progression of SV40-immortalized rat hepatocytes during in vitro passaging was paralleled by enhanced expression of the c-Ha-ras oncogene. Transgenic mice harboring the SV40 T-gene under the control of liver-specific promoters showed progressive histological alterations in the liver (15–17). The development of neoplastic nodules and multiple macroscopic HCCs was preceded sequentially by megalocytic change and regenerative focal growth. The point mutation on codon 61 of c-Ha-ras has been recently identified in 10 of 25 HCCs from plastic liver tissue samples showed the mutated c-Ha-ras (40).

The fact that untransfected ALB-8 cells were weakly tumorigenic enabled us to compare histological appearance of tumors derived from ALB-8 cells before and after ras introduction. As illustrated in Fig. 4, the tumors developing from ras-transformed ALB-8 cells were less differentiated in comparison to the tumors from untransfected or pSV2neo-transfected ALB-8 cells. The alteration in morphology may be a reflection of the high degree of malignancy of ras-transformed cells.

Despite the poor or moderate degree of histological differentiation, the tumors from ras-transformed cells retained the capacity to synthesize liver-specific mRNAs. The maintenance of expression of these hepatic markers in tumors developing from ras-transformed cells suggested that the ability of cells to express the liver-specific genes examined were not lost during ras transformation. Our finding is consistent with two recent reports addressing the effect of c-Ha-ras oncogene on hepatic differentiation. By introduction of the activated c-Ha-ras oncogene into a human hepatoma cell line, HuH-7, Nakao et al. (45) showed that albumin gene expression was not blocked although the expression of α-fetoprotein was suppressed. The second report involved rat liver oval cell lines, transformation of which with the c-Ha-ras was paralleled by induction of some hepatocyte-specific antigens (46), suggesting that the expression of c-Ha-ras induced oval cells to differentiate into the hepatocyte lineage.

The successful transformation of hepatocytes by sequential exposure to the SV40 T- and the activated ras oncogenes suggests that this approach could be extended to study other recognized oncogenes, undefined transforming sequences, as well as chemical carcinogens. Because they expressed many hepatocyte-specific functions, SV40-immortalized hepatocyte cell lines will be particularly useful in evaluation of the oncogenic effect of liver-specific carcinogens, such as aflatoxin, on hepatocytes. Conversion of aflatoxin to its active form requires cytochrome P-450 enzymes present in differentiated hepatocytes (47). ALB-6 and ALB-8 cells may also be useful to study preservation of differentiation of primary granulosa cells.

Transformation by ras oncogenes could either induce or block differentiation depending on the genetic and developmental backgrounds of the cells (41–44). v-Ha-ras transformation caused dedifferentiation of terminally differentiated human bronchial epithelial cells (41) while transformation of a rat neuronal cell line (PC12) by v-Ha-ras induced the morphological and synthetic development to a differentiated sympathetic neuron (42, 43). In primary rat granulosa cells, Amsterdam et al. (44) found that cotransfection with SV40 DNA and an activated c-Ha-ras oncogene generated stable lines in which steroidogenesis could be induced. In contrast, transfection with SV40 DNA alone generated only undifferentiated clones. This finding suggests that expression of the c-Ha-ras oncogene favors
oncogenicity of HBV. It has been demonstrated recently that the MCA-RH7777 cells are competent for human HBV replication (48). Preliminary studies in our own laboratory have revealed that ALB-6 and ALB-8 cells are also capable of supporting HBV replication. We are currently testing whether the McA-RH7777 cells are competent for human HBV replication.4

ACKNOWLEDGMENTS

The authors wish to thank Y. Chen and Z. Liu for their technical assistance and Drs. I. Wanless and E. Farber for their comments and discussion about the tumor histology.

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