EJ/ras Neoplastic Transformation of Simian Virus 40-immortalized Human Uroepithelial Cells: A Rare Event

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ABSTRACT

To determine if expression of mutant p21 ras could convert Simian Virus 40-immortalized human uroepithelial cell line (SV-HUC) to tumorigenicity, SV-HUC cells were transfected with pSV2-neo (a neomycin-resistant gene) or PREJ/ras (c-HA-ras-1 with the 12th codon mutation and neo). Seven independent G418-resistant clones (A—»G) were isolated from each group (SV-HUC/ras and SV-HUC/neo). SV-HUC/neo clones were morphologically altered, while SV-HUC/neo clones retained a typical SV-HUC epithelial morphology. Electrophoretic analysis of immunoprecipitated ras proteins detected altered p21 ras protein in four of seven SV-HUC/neo clones at passage (P2) and in five of seven clones at P12 posttransfection. The relative levels of ras p21 differed among the clones and appeared to increase with passage in culture. RNA and DNA dot blot analyses showed that clones with more abundant mutant p21 also had higher ras RNA levels and, in one case, increased ras gene copy number. No altered ras protein was detected in any SV-HUC/neo clones. ras- and neo-transfected clones were tested for tumorigenicity at P2 posttransfection and again at P12 by four s.c. inoculations each into athymic nude mice. None of 56 inoculations of SV-HUC/neo clones, ras- and neo-transfected clones were tested for tumorigenicity at P2 posttransfection and again at P12 by four s.c. inoculations each into athymic nude mice. None of 56 inoculations of SV-HUC/neo clones, ras- and neo-transfected clones were tested for tumorigenicity at P2. Two ras-transfected clones, SV-HUC/ras-B and SV-HUC/ras-F, produced one tumor each. One clone, SV-HUC/ras-D which produced abundant mutant p21, was negative when inoculated at P2, but produced tumors in four of four sites when inoculated after ten passages in vitro. All tumorigenic clones had detectable levels of mutant ras p21. However, the relative levels of altered p21 ras protein among the SV-HUC/neo clones did not directly predict their tumorigenic potential, as several nontumorigenic SV-HUC/neo clones had protein levels equal to or higher than the most tumorigenic clone (SV-HUC/neo-D at P12). Cell lines established from the tumor explants exhibited higher ras gene copy numbers, higher RNA levels, and more abundant p21 than was seen in the clones at the time of inoculation. Therefore, increases in ras protein abundance occurred during tumor formation in vivo, as well as during passage of cells in culture, and such cells apparently had a selective growth advantage. However, expression of abundant mutant ras protein was not in itself sufficient for neoplastic transformation of SV-HUC. These data are consistent with a model of transformation in which mutant ras protein in combination with at least one additional, rare, and stochastically occurring event contributes to neoplastic transformation of SV-HUC and, in high abundance, provides cells with a selective growth advantage in vitro and in vivo.

INTRODUCTION

The high incidence of ras gene alterations in human and rodent tumors strongly implicates activation of these genes in mammalian cell carcinogenesis (1–7). However, the presence of altered ras genes in cancer cells does not per se prove a causal relationship. Therefore, numerous studies using diverse systems both in vivo and in vitro have attempted to address the role of ras gene alterations in multistep neoplastic transformation of mammalian cells.

An important approach in addressing this question has been to study the properties of cultured cells after transfection with ras genes (3). There are many reports of studies examining the characteristics of ras genes transfected into rodent cells in culture. Mutant ras can neoplastically transform some immortal cell lines, including NIH/3T3 and C3H/10T1/2 (8–11). However, mutant ras does not neoplastically transform all established cultures (12, 13), possibly reflecting important differences in the target cells. The quantity of mutant ras protein affects the transformed phenotypes (14–18). For example, mutant ras extends the life of diploid rodent cells in cultures, but results in neoplastic transformation only when expressed in high abundance (19). The presence of a second nuclear acting oncogene may cooperate with ras in transforming cells (20–24). For example, ras with myc or adeno-E1A neoplastically transforms diploid rodent cells, while ras alone is not transformed (20, 21, 23, 24). However, some evidence suggests that transformation of diploid rodent cells by two cooperating oncogenes may require an additional event. For example, tumorigenic transformation of diploid hamster cells by myc and ras is highly correlated with segregation of a specific chromosome (25). Normal ras protein, when expressed in high abundance, also has transforming ability for rodent cells, but this is limited compared with mutant ras (14, 19, 26).

In general, human cells, particularly epithelial cells, have been more difficult to transform in vitro than rodent cells (27). There are a few reports of neoplastic transformation of human epithelial cells by viral ras oncogenes. For example, human bronchial epithelial cells were transformed to tumorigenicity after transfection with v-Ha-ras (28). Human keratinocytes immortalized after infection with an adeno-12-SV40 hybrid and human amniocytes immortalized by SV40 converted to tumorigenicity after infection with KISV (29, 30), which contains a ras oncogene related to v-Ha-ras (31). Recently, it has been reported that human keratinocytes immortalized by HPV-16 DNA were converted to tumorigenicity after transfection with v-Ha-ras (32). These results taken together support a model in which mutant ras may be sufficient for transformation of certain immortalized human epithelial cells. In contrast, it has been reported that v-myc and v-ras do not transform human lymphocytes (33).

In this paper, we present the first example of transformation of immortalized human epithelial cells with a ras oncogene isolated from a human carcinoma, rather than from a retrovirus. For these studies, we have used the well-characterized ras oncogene from the EJ(T24) human bladder cancer cell line. EJ/ras carries point mutations at the 12th codon (9, 34) and in the last intron (35). These mutations are thought to be responsible for its transforming activity and increased gene expression. Significantly, we have for the first time transfected the EJ/ras

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3 The abbreviations used are: v-Ha-ras, Harvey viral ras; SV40, Simian Virus 40; KISV, Kirsten sarcoma virus; HPV-16, human papilloma virus 16; SV-HUC, SV40-immortalized human uroepithelial cell; HUC, human uroepithelial cell; P1, passage 1; P2, P12, and P100 defined similarly; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, fetal bovine serum.
gene back into a cell type from which it was originally isolated, namely, human uroepithelial cells. The cell line that was used is an SV40-immortalized-HUC (SV-HUC) that was clonal in origin and pseudodiploid (36, 37). We have previously shown that SV-HUC can be neoplastically transformed after treatment with the polycyclic hydrocarbon carcinogen, 3-methylcholanthrene (38), and/or the human bladder carcinogen, 4-amino-biphenyl (39). In this paper we present an analysis of the capability of the ras oncogene to neoplastically transform SV-HUC. These results show that expression of abundant mutant ras-encoded protein is insufficient to neoplastically transform SV-HUC but, in combination with an additional stochastically occurring event, contributes to neoplastic transformation.

**MATERIALS AND METHODS**

Cells and Culture Methods. A clonal line of SV-HUC was used in this study. The characteristics of SV-HUC have been described (36, 37). Briefly, SV-HUC was derived from a culture of normal HUC after infection at P1 with SV40. SV-HUC cells have remained nontumorigenic when tested in nude mice (>150 inoculations using 10^6 to 10^7 cells/site) after 80 serial passages and more than 2 yr in continuous culture. SV-HUC cells are 100% positive for human epithelial keratins and for SV40 T-antigen, but do not produce infectious SV40. Southern blot analysis shows a single integration site of SV40 in SV-HUC. Cytogenetic analysis shows that SV-HUC maintains a near balanced karyotype (37). In the present study, SV-HUC were grown on plastic dishes (Corning, Corning, NY) in 1% FBS-F12+, a supplemented medium developed for human uroepithelial cells (40). The basal medium contains the 6.6-kilobase pair BamHI restriction fragment of the mouse hepatitis virus RNA (MHV). The medium developed for human uroepithelial cells (40). The basal medium contains the 6.6-kilobase pair BamHI restriction fragment of the murine sarcoma virus (MSV). The medium for human uroepithelial cells (40) contains 100 mg/ml of streptomycin (Pfizer), and 1% FBS (HyClone, Logan, UT) added. Cultures were grown in humidified incubators at 37°C in

Analysis of p21 ras Proteins. Analysis of the electrophoretic mobility of immunoprecipitated ras proteins was performed according to the method of Srivastava et al. (45). Briefly, proliferating clones of pSV2neo- and PREJras-transfected SV-HUC were labeled for 4 h with 200 μCi per ml of [35S]methionine (1100 Ci/mmol; Amersham, Arlington Heights, IL). Labeled cells were then lysed and immunoprecipitated with antibody Y13-259 (Oncogene Science, Inc., Manhasset, NY) which binds to Ha-, K-, and N-ras-encoded p21. The immunoprecipitated samples were analyzed by 15% SDS-PAGE and fluorographed. To demonstrate that p21 with altered mobility could be detected in our gels, the T24 bladder carcinoma cell line, which has a mutation in the 61st codon of the c-Ha-ras gene resulting in increased mobility of p21, was used as control. Each dot blot was performed in triplicate, and the mobility of p21 was the same in all triplicate blots. The mobility of p21 was determined by scanning the blots with a densitometer and comparing the relative mobility of p21 to a standard curve prepared with p21 from the T24 cell line.

Karyotypic Analyses. Karyotypic analyses were performed using published techniques (37, 47) on cell lines initiated from tumor explants as soon as sufficient cells were available (P2). For comparison, kary-
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Typical analyses were also performed on the clones at the passages which resulted in tumor production. Samples of these clones were retrieved from the liquid N\textsubscript{2} freezer, cultured, and karyotyped as soon as possible. For each karyotypic analysis, 50 Giemsa-banded metaphase spreads were analyzed.

RESULTS

Clones of ras-transfected SV-HUC Show Morphological Changes. Seven independent G418-resistant clones (A—>G) of ras-transfected SV-HUC were isolated. Compared with the parental clonal cell line which shows a flat, tightly adherent epithelial morphology (Fig. 1a), SV-HUC/ras clones were loosely packed, rounded, and more refractile. SV-HUC/rai clones C and G also showed criss-crossing of cells with bipolar morphology (Fig. 1b). Thus, all ras-transfected cells showed morphological alterations. In contrast, seven independent G418-resistant neo-transfected SV-HUC clones showed morphologies indistinguishable from the parental SV-HUC.

Tumorigenic Transformation of SV-HUC after ras-Transfection Is Rare. The parental clonal SV-HUC cell line has remained nontumorigenic through 80 passages and more than 150 inoculation sites (33, 38, 40). The 7 independent neo-transfected SV-HUC control clones were inoculated at P2 and P12 into nude mice using 4 sites at each passage. All of the 56 control inoculation sites remained negative (Table 1). To determine if mutant ras could convert SV-HUC to tumorigenicity, seven independent clones (A—>G) of ras-transfected SV-HUC were isolated and inoculated into nude mice using 4 sites per group (Table 1). Control inoculations into mice were done using tumorigenic cell lines carrying mutant ras genes (HS242 and T24), and all gave tumors at 4 of 4 sites (2- to 6-wk latent periods). However, none of the seven ras-transfected clones gave tumors at all 4 sites of inoculation. One clone, SV-HUC/rai-B, gave one tumor from 4 sites injected after a 4-wk period. Another clone gave one tumor from 4 sites inoculated after a longer latent period of 20 wk. Neither of these clones produced tumors at any of the other 3 inoculation sites, and neither produced tumors when reinoculated at P12, suggesting that tumors obtained from the first inoculations resulted from rare transformants in the population. Cell lines initiated from these tumors all produced tumors at P2 with a short latent period at all 4 sites inoculated, which shows that the lack of tumors at all sites in the original inoculations was not related to insensitivity in the assay. One clone, SV-HUC/rai clone D which was negative for tumorigenicity at P2, produced tumors at 4 of 4 sites at P12, all with a short latent period. This result suggests that a neoplastic transformant which emerged in this clone between P2 and P12 became expanded during the period of cell culture, resulting in tumorigenicity at all sites. These results are consistent with a model in which neoplastic transformation of SV-HUC by EJ/ras requires a rare additional, randomly occurring event.

Although the latent periods to first appearance of tumors among the clones varied, once the tumors appeared, all grew rapidly and progressively, reaching approximately 1-cm diameter in size within 3 wk (Fig. 2a). Likewise, the histopathological characteristics of all the tumors were similar. These were classified as high-grade poorly differentiated transitional cell carcinomas (Fig. 2b). These tumors showed a high mitotic

Table 1 Tumorigenicity of PREJ/ras and pSV2/neo clones

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<th>Clone</th>
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<td>SV-HUC/ras</td>
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<td>A</td>
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* Numbers in parentheses, latent period (wk).

ND, not determined. These clones were lost at late passage.

Fig. 1. Phase-contrast photomicrographs showing the morphologies of SV-HUC/neo and SV-HUC/ras cells at P4 posttransfection. SV-HUC/neo clone A (a) appeared as a monolayer of uniformly shaped epithelial cells indistinguishable from the SV-HUC parent culture. SV-HUC/ras clones grew in a more loosely packed fashion than did SV-HUC, and some exhibited a polar morphology with criss-crossing of cells as illustrated by clone C (b). × 150.
Fig. 2. Characteristics of SV-HUC/ras tumors as illustrated by Tumor D. Subcutaneous tumors grew to approximately 1 cm in diameter within 3 wk after first appearance (a). The SV-HUC/ras-induced tumors were classified as Grade IV poorly differentiated transitional carcinomas with high mitotic activity (b). Tumor cell lines derived from the ras-induced tumors formed colonies in the lung (c) when inoculated i.v. into athymic nude mice.

index, occasional foci of ischemic necrosis, and invasion into adjacent muscle and adipose tissue. None of these subcutaneous tumors spontaneously metastasized to the liver, lung, spleen, or kidneys. The cell lines initiated from the tumor explants (SV-HUC/ras clones B, F, and D) produced tumors with the same histopathology as the parental tumors at 4 of 4 sites after s.c. inoculation. When inoculated i.v., all three tumor cell lines formed colonies in the lungs within 2 wk (Fig. 2c).

Mutant p21 Contributes to, but Alone Is Insufficient for SV-HUC Tumorigenicity. To test if differences in expression of altered ras-encoded p21 in the different clones explained the differences in their tumorigenicity at the different passages, the electrophoretic mobilities of p21 were examined in SV-HUC/ras and SV-HUC/neo at P2 soon after the clones were ring isolated, and at P12 after a 5-mo period of cell culture (Fig. 3). Results showed that 4 of the 7 ras-transfected SV-HUC clones (clones A, B, D, and F) showed mutant p21 with the expected decreased mobility at P2 (Fig. 3a). One clone, SV-HUC/ras G, appeared negative for mutant p21, but showed the altered protein at P12 (Fig. 3b), demonstrating an increase in ras expression after a period of in vitro culture. Altered p21 was not detected in SV-HUC/ras C even after passage in vitro (Fig. 3b), even though this was one of the two most morphologically altered clones (Fig. 1b). Neither the parental SV-HUC cell line, nor any of the SV-HUC/neo clones produced altered p21 at P2 or P12. These results were reproducible in 4 gels at each passage.

All clones which were tumorigenic expressed detectable ras protein, but none of the clones which did not express mutant p21 was tumorigenic. However, if simple expression of a detectable level of altered p21 was sufficient for tumorigenicity, 4 of the 7 clones should have produced tumors at P2 and one more at P12. In fact, only 2 clones, SV-HUC/ras-B and SV-HUC/ras-F, produced tumors at P2, and at only one site each. Furthermore, there was no obvious correlation between the level of p21 and the tumorigenicity of some clones compared with other clones. The presence of altered p21 was barely detectable in clone B, which produced a tumor with a relatively short latent period, while SV-HUC/ras clone F showed a high
level of expression, but produced only one tumor with a long 20-wk latent period. SV-HUC/ras-A and SV-HUC/ras-D had protein levels similar to the tumorigenic clone F and much greater than tumorigenic clone B, but neither of these clones was tumorigenic. Thus, the abundance of altered p21 at P2 in the independent clones did not predict their tumorigenic potential. The same result was observed at P12 (Fig. 3b). One clone (SV-HUC/ras-D) showed abundant protein at P12 and gave rise to tumors at 4 of 4 sites. However, SV-HUC/ras clones A and G showed equally abundant protein, but yielded no tumors.

The levels of p21 were examined in three tumor cell lines (TB, TD, and TF) initiated from explants of tumors obtained after inoculation of ras-transfected clones into nude mice. In all cases, the level of expression of p21 ras-encoded protein was elevated compared with the cell line which was inoculated (Fig. 4). The results are consistent with a model in which increased expression of ras occurs in vitro and/or in vivo and renders a selective growth advantage.

Increased p21 Correlates with Increased ras DNA and/or RNA Levels. Dot blot analyses and quantitative densitometric determinations for DNA and RNA levels were done on SV-HUC/ras and SV-HUC/neo clones to support SDS-PAGE results by determining if cells with increased mutant p21 also showed increased ras DNA and/or RNA levels (Fig. 5). DNAs and RNAs were prepared from neo- and ras-transfected clones and the tumor cell lines as soon as sufficient cells were available. Results of RNA analysis showed a good correlation between p21 abundance and ras RNA levels, with clones with the most abundant protein (i.e., SV-HUC/ras clones A, B, D, and F) showing 1.6 to 3.6 times the ras RNA levels seen in the SV-HUC/neo controls. The SV-HUC/ras TB and TD tumor cell lines (which showed even greater p21 abundance than any of the 7 original clones) were 9 and 5 times amplified, respectively, for ras RNA when examined at early passage. DNA dot blot analysis showed that only one of the ras-transfected clones (SV-HUC/ras-D) had an increased ras gene copy number at P2 (6 times compared to SV-HUC/neo controls), probably resulting from an efficient transfection. This increased gene copy number was also seen in the tumor (TD) obtained after inoculation of this clone. One tumor line (TB) showed a 5-fold amplification of ras DNA in the tumor compared with the control. Therefore, the increased p21 observed in the tumors, as well as in SV-HUC/ras clone D, can be explained by increased ras gene copy number. In the other clones, the increased p21 could be attributed to either the 2- to 3-fold increased transcription reported for the EJ/ras gene (35) or increased mRNA stability. The levels of ras RNA and DNA in control SV-HUC/neo clones A—G varied by a maximum of 0.1-fold and 0.3-fold, respectively. When DNA and RNA dot blots were also probed using β-actin, no increases in β-actin gene copy number were seen in any clones including SV-HUC/ras-D, which showed a 6-fold increase in ras gene copy number. Furthermore, there were no increases in β-actin RNA levels in the 3 independent SV-HUC/ras clones (B, D, and F) which showed a 1.6-, 3.1-, and 3.6-fold increase in ras RNA, respectively. One clone (A) which was 1.6-fold amplified for ras RNA was also 1.5-fold amplified for actin RNA.

Tumor Cell Lines Show SV-HUC Karyotypes with Deletions. To confirm that the tumors were human and derived from the inoculated SV-HUC/ras clones, karyotypes of the tumors were done and were compared with the inoculated clones and the parental SV-HUC cell line. The parental SV-HUC cell line showed a monosomic segment, 7q[32–34] and 3 trisomic arms (8p, 11q, and 14q). All of the ras- and neo-transfected clones showed the same monosomic and trisomic segments, demonstrating the clonal origin of the cells used for transfection. At early passage (P2), most of the transfected clones showed no chromosomal losses or gains (Table 2). Thus, gross chromosomal alterations were not associated with the transfection or cloning of SV-HUC. However, after serial passage, all of the clones examined lost one or more chromosomal segments.

The three tumor lines showed additional chromosomal losses compared with the clones at the time of inoculation. Two of these lost the extra 8p seen in the parental line. These same lines both showed a net loss of 1 or 1p, and 3 or 3p (Table 2). Neither of the parental clones showed losses of these chromosomes, which were the only apparently nonrandom chromosomal losses associated with tumorigenicity. The third cell line SV-HUC/ras TD did not show gross losses on chromosome 1 or 3 by cytogenetic analysis, but showed a loss of 21q, which
results would simply have confirmed the results of others, whose animals inoculated with pooled ras-transfected cells. Thus, our tumor-producing clones, or all of the cells in the expanded altered ras alone or from a specific integration site of ras within (Table 1). This tumorigenicity could not have resulted from of mutant ras alone is insufficient to neoplastically transform
independent clones gave rise to tumors at early passage, and tumorigenic (Fig. 3). Instead, only one of 4 sites in 2 of the 7 HUC, then most of the SV-HUC/ras clones should have been tumorigenic. The results also show that the addition of mutant p21 ras may have a tumor growth advantage in vivo. These results also show that “spontaneous” amplification of ras occurred in one tumorigenic clone (SV-HUC/ras-B), and that these cells were subsequently selected in vivo. This interpretation is consistent with the idea of clonal selection within tumors (49) and with observations that high ras expression is correlated with high tumorigenicity (14, 16, 17).

The SV-HUC/ras transformants, although rare, produced malignant, rapidly growing carcinomas (Fig. 2). In contrast, the nontumorigenic parental SV-HUC cell line and the neo-transfected control clones did not produce tumors or nodules of any nature in any animal on inoculation, even after cloning and serial passage in vitro. The tumor cell lines when inoculated into nude mice rapidly formed tumors at all sites of inoculation with a short latent period. These results show that the lack of tumorigenicity at all injection sites in the original inoculations of the SV-HUC/ras clones (Table 1) cannot be explained by a low tumorigenic potential of ras transformants in the nude mouse system or by insensitivity in the assay.

All of the above results taken together suggest a model of transformation in which mutant ras together with at least one additional event, which apparently occurs stochastically and rarely, leads to neoplastic transformation of SV-HUC. It would be of great interest to determine the nature of this event. The karyotypic analysis of ras transformants fortuitously may have provided a possible clue to such a mechanism. Cytogenetic analysis shows very few chromosomal alterations associated with transfection, cloning, and serial culture of SV-HUC (Table 2). It is, therefore, significant that 2 of the 3 independent tumor cell lines showed a net loss of 2 of the same chromosomal arms, namely 1p and 3p, and the third line showed a loss of 21q, which has been described as the sole cytogenetic change in some bladder cancer subtypes (48). The parental clones from which these tumors derived showed no such losses. Losses of regions on chromosomal arms 1p and 3p have been observed in an increasing number of human carcinomas (50) and, therefore, it has been hypothesized that these regions may contain tumor suppressor genes (51). While it is premature to suggest that loss of such genes is a necessary step in ras transformation of SV-HUC, it is interesting that similar losses (i.e., 3p) have also been observed in chemically transformed SV-HUC (38, 40). Furthermore, several lines of evidence suggest that suppressor(s) of ras transformation may be present in nontumorigenic cells. For example, somatic cell hybrids between tumorigenic ras-transfected cells and nontumorigenic cells in our system and in other systems (52, 53) are nontumorigenic. Furthermore, neoplastic transformation of diploid hamster cells after trans-
fection with myc and ras is correlated with specific chromosome loss (25). In addition, there are several reports of failure of mutant ras to transform immortalized cell lines (12, 13). It is possible that such cell lines have not lost (or do not easily lose) the hypothesized cancer suppressor gene(s), in contrast to ras-transformable cell lines.

In summary, this paper presents a model of transformation in which mutant ras participates in neoplastic transformation of SV40-immortalized human epithelial cells, but of itself is insufficient. The data suggest that an additional event is required for tumorigenic conversion. The nature of this event has not been identified. However, results suggest a possibility that loss of suppressor genes may be involved. Studies to address this hypothesis are in progress in our laboratory.

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