Derivation of Human Tumor Cells in Vitro without Widespread Genomic Instability

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ABSTRACT

The majority of adult human epithelial cancers exhibit evidence of genetic instability, and it is widely believed that the genetic instability manifested by aneuploidy or microsatellite instability plays an essential role in the genesis of these tumors. Indeed, most experimental models of cancer also show evidence of genomic instability. The resulting genetic chaos, which has widespread effects on many genes throughout the genome, confounds attempts to determine the precise cohort of genetic changes that are required for the transformation of normal human cells to a tumorigenic state. Here we show that genetic transformation of human kidney epithelial cells can occur in the absence of extensive aneuploidy, chromosomal translocations, and microsatellite instability. These observations demonstrate that the in vitro oncogenic transformation of human cells can proceed without widespread genomic instability.

INTRODUCTION

Cancer arises from a stepwise accumulation of genetic changes that afford an incipient cancer cell the properties of unlimited, self-sufficient growth and resistance to normal homeostatic regulatory mechanisms (1). Over the past 2 decades, the involvement of an array of oncogenes and tumor suppressor genes in cancer pathogenesis has been demonstrated through the study of the effects of such genes on cultured cells and in animal models of cancer development (2, 3). Somatic mutation of these genes as well as germ-line transmission of already mutated alleles both contribute to the formation of the highly mutated genomes found in late stage, aggressively growing tumor cells. Although the total number of mutant genes required to create a malignant human cell remains unclear, studies of the kinetics of tumor appearance in human populations (4) and of colonic tumor pathogenesis (5) suggest that at least four to six successive mutations are required before an adult malignant tumor appears.

The somatic mutational mechanisms that affect the cell genome and contribute to cancer pathogenesis may include, in addition to well-characterized processes such as point mutations, deletions, translocations, and gene amplification, changes in chromosome number and configuration that affect the relative copy number of a large number of still-unknown genes (6, 7). These alterations in karyotype and attendant changes in gene number (aneuploidy or CIN) may, in turn, perturb the regulatory mechanisms that govern normal cell growth. In support of this notion, the majority of human cancer cells have been found to harbor karyotypic alterations that result in various degrees of aneuploidy (6). Furthermore, neoplastic transformation of both human and rodent cells in vitro through a variety of chemical or physical agents, oncogenic viruses, and oncogenes, singly or in combination, has resulted in transformed cells characterized by aneuploidy and obvious chromosomal alterations (8–18). Such observations have led some to suggest that the mechanisms generating aneuploidy are intrinsic to tumorigenesis and operate as an essential causal force in tumor progression through the simultaneous disruption of a large array of normal genes (reviewed in Refs. 14 and 19).

An alternative scenario is inspired by the suggestion of some that the number of mutant genes required to create a malignant cell, when taken together with the normally low rate of mutation per cell generation, makes it highly unlikely that the process of tumor progression can reach completion within a human life span (20). Viewed in this way, genetic instability, including those types that destabilize karyotype, may be nothing more than an effective means of rapidly generating the substantial number of mutant alleles required for the programming of neoplastic growth, and hypermutability may not itself be an essential component of the malignant state. Aneuploidy would then represent an epiphenomenon of cancer pathogenesis, a by-product of the hypermutable state exploited by tumor cells during the process of tumor progression to accumulate the mutant alleles essential for malignant proliferation. If this second scenario was correct, the experimental introduction of the requisite mutant alleles into a normal human cell would obviate the genomic instability needed to acquire the mutant alleles necessary for the neoplastic phenotype. Human tumor cells produced in this manner may therefore not exhibit the widespread genetic chaos that occurs as a consequence of genetic instability.

We have reported recently that the introduction of SV40 ER together with a ras oncogene and hTERT, the telomerase catalytic subunit, is sufficient to transform normal human fibroblasts (BJ), kidney epithelial (HEK), and mammary epithelial cells into tumorigenic cells (21, 22). The need for the introduction of this particular combination of genes demonstrated that, as had long been suspected, human cell transformation requires a larger number of genetic changes than are needed for the transformation of similar types of rodent cells. Moreover, this experimental system afforded us the opportunity to gauge the importance of widespread genetic instability to human cell transformation and, in turn, to define more accurately the number of genetic changes required for this process to reach completion.

MATERIALS AND METHODS

Generation and Biochemical Characterization of Cell Lines. The transformed BJ fibroblasts and HEK cells used in this study were derived by the introduction of the SV40 ER, oncogenic Ras, and hTERT using amphotrophic retroviruses as described (21). The vector used to introduce SV40 encodes the entire SV40 ER and directs the expression of both LT and small t antigens (21,
C. Electrophoresis was performed with a final 10-min extension at 72°C, 2°C at 54°C, during which the annealing temperature was decreased 1°C for each °C.

Cy5.5 antibodies (Sigma-Aldrich Chemical Co.). Fluorescent was carried out using avidin-Cy5 (Amersham-Pharmacia) and antidigoxigenin-probe cocktail, labeled by Spectrum Orange, Texas Red, CY5, Spectrum... mitotic 0.75 M KCl solution resulted in a high percentage of broken metaphases excluded by cell size and fluorescence intensity. The Modfit LT v2.0 for MAC cytometer (Becton Dickinson Immunocytometry Systems). Doublets were...

23). Single-cell clones were isolated by ring cloning. Cells were explanted from tumors after digestion with dilute collagenase. Because the retrovirus used to introduce the ras oncogene into these transformed HEK cells also carried a puromycin resistance marker (24), constituent tumorigenic human cells were isolated in vitro using puromycin drug selection. Immuno blotting using standard methods with antibodies for LT (Pab 101; Santa Cruz Biotechnology) and H-Ras (C-20; Santa Cruz Biotechnology), as well as the telomerase repeat amplification protocol (25), was used to confirm protein expression.

SKY. Because treatment of both BJ and HEK cells with a standard hypotonic 0.75 M KCl solution resulted in a high percentage of broken metaphases unsuitable for chromosome counts and hybridization, metaphase spreads were obtained using an improved procedure for chromosome preparation from solid tumors (26). For SKY analysis, slides were incubated at room temperature for 5–6 days, hybridized, and analyzed as described (22, 27, 28). The chromosome probe cocktail, labeled by Spectrum Orange, Texas Red, CY5, Spectrum Green, and Cy5.5, was denatured and hybridized to denatured target slides. Visualization of biotin- and digoxigenin-labeled DNA of the probe cocktail was carried out using avidin-Cy5 (Amer sham-Pharmacia) and antidigoxigenin-Cy5.5 antibodies (Sigma-Aldrich Chemical Co.). Fluorescent in situ hybridization with biotin- and digoxigenin-labeled painting probes (AL Technologies) was used to confirm certain rearrangements detected by SKY. For each cell clone, 25 metaphases were analyzed by SKY, which included spectral, classified, and DAPI-generated G-banding karyotypes, and 50 metaphases were analyzed for chromosome number, except for HA1ER-3, where only 40 metaphases could be counted.

Flow Cytometry. Cells were fixed in 70% ethanol, filtered through a 50-μm nylon mesh screen, resuspended at a concentration of 4 × 10⁶ cells/ml in propidium iodide (50 μg/ml, Sigma-Aldrich Chemical Co.) and DNase-free RNase (100 units/ml, Sigma-Aldrich Chemical Co.), and incubated for 20 min at 37°C. Samples (3.5–4 × 10⁶) were analyzed using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems). Doublets were excluded by cell size and fluorescence intensity. The Modfit LT v2.0 for MAC cell cycle analysis program (Verity Software House, Inc.) was used for cell cycle and ploidy analysis. DNA content was calibrated by performing flow cytometry on experimental samples mixed with either normal human fibroblasts or leukocytes.

MIN. To look for the presence of MIN, we isolated genomic DNA from cell clones and amplified specific microsatellite repeats by PCR using 11 defined markers constituting the MIN consensus panel (BAT25, BAT26, D2S123, D5S346, and D17S250; Refs. 29 and 30), as well as the University of Utah Genomics Core Facility MIN panel (D1S405, D5S592, D8S184, D11S1918, D18S51, and D18S47; Ref. 31). Samples were amplified in 20-μl reactions, and the conditions for thermocycling included a 5-μl initial incubation at 95°C followed by eight cycles (20 s at 95°C, 20 s at 62°C, and 40 s at 72°C), during which the annealing temperature was decreased 1°C for each cycle. Thirty additional cycles (20 s at 95°C, 20 s at 54°C, and 40 s at 72°C) were performed with a final 10-min extension at 72°C. Electrophoresis was performed with a Perkin-Elmer Biosystems 373A Sequencer and analyzed using ABI Prism GeneScan and Genotyper software.

Anchorage-independent Growth and Tumorigenicity Assays. Growth of cells in soft agar and in immunodeﬁcient animals was performed as described previously (21). For tumorigenicity assays, 2 × 10⁶ cells were injected s.c. in each experiment. Anchorage-independent colonies were counted using an automated colony counter (Gel-doc system; UVP, Inc.).

RESULTS AND DISCUSSION

We examined the karyotype of these human BJ fibroblast and HEK cell populations transformed by the successive introduction of the SV40 ER, the hTERT gene, and an oncogenic allele of the H-ras gene. For the human fibroblasts, we introduced hTERT first followed by SV40 ER and ras, whereas in HEK cells, SV40 ER was expressed first followed sequentially by hTERT and ras. Because we used retroviral vectors to produce these cells, both the transformed fibroblasts and HEK cells were polyclonal, and we found no evidence for secondary mutations that cooperated with these introduced genetic elements to achieve transformation (see below and Ref. 21).

When we analyzed the karyotype of these transformed cells by chromosome G-banding and SKY, we found that all of the transformed BJ fibroblasts examined were highly aneuploid. Furthermore, we found no evidence of specific chromosomal rearrangements shared by substantial numbers of these cells (data not shown and Ref. 32). In stark contrast, we found that 62–70% of HEK cells derived from both immortal, nontumorigenic (expressing SV40 ER, hTERT, and a control vector, termed HA1EB), and tumorigenic (expressing SV40 ER, hTERT, and Ras, termed HA1ER) HEK cells carried a near-diploid, possibly euploid karyotype, as evidenced by counting chromosomes (data not shown) and by determining cellular DNA content using flow cytometry (Fig. 3, A and B). Individual cells within these two populations contained chromosome numbers ranging from 45 to 92 (HA1EB, 44% diploid, 42% tetraploid, and 14% intermediate) and 44

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to 92 (HA1ER, 54% diploid, 6% tetraploid, and 40% intermediate). This difference in the degree of aneuploidy between fibroblasts and epithelial cells is reminiscent of prior observations, which suggested that some cell types, largely of epithelial origin, are more resistant to the genomic instability induced by LT (22, 33).

To analyze further these transformed cells, we isolated single-cell clones and performed the experiments depicted schematically in Fig. 1. Single-cell clones of the transformed HA1ER cells were first compared to find clones that expressed comparable levels of the ectopically expressed gene products (Fig. 2). Two HEK cell clones (HA1ER-2 and HA1ER-3) that expressed similar levels of these proteins were then analyzed by flow cytometry (Fig. 3, C and E), SKY (Fig. 4, A and E), and DAPI-based chromosome G-banding (Fig. 4, B and F). These complementary methods confirmed the presence of structurally normal, diploid complements of chromosomes. We note, however, that subtle deletions or inversions undetectable at this level of G-banding resolution may exist. Although the majority of the individual cells analyzed from each clonal population was diploid, we
noted that 7–23% of individual metaphase spreads were tetraploid (Fig. 3, C and E). We suspect that these tetraploid cells represented the minority of HEK cells that we have observed to be markedly larger than the average cell in these HEK cultures. We note that although we did not detect evidence of any chromosomal rearrangements in the great majority of the diploid cells (Fig. 4, A, B, E, and F), we did observe translocations in all of the tetraploid cells and in a minority of the diploid cells (Table 1). Translocations were occasionally seen in more than one metaphase, but specific translocations observed in chromosomes derived from the HA1ER-2 cell clone were never seen in the HA1ER-3 cell clone (Table 1). Furthermore, in contrast to what we observed previously when we analyzed human mammary epithelial cells that had been transformed in a similar manner to these HEK cells (22), we did not observe rearrangements involving the MYC locus.

These two predominantly diploid HEK cell clones were then analyzed for their ability to grow in an anchorage-independent manner (Fig. 5A) and to form tumors in immunodeficient animals (Fig. 5B). Each of these clones formed colonies in soft agar (Fig. 5A) and, when implanted into murine hosts, formed tumors at comparable rates of growth and size to those shown by the parental polyclonal population of cells (Fig. 5B) and those formed by control cell lines derived from
human cancers (data not shown). Tumors derived from each of these implanted clones were then explanted (HA1ER-2T and HA1ER-3T). Similar to what we had observed previously (21), these explanted tumor-derived cells grew in an anchorage-independent manner (Fig. 5A) and formed tumors at a rate similar to that observed in the parental clones that had never been injected into mice (Fig. 5B). These observations indicated that these HEK cell populations and clones did not sustain infrequent alterations, such as somatic mutations and changes in methylation status, that were required, in conjunction with these introduced genetic elements, for their tumorigenicity. We concluded, furthermore, that these diploid-transformed HEK cells retained the ability to form tumors and, in this respect, exhibited a behavior indistinguishable from that of the original polyclonal population of transformed HEK cells.

We proceeded to analyze the karyotype of these explanted, tumor-derived cells. If karyotypic instability had been required for their tumorigenic growth, we expected to find that these cells would exhibit widespread genomic instability. Strikingly, the great majority of each of the explanted tumor cells retained a structurally normal diploid karyotype based on SKY (Fig. 4, C and G) and the corresponding DAPI-based chromosome G-banding (Fig. 4, D and H). Similar to what we observed with the original transformed HEK clones (HA1ER-2 and HA1ER-3), 17–18% of the cells were tetraploid (Fig. 3, D and F). In addition, we found that a small minority of the diploid and all of the tetraploid cells carried translocations that were different from those observed in these cell clones before the formation of tumors (Table 1). In each case, the karyotypically normal tumor cells clearly predominated, and neither diploid nor tetraploid cells harboring translocations achieved a selective survival advantage over these karyotypically normal cells during the process of forming a tumor. This observation that recurring translocations were not seen after tumor formation makes it highly unlikely that a specific abnormal karyotype was required for the observed tumorigenicity. However, we cannot exclude the possibility that the generation of the small population of diploid and tetraploid cells harboring chromosomal rearrangements cooperated with the majority diploid, normal HEK cells during the process of tumor formation. Indeed, aneuploid HEK variants appear to be generated continuously but either suffer cell death or are at a selective proliferative disadvantage. Taken together, these observations demonstrate that the accumulation of neither widespread aneuploidy nor specific chromosomal rearrangements detectable by the methods used here is required for the tumorigenic behavior of these transformed HEK cells.

These observations increased the likelihood that the introduced genes were sufficient on their own to accomplish malignant cell transformation. The observation that explanted tumor cells subsequently formed tumors with the same kinetics as did those cells that had never been passaged in vivo appeared to exclude the necessary involvement of additional rare mutations that were acquired by these cells during their growth in vivo and were essential, together with the introduced genes, for tumorigenicity. Nonetheless, it remained possible that widespread genomic instability in these HEK cells induced by defects in the DNA replication error repair system, as has been described for many diploid human colorectal carcinomas (29, 34–36), would generate, at a high rate, the additional mutant alleles required for tumorigenesis without affecting the karyotype of these cells. Such defects result in random single bp errors throughout the genome. This

![Fig. 5. Tumorigenicity of HEK cells. A, anchorage-independent growth of HEK cells in 0.4% Noble agar. Mean and SD for three experiments is shown. In each experiment, 10^6 cells were plated, and colonies were counted by an automated colony-counting apparatus after 21 days. B, growth of indicated HEK cells in immunodeficient mice. For HA1ER, HA1ER-2, and HA1ER-3, results shown represent mean and SD for nine experiments; for HA1ER-T, HA1ER-2T, and HA1ER-3T, results shown represent the mean and SD for three experiments. We did not observe tumor growth in HA1EB cells in 12 separate experiments (data not shown). In each experiment, 2 x 10^6 cells were injected s.c. | HA1ER; •, HA1ER-2; ▲, HA1ER-3; □, HA1ER-T; ○, HA1ER-2T; △, HA1ER-3T.](image-url)
condition, termed MIN, can be detected by searching for specific alterations of microsatellite repeats.

To address the possible contribution of MIN to the observed tumorigenic phenotype, we isolated genomic DNA from clones both before and after they formed tumors in animals and analyzed these DNAs using well-characterized sets of microsatellite markers (29–31). For this analysis, we elected to use two complete sets of clinically validated microsatellite markers rather than a single set to ensure that MIN, if present, would be detected (29). Using 11 marker sets, we found no evidence for MIN in the electropherograms of either of the diploid HEK cell clones both before and after tumor formation (Table 2), providing strong evidence that MIN did not contribute to the tumorigenicity of these cell clones.

Taken together, these observations made it highly unlikely that these diploid-transformed HEK cells sustained widespread genomic instability and the attendant acquisition of large numbers of mutant alleles. Such observations strongly support the notion that the collaborative actions of the experimentally introduced genes sufficed on their own to transform these HEK cells. We conclude that genetic instability, resulting from either CIN or MIN, is not essential to the transformation of at least one type of normal human cell into a tumor cell when the appropriate mutant alleles are introduced experimentally into a normal cell. Although the methods used here cannot exclude the presence of subtle deletions or inversions in these diploid human tumor cells, these findings extend prior observations, which showed that many spontaneously arising human cancers (37) and some chemically transformed rodent cell lines (11) lack evidence of visible chromosome changes using conventional banding analyses.

We acknowledge that the transformation mechanism described here contrasts with those operating during the formation of most autonomously arising human cancers. In the latter, certain types of acquired genetic instability appear to play an essential role in the genesis of large numbers of genetic abnormalities, some of which then act in concert to program the malignant growth of these neoplasms (20, 38, 96). The presence of widespread genomic alterations in human cancer cells has confounded all prior attempts at determining the precise number of mutant genes required in aggregate for neoplastic transformation of normal human cells into tumorigenic derivatives. Whereas the transformation procedure described here does not recapitulate the process of spontaneous mutation and transformation that occurs during human tumor progression, these observations nonetheless provide a foundation for future experiments that will delineate with precision the number of mutant genes and disrupted regulatory pathways that are required for the formation of human cancer cells.

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