Suppression of the Simian Virus 40 Tumorigenic Phenotype in Hybrid Cells Formed from Simian Virus 40- and Adenovirus 2-transformed Hamster Embryo Cells

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ABSTRACT

Hamster cells transformed by adenovirus 2 (Ad2) or simian virus 40 (SV40) have different tumorigenic phenotypes. In the present study, somatic cell hybrids formed from Ad2- and SV40-transformed hamster cells were used to determine whether possible interactions between the integrated viral genomes would influence the tumorigenic phenotype of hybrid transformed cells. These somatic cell hybrids were of two types, one expressing both Ad2 and SV40 T-antigens and the other expressing only SV40 T-antigens. Tumor induction by hybrid cells that expressed both Ad2 and SV40 T-antigens was reduced in adult syngeneic hamsters and allogeneic hamsters. These results indicate that the tumorigenic phenotype of transformed somatic cell hybrids that contain both the Ad2 and SV40 genome is governed by the genetic expression of Ad2. This expression may alter the ability of SV40-transformed hamster cells to resist the immunologically nonspecific defenses of the host.

INTRODUCTION

SV40 and Ad2 viral functions interact in dually infected monkey cells. The effect of these interactions, which involve early functions of both viruses, is to reverse the expected pattern of viral production in such cells. When singly infected, monkey cells are permissive for SV40 and only semipermissive for Ad2. When infected with both viruses, monkey cells are fully permissive for Ad2, while SV40 propagation is aborted (16, 26). There is evidence from studies with Ad2-SV40 hybrid viruses that the early functions of these 2 viruses, which act to initiate and maintain the transformed state, may also interact in nonpermissive infection (17). In order to examine these possible viral interactions in transformed cells in more detail, we have prepared somatic cell hybrids from LSH HE cells transformed by SV40 or Ad2 and characterized them with respect to their tumorigenic phenotype, a property in which SV40-transformed cells are clearly different from Ad2-transformed cells. HE cells transformed in tissue culture by Ad2, a nononcogenic human adenovirus serotype, possess many of the characteristics known to be associated with tumorigenicity in vivo; however, they are poorly oncogenic in that they usually produce tumors only in immunologically suppressed or immunologically immature hosts (4). Although the early E1 region of the Ad2 genome is known to be essential for transforming HE cells to a neoplastic immortal state (4), the role of the Ad2 genome in determining the tumor-inducing capacity (i.e., the tumorigenic phenotype) of transformed HE cells has not been elucidated.

In contrast to Ad2-transformed HE cells, SV40-transformed HE cells induce tumors not only in newborn and adult syngeneic hamsters but adult allogeneic hamsters. Hybrid cells that expressed only SV40 antigens resembled the parental Ad2-transformed cells morphologically; they induced tumors in newborn syngeneic hamsters and only occasionally in adult syngeneic hamsters, but not in allogeneic hamsters. Hybrid cells that expressed only SV40 antigens resembled the parental SV40-transformed cells morphologically and induced tumors in both syngeneic and allogeneic immunocompetent hamsters as well as in newborn hamsters. These results indicate that the morphological and tumorigenic phenotype of hybrid transformed cells which express both Ad2 and SV40 T-antigens appears to be governed by Ad2 gene expression.

MATERIALS AND METHODS

Cell Lines. SV40HE1 and SV40HE2 were derived from Syrian LSH HE cells transformed by UV-inactivated SV40 (strain 777). Ad2HE1 and Ad2HE3 were derived from LSH HE cells transformed by UV-inactivated prototype Ad2; all cell lines were started from single foci growing under agar on plastic dishes (4). These transformed cell lines have been shown to be free of a number of potential contaminants (4). Untransformed HE cell cultures were prepared by mincing and trypan blueing whole 14-day-old LSH HEs. All cell lines were carried in DMEM (Grand Island Biological Co.) with 10% FBS.

Hybrid Cell Lines. TK- or HPRT- mutant cell lines were established by culturing transformed cells in DMEM (10% FBS) with 5-bromoodeoxyuridine (100 μg/ml; Sigma Chemical Co.) or 6-thioguanine (100 μg/ml;
Cloned lines of TK⁻ and HPRT⁻ mutant cells were fused by suspending cell mixtures in 50% polyethylene glycol (J. T. Baker; M, 950 to 1050) in serum-free DMEM for 1 min followed by incubation (15 to 20 hr) in DMEM (10% FBS) (19). After fusion, hybrid cell foci were selected in HAT medium. The frequency of reversion of mutant cell lines to wild type as determined by growth in HAT medium was between 10⁻⁷ and 10⁻⁸. Clonal cell lines were established from selected hybrid foci. All cell lines were periodically shown to be free of Mycoplasma. A more complete description of the formation and characterization of these hybrid cells will be presented elsewhere.

**Immunofluorescent Staining for Viral T-Antigens.** Cells were grown on glass coverslips, fixed for 10 min in cold acetone, and incubated (30 min at 37°C) with serum from hamsters bearing tumors induced by either SV40 or Ad2. The cells were washed extensively with PBS and reincubated with fluorescein-conjugated rabbit anti-hamster IgG containing rhodamine as a counterstain. After a second washing with PBS, the coverslips were mounted on microscope slides with glycerin and examined in a UV microscope. To test for viral-specific T-antibodies in serum from hamsters bearing cell-induced tumors (50 mm), the serum was diluted 1:5 in PBS and incubated with SV40-infected and uninfected Vero cells or with Ad2-infected and uninfected embryonic kidney cells. All cells were grown on glass coverslips, stained, and evaluated as described above.

**Karyotyping.** Near confluent monolayers were incubated with DMEM (10% FBS) containing colchicine (Grand Island Biological Co., 0.1 μg/ml) for 2 to 4 hr at 37°C. Metaphase cells were shaken free, centrifuged, resuspended in 75 mM KCl (Grand Island Biological Co.), fixed with absolute methanol:acetic acid (3:1), mounted on microscope slides, and stained with Giemsa. The stained metaphase cells were photographed in a microscope (×600), and the number of chromosomes in 25 to 50 cells was determined.

**Animal Studies.** The inbred LSH and CB strains of Syrian hamsters (Charles River Lakeview, Vineland, N. J.) differ by one or more histocompatibility loci (24). Newborn animals were no more than 4 days old, and adult animals were at least 28 days old. Cells to be tested for tumor induction were trypsinized and resuspended in serum-free DMEM at a concentration of 5 x 10⁷ cells/ml. Groups of 4 to 20 hamsters were inoculated (s.c. between the scapulae) with 0.2-ml aliquots of suspended cells. All cell lines established themselves.

**Characterization of Nonhybrid TK⁻ and HPRT⁻ Parental Transformed HE Cells.** The purpose of this study was to determine the tumorigenic phenotype of hybrid cells formed from SV40- and Ad2-transformed HE cells. In order to form these hybrid cells, we first selected TK⁻ and HPRT⁻ mutants as described in "Materials and Methods." The mutant cell lines were cloned, and the clonal lines were used to inoculate newborn and adult LSH and CB hamsters. The data in Table 1 show that the mutant clonal lines have retained the same tumorigenic phenotypes as the nonmutant lines from which they were derived (12, 13). Moreover, the tumorigenic phenotype of SV40HE1(TK⁻) is very stable as indicated by the similarity of the TPD₅₀S of clonal lines derived from an uncloned mutant population (Table 1). Both SV40- and Ad2-transformed mutant cells readily induce tumors in newborn syngeneic hamsters. Adult LSH hamsters occasionally develop tumors when challenged with high doses (10⁷ cells) of Ad2-transformed mutant HE cells; however, tumors have not been observed in allogeneic CB hamsters inoculated with these cells (Table 1). In contrast, we have always observed tumors in adult LSH and CB hamsters inoculated with SV40-transformed mutant HE cells.

**Characterization of Hybrid Cells Formed from Ad2- and SV40-transformed HE Cells.** Two groups of hybrid cells were prepared by fusing SV40HE1(TK⁻) C1 with Ad2HE1(HPRT⁻) C1 or Ad2HE3(HPRT⁻) C1. The 2 Ad2-transformed lines are identical with respect to their tumorigenic phenotypes (Table 1), but they differ with respect to their expression of Ad2 proteins. Ad2HE1 appears to express 4 viral proteins (having molecular weights of 75,000, 67,000, 58,000, and 45,000) that are precipitated with serum raised against early Ad2 proteins, while Ad2HE3 appears to express 2 Ad2 early proteins with molecular weights of 15,000 and 58,000 (10, 18).

Since hybrid cells frequently undergo chromosomal loss, the hybrid foci were recloned after several passages to insure a more uniform cell population. Hybrid cells formed with Ad2HE1(HPRT⁻) C1 (Table 2) were of 2 types. Some hybrid lines (S1+A1⁻ 2.4 and S1+A1¹ 11.4) contained small, round cells resembling the parental Ad2-transformed cells and expressed both SV40 and Ad2 antigens. Cells in the other hybrid lines (S1+A1⁻ 8.2, S1+A1¹ 9.4, and S1+A1¹ 10.3) were morphologically indistinguishable from the parental SV40-transformed cells (spindle or stellate morphology) and contained only SV40 T-antigens. Cells from 4 of 5 hybrid lines formed with Ad2HE3(HPRT⁻) C1 (Table 2) had an adenovirus-like morphology resembling the parental Ad2-transformed cells and expressed both SV40 and Ad2 antigens; S1+A3 9.2, which does not accumulate detectable amounts of Ad2 antigens, had an SV40-like morphology.

All hybrid cell lines examined contained more chromosomes than either of their parents, indicating that they were true hybrid cells (Tables 1 and 2). Attempts to identify parental chromosomes in the hybrid cells by band staining were unsuccessful due to the absence of any distinctive chromosomal markers in any parent. However, the very low frequency of reversion (10⁻⁷) of the mutants in HAT medium makes it very unlikely that our hybrids, including those that lacked Ad2 antigens, could have arisen from wild-type revertants.

The Ad2 DNA content in selected hybrid cell lines was determined by hybridizing single-stranded radioactive Ad2 DNA with cellular DNA (data not shown). Ad2 DNA was detected only in those hybrid cells that expressed Ad2 antigens. Evidently, the chromosome(s) in which Ad2 DNA was integrated in the heterokaryon progenitors was among those lost as these hybrid cell lines established themselves.

The 10 hybrid foci were tested for tumor induction in newborn and adult LSH hamsters and in adult CB hamsters (Table 2).
Tumor induction by nonhybrid parental SV40- and Ad2-transformed HE cell lines

<table>
<thead>
<tr>
<th>Nonhybrid mutant cell line</th>
<th>Chromosome no.</th>
<th>Viral antigens</th>
<th>Newborn LSH</th>
<th>Adult LSH</th>
<th>Adult CB</th>
<th>Passage no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SV40HE1(TK) UC</td>
<td>NA</td>
<td>S+</td>
<td>(11/11)</td>
<td>2.2</td>
<td>2.5</td>
<td>6</td>
</tr>
<tr>
<td>SV40HE1(TK) C1</td>
<td>57 ± 3</td>
<td>S+</td>
<td>(20/20)</td>
<td>2.0</td>
<td>2.0</td>
<td>23</td>
</tr>
<tr>
<td>SV40HE1(TK) C3</td>
<td>NA</td>
<td>S+</td>
<td>(13/13)</td>
<td>2.5</td>
<td>4.0</td>
<td>8</td>
</tr>
<tr>
<td>SV40HE1(TK) C6</td>
<td>NA</td>
<td>S+</td>
<td>(20/20)</td>
<td>2.0</td>
<td>2.5</td>
<td>8</td>
</tr>
<tr>
<td>SV40HE1(HPRP) C3</td>
<td>NT</td>
<td>S+</td>
<td>(14/14)</td>
<td>3.5</td>
<td>3.8</td>
<td>12</td>
</tr>
<tr>
<td>Ad2HE1(HPRP) C1</td>
<td>55 ± 2</td>
<td>A+</td>
<td>(20/20)</td>
<td>(0/8)</td>
<td>&gt;7.5</td>
<td>16</td>
</tr>
<tr>
<td>Ad2HE3(HPRP) C1</td>
<td>66 ± 2</td>
<td>A+</td>
<td>(16/16)</td>
<td>&gt;7.5</td>
<td>&gt;7.5</td>
<td>14</td>
</tr>
</tbody>
</table>

* Determined as described in "Materials and Methods."

† Viral antigens were detected (+) or not detected (−) as determined by immunofluorescent staining using sera from hamsters bearing tumors induced by SV40 (S) or Ad2 (A).

‡ See "Materials and Methods."

§ Passage number of cell line tested.

Table 2

<table>
<thead>
<tr>
<th>Hybrid cell line no.</th>
<th>Chromosome no.</th>
<th>Viral antigens</th>
<th>Newborn LSH</th>
<th>Adult LSH</th>
<th>Adult CB</th>
<th>Passage no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1<em>A1</em> 2.4</td>
<td>74 ± 6</td>
<td>S+ A+</td>
<td>(20/20)</td>
<td>4.0</td>
<td>5.8</td>
<td>&gt;7.5</td>
</tr>
<tr>
<td>S1<em>A1</em> 8.2</td>
<td>NT</td>
<td>S+ A−</td>
<td>(20/20)</td>
<td>4.0</td>
<td>5.2</td>
<td>20</td>
</tr>
<tr>
<td>S1<em>A1</em> 5.4</td>
<td>71 ± 5</td>
<td>S+ A−</td>
<td>(20/20)</td>
<td>2.8</td>
<td>5.0</td>
<td>23</td>
</tr>
<tr>
<td>S1<em>A1</em> 10.3</td>
<td>70 ± 3</td>
<td>S+ A−</td>
<td>(20/20)</td>
<td>2.0</td>
<td>1.8</td>
<td>17</td>
</tr>
<tr>
<td>S1<em>A1</em> 11.4</td>
<td>64 ± 9</td>
<td>S+ A+</td>
<td>(20/20)</td>
<td>(0/8)</td>
<td>(0/8)</td>
<td>17</td>
</tr>
<tr>
<td>S1<em>A3</em> 1.1</td>
<td>78 ± 3</td>
<td>S+ A+</td>
<td>(20/20)</td>
<td>&gt;7.5</td>
<td>&gt;7.5</td>
<td>6</td>
</tr>
<tr>
<td>S1<em>A3</em> 3.3</td>
<td>86 ± 5</td>
<td>S+ A+</td>
<td>(17/17)</td>
<td>&gt;7.5</td>
<td>&gt;7.5</td>
<td>6</td>
</tr>
<tr>
<td>S1<em>A3</em> 4.2</td>
<td>71 ± 6</td>
<td>S+ A+</td>
<td>(18/18)</td>
<td>&gt;7.5</td>
<td>&gt;7.5</td>
<td>7</td>
</tr>
<tr>
<td>S1<em>A3</em> 6.3</td>
<td>125 ± 7</td>
<td>S+ A+</td>
<td>(0/14)</td>
<td>&gt;7.5</td>
<td>&gt;7.5</td>
<td>8</td>
</tr>
<tr>
<td>S1<em>A3</em> 9.2</td>
<td>68 ± 4</td>
<td>S+ A−</td>
<td>(20/20)</td>
<td>3.8</td>
<td>3.8</td>
<td>11</td>
</tr>
</tbody>
</table>

$ Hybrid cell lines are designated by combining symbols for the parental lines: S1, SV40HE1(TK) C1 × SV40HE1(HPRP) C3; A1, Ad2HE1(HPRP) C3; A3, Ad2HE3(HPRP) C3; HE, untransformed hamster embryo; the superscript by the symbol for the parental line indicates that the viral antigens of the parental line are expressed (') or not expressed ('') in the hybrid cell line. The number at the end of the designation indicates the focus number (before the period) and clonal number (after the period), e.g., S1*A1* 2.4 = hybrid cell line from SV40HE1(TK) C1 and Ad2HE1(HPRP) C1. This hybrid line expresses both SV40 and Ad2 T-antigens and is the fourth clone isolated from focus 2.

* Determined as described in "Materials and Methods." Only the S1*A3*1.1 line exhibited a bimodal distribution, with a minor population of cells containing 53 ± 2 chromosomes.

† Viral antigens were detected (+) or not detected (−) as determined by immunofluorescent staining using sera from hamsters bearing tumors induced by SV40 (S) or Ad2 (A).

‡ See "Materials and Methods."

§ Passage number of cell line tested.

None of the 6 hybrid cell lines that expressed both Ad2 and SV40 T-antigens induced any tumors in adult allogeneic (CB) hamsters. One line, S1*A1* 2.4, induced a few tumors in adult syngeneic (LSH) hamsters, and all but one line, S1*A3* 6.3, readily induced tumors in newborn hamsters (Table 2). The 4 hybrid lines that accumulated only SV40 antigens induced tumors in all test animals, with TPD̄ comparable to those observed for nonhybrid SV40-transformed cells (Tables 1 and 2).

Sera from adult LSH hamsters bearing tumors induced by S1*A1* 2.4 contained antibodies against both SV40 and Ad2 T-antigens, indicating that the SV40 antigens are expressed in vivo as well as in vitro. Tumor cells established in tissue culture from

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an S1*A1 2.4 tumor continued to accumulate both Ad2 and SV40 T-antigens. In general, the histomorphology of tumors induced by adenovirus-like hybrids resembled that of tumors induced by nonhybrid Ad2-transformed cells, while the histomorphology of tumors induced by SV40-like hybrids resembled that induced by nonhybrid SV40-transformed cells.

In other experiments, we fused uncloned SV40-transformed HE cells with 2 lines of uncloned Ad2-transformed cells. Hybrid foci were selected in HAT medium, tested for viral T-antigens, and inoculated into hamsters. Three hybrid foci contained both Ad2 and SV40 antigens and resembled Ad2-transformed cells. These cells induced tumors in newborn hamsters but not in adult hamsters. Three hybrid foci contained only SV40 antigens and resembled SV40-transformed cells; these cells induced tumors in adult LSH and CB hamsters (data not shown). Thus, the tumorigenic phenotypes of 7 hybrid cell lines (cloned and uncloned) that express both Ad2 and SV40 T-antigens were similar to that of Ad2-transformed cells, while the tumorigenic phenotypes of 7 hybrid cell lines which lack Ad2 T-antigens were nearly identical to that of SV40-transformed cells. The probability of obtaining this result by chance is less than 0.01%. Thus, there is a significant association between the expression of Ad2 T-antigen and a reduction in the tumor-inducing capacity of these somatic cell hybrids. Since some of these hybrid cell lines (those derived from Ad2HE3) appear to express only 2 Ad2 proteins (10, 18), the action of one or both of these proteins may be sufficient to determine the tumorigenic phenotype of these hybrid cells.

Characterization of Hybrid Cells Formed from SV40-transformed and Normal Untransformed HE Cells. Somatic cell hybrids formed from highly oncogenic cells and nononcogenic or weakly oncogenic cells from the same or different species have been reported to be either nononcogenic or less oncogenic than the more tumorigenic of the parental cells (8, 9). There is no evidence in these or other studies (5, 6, 15) suggesting the attenuation of oncogenicity by the hybridization process per se. However, as additional controls for our experiments, we formed a series of hybrids between SV40HE1(TK') C1 and untransformed HE cells or SV40HE1(Hprt') C3 (Table 2). Clonally derived cells were established by procedures identical to those used to obtain the Ad2:SV40 somatic cell hybrids. These hybrid cell lines all retained the SV40 tumorigenic phenotype in that they readily induced tumors in both syngeneic and allogeneic adult hamsters. These results imply that the alteration of the SV40 tumorigenic phenotype in hybrid cells expressing both Ad2 T- and SV40 T-antigens is not due to the hybridization process nor to the genetic expression of normal cells.

DISCUSSION

These studies of somatic cell hybrids formed between Ad2- and SV40-transformed HE cells indicate a correlation between virus-specific antigen expression and the morphological and tumorigenic phenotypes of the hybrid cell. Retention of Ad2 T-antigen expression in such hybrid cells is associated with an abrogation (in the allogeneic host) or reduction (in the syngeneic host) of the tumor-inducing capacity of highly oncogenic SV40-transformed cells. We believe that suppression of the SV40 tumorigenic phenotype in our hybrid cells may define a dominant function for one or more of the early Ad2 proteins that determine the tumorigenic phenotype of Ad2-transformed hamster cells.

One possible explanation for the mechanism by which early Ad2 proteins influence the tumor-inducing capacity of the transformed cell is the expression of virus-specific transplantation antigens (14). However, several lines of evidence suggest that differences in the immunogenicities of DNA virus-transformed cells are an unlikely explanation for differences in their tumor-inducing capacities. We have shown that there is no correlation between the tumor-inducing capacity of Ad2- and SV40-transformed HE cells (including the Ad2HE1 and Ad2HE3 lines used herein) and the ability to induce immunity to virus-specific transplantation antigens in bioassays (13). A similar lack of correlation between the tumor-inducing capacities and the immunogenicities of Ad2- and SV40-transformed cells from other species has been reported (7, 25). Furthermore, the inability of immunocompetent newborn hamsters (which are unable to respond to specific antigens) to reject large numbers of certain types of Ad2-transformed cells suggests that host defenses, unassociated with immunity to virus-specific transplantation antigen, play a predominant role in tumor rejection. Histopathological studies of Ad2-transformed cell-induced tumors harvested at various times after challenge also suggest that the inflammatory response associated with tumor cell destruction occurs earlier than would be predicted in the case of a specific immune response to viral-induced transplantation antigens. Moreover, recent studies have shown that our somatic cell hybrids which express Ad2 T-antigens, as well as nonhybrid Ad2-transformed cells, are much more sensitive to lysis in vitro by unprimed lymphoid cells and by activated macrophages than are hybrid cells which express only SV40 T-antigen or nonhybrid SV40-transformed cells. Thus, it seems probable that sensitivity or resistance to nonspecific immune effector cells plays a critical role in determining the tumorigenic properties of our hybrid cells. Other factors might also be important. For example, the ability of transformed cells to proliferate in vivo may depend on the presence of autonomous tumor cell growth factors and/or on the availability of host growth factors, and whether these factors can be utilized by transformed cells (20, 22).

Although the mechanism of host rejection of Ad2-transformed cells is uncertain, the results reported here indicate that early Ad2 proteins are dominant in determining the morphological and tumorigenic phenotypes of hybrid cells that also express SV40 antigens. Possibly, Ad2 proteins interfere with the functions of SV40 proteins (i.e., protein kinase specificity) or perhaps compete more effectively for host proteins essential for some of these functions. Recent evidence indicates that both the M, 58,000 protein encoded in the transforming region of the adenovirus type 5 genome (E1) and the M, 94,000 SV40 T-antigen are coprecipitated with the host M, 54,000 protein by monoclonal antibody specific for the M, 54,000 protein induced in mouse cells transformed by adenovirus type 5 and SV40 as well as other agents (21). Presumably, both viral T-antigens associate with the cellular protein in transformed cells. It is possible that the tumorigenic phenotype of our hybrid cells depends on which viral protein forms the more stable complex with the M, 54,000 cellular protein.

4 A. M. Lewis, unpublished results.

Finally, we have begun to examine the properties of viral proteins expressed in these hybrid cells. Preliminary results obtained from electropherograms of immunoprecipitated viral antigens indicate that the molecular weights of the SV40 and Ad2 T-antigens are not altered by hybrid formation. However, SV40 T-antigens appear to be present in reduced amounts in hybrid cells that also express Ad2 T-antigens, whereas the expression of Ad2 T-antigens is not significantly altered by hybrid formation.

The data in this report add to the mounting evidence that factors in addition to those generally associated with transformation per se determine the tumor-inducing capacity of transformed cells (23). Elucidation of the mechanism by which the expression of Ad2 early gene products in these hybrid cells is correlated with tumorigenicity is needed for further study.
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