Neoplastic Transformation of Human Epidermal Keratinocytes by AD12–SV40 and Kirsten Sarcoma Viruses

Abstract. *Recent investigations have begun to dissect the number and nature of genetic alterations associated with cancer cells. In the present study, primary human epidermal keratinocytes acquired indefinite life-span in culture but did not undergo malignant conversion in response to infection with a hybrid of adenovirus 12 and simian virus 40. Addition of Kirsten murine sarcoma virus, which contains a K-*ras *oncogene, to these cells induced morphological alterations associated with the acquisition of neoplastic properties. These findings demonstrate the malignant transformation of human primary epithelial cells in culture and support a multiple-step process for neoplastic conversion.*

Clinical observations have implied that cancer is a multiple-step process (*1*). Efforts to elucidate the number and nature of genetic alterations associated with cancer have been aided by investigations of tumor viruses. A small group of RNA-containing viruses has transduced cellular genes, termed proto-oncogenes, which acquire oncogenic properties when incorporated by these viruses. Some of these cellular genes have been implicated as frequent targets for genetic alterations that can lead to malignancy of human cells under natural conditions.

In studies to develop models in tissue culture for malignant transformation of cells by oncogenes, several investigators have reported that primary rodent fibroblasts can undergo neoplastic conversion in response to the combined actions of two transforming genes from viral or cellular oncogenes or from the transforming genes of DNA tumor viruses, but that one transforming gene by itself was not sufficient (*2–4*). However, there have been only a few investigations of the nature and minimum number of genes that could induce malignant transformation of human cells, particularly those of epithelial origin, in culture (*5*). We used newly developed tissue culture methods to ascertain whether prototype RNA or DNA tumor viruses containing well-defined transforming genes could confer the malignant phenotype to human epithelial cells. Our study is apparently the first to show neoplastic conversion of human epithelial cells in tissue culture and to define the minimum number of transforming genes that appears to be required.

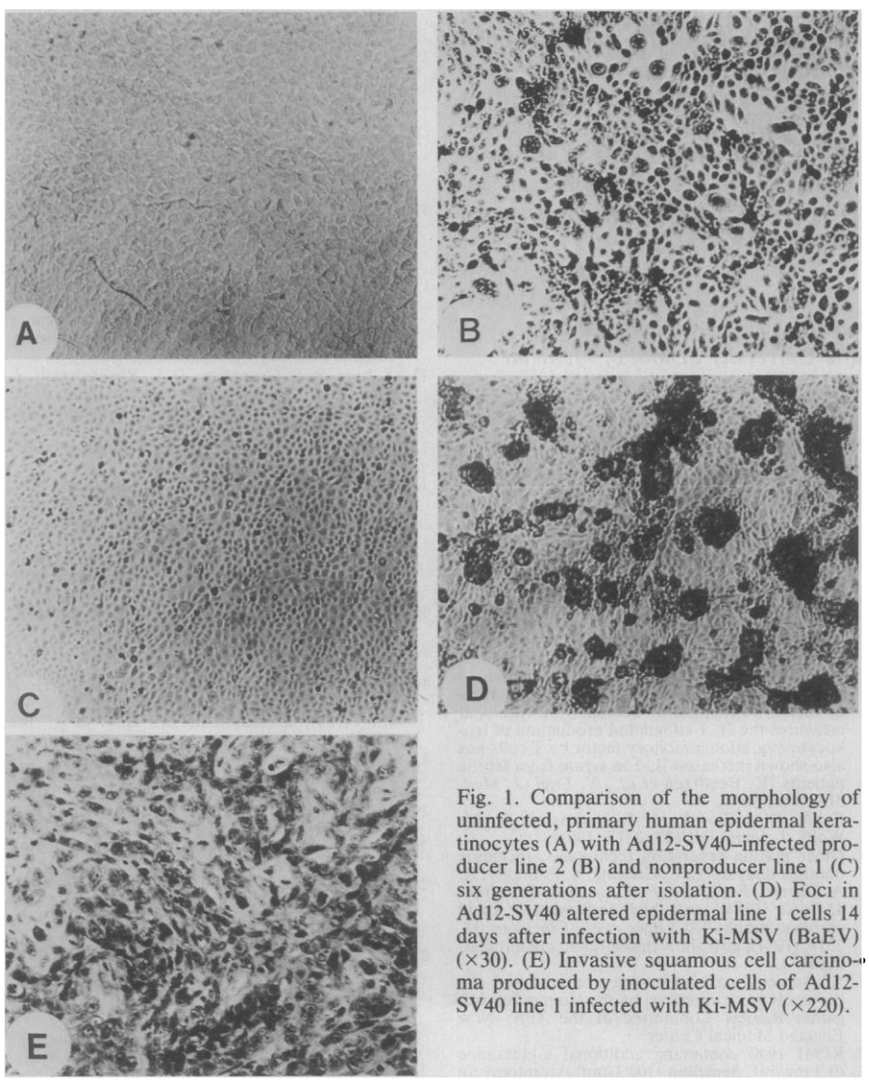

Fig. 1. Comparison of the morphology of uninfected, primary human epidermal keratinocytes (A) with Ad12-SV40–infected producer line 2 (B) and nonproducer line 1 (C) six generations after isolation. (D) Foci in Ad12-SV40 altered epidermal line 1 cells 14 days after infection with Ki-MSV (BaEV) (×30). (E) Invasive squamous cell carcinoma produced by inoculated cells of Ad12-SV40 line 1 infected with Ki-MSV (×220).

Primary human epithelial cells from the foreskins of several day-old Caucasian males were initiated in NCTC 168 medium with 10 percent horse serum containing antibiotics (100 μg of gentamicin and 25 μg of fungizone per milliliter) (*6*). In attempts to alter the growth properties of these cells, we used Kirsten murine sarcoma virus (Ki-MSV), a prototype retrovirus whose K-*ras* oncogene has been detected in many human epithelial malignancies (*7*), and Ad12-SV40 hybrid virus, which contains adenovirus 12 and simian virus 40 transforming genes (*8*) and induces malignant transformation of fibroblasts in culture (*9*). Neither control nor Ki-MSV–infected human epithelial cultures could be propagated serially beyond two or three subcultures (Table 1). In contrast, Ad12-SV40 infection led to the appearance of actively growing colonies by weeks 3 to 4. By week 6, SV40 tumor (T) antigen was revealed in the nuclei of a large fraction of the infected cultures by complement fixation and indirect immunofluorescence staining.

A number of cell lines was obtained from colonies that proliferated at a growth-limiting dilution of cells. All lines but one released Ad12-SV40 virus, as indicated by the induction of cytopathic effect in Vero cells. Although efforts to obtain infectious hybrid virus from this line by cocultivation with Vero cells were unsuccessful, this cell line remained positive for SV40 T antigen as determined by complement fixation and fluorescent antibody assays. We selected the nonproducer line, designated Ad12-SV40 line 1, and one representative producer line, Ad12-SV40 line 2, for further characterization.

In contrast to the organized growth of uninfected human epidermal keratino-

cytes (Fig. 1A), the Ad12-SV40–infected cells were pleomorphic, varying in size and shape (Fig. 1, B and C). A small proportion of the cells was multinucleated. The nonproducer Ad12-SV40 line 1 maintained a flatter, more uniform morphology (Fig. 1C). The cell lines had apparently unlimited life-spans, and each line has been successively subcultured for more than 50 passages over the course of 1 year with no evidence of decreased proliferative capability.

Evidence of the human origin of the two lines was obtained by isoenzyme analysis and cell membrane species-specific immunofluorescence. Moreover, both cell lines showed human karyotypes with a Y chromosome. When first analyzed at passage 6, both lines were aneuploid. By passage 10, Ad12-SV40 line 1 showed two marker chromosomes. Keratin, an epithelial cell marker, was revealed by Kreyberg stain (*10*) and indirect immunofluorescence with antiserum to human keratin (*11*). As revealed by electron microscopy, the cells contained intermediate filaments, tonofilaments, and defined desmosomes also characteristic of epithelial cells. When we analyzed their biologic properties, we observed that neither cell line grew in soft agar or produced tumors in 129J nude mice even when the mice were inoculated with 10^7 cells (Table 1). In some cases, small cystic nodules containing epidermoid cells appeared at the site of inoculation, but these nodules regressed within 1 to 2 weeks. Thus Ad12-SV40 infection was associated with the continued proliferative capacity of human epithelial keratinocytes in culture without the concomitant acquisition of the neoplastic phenotype.

Table 1. Biologic properties of human epidermal keratinocytes exposed to Ki-MSV or Ad12-SV40 virus. Ki-MSV (BaEV) was produced in human nonproducer cells (*12*) by superinfection with baboon endogenous virus (*13*). Ad12-SV40 virus (*8*) was grown in Vero cells. Nude mice were inoculated with 10^6 or 10^7 cells as indicated.

Cells	Passage in culture (number)	Agar colony formation (percent)	Nude mice with tumors	
			10^7	10^6
Primary human keratinocytes	<3	<0.01		
+ Ki-MSV	<3	<0.01		
+ Ad12-SV40	>50	<0.01	0/20	0/4
+ Ad12-SV40 + Ki-MSV	>50	0.1 to 0.5	16/18*	3/4*

*Tumors were re-established in tissue culture and confirmed as human; their resemblance to the cells of origin was determined by karyologic analysis.

In experiments to determine which, if any, of the transforming genes in the Ad12-SV40 hybrid virus genome was actively transcribed in the altered human epithelial cells, polyadenylated [poly-(A)$^+$] RNA from these cells was fractionated in an agarose gel and then successively hybridized to one of three ^{32}P-labeled DNA probes (Fig. 2A): pAT153-Ad12 E1A DNA, which carries the Ad12 E1A gene (panel a); pAT153-Ad12 E1B DNA, which carries the Ad12 E1B gene (panel b); and SV40 DNA, which includes the genes for both large T and

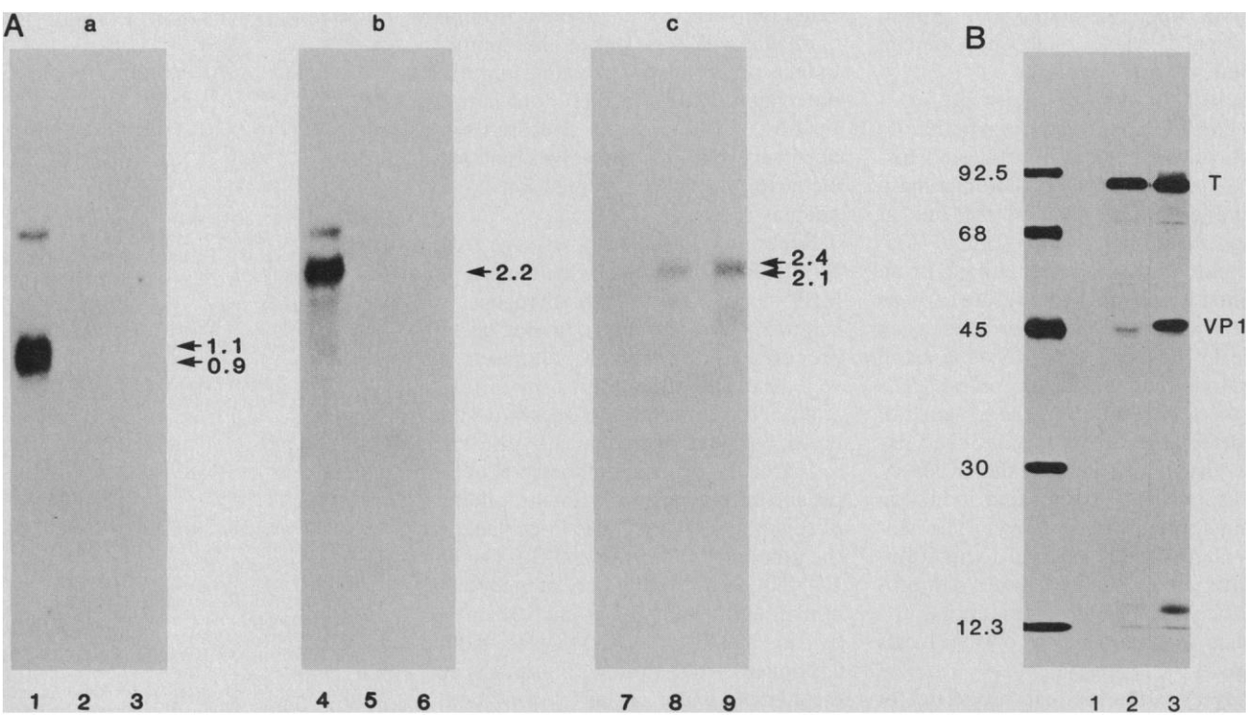

Fig. 2. Characterization of Ad12-SV40 early gene expression in Ad12-SV40–altered human epidermal cell line. (A) Detection of virus-specific mRNA's (*15*). Poly(A)$^+$ RNA from the Ad12-transformed cell line (lanes 1, 4, and 7), the Ad12-SV40 human epithelial cell line (lanes 2, 5, and 8), or the SV40-transformed cell line (lanes 3, 6, and 9) was fractionated by electrophoresis on a 0.9 percent agarose gel in the presence of formaldehyde. The RNA was then transferred to a nitrocellulose membrane and hybridized to one of three ^{32}P-labeled DNA probes (*15*): Ad12 E1A (*16*) (panel a), Ad12 E1B (*16*) (panel b), or SV40 (panel c). The positions of the viral transcripts are indicated (kilobases). (B) Immunoprecipitation of SV40 T antigen (*17*). Extracts from cells that had been labeled with [^{35}S]methionine for 4 hours were subjected to indirect immunoprecipitation with either control hamster serum (lane 1) or hamster SV40 tumor antiserum (lanes 2 and 3). The cell extracts used were from either the Ad12-SV40 human epithelial cell lines (lanes 1 and 2) or SV40-infected African green monkey kidney cells (lane 3). The precipitates were analyzed on a 10 percent SDS-polyacrylamide gel. The molecular weight markers (in thousands) were phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, and cytochrome c.

small t antigens (panel c). Besides the RNA from the human Ad12-SV40 epithelial cell line 1 (lanes 2, 5, and 8), two other RNA's were included as positive controls: C3AT1 RNA (lanes 1, 4, and 7) from a C3H mouse cell line that has been stably transformed by Ad12; and 3T3WT RNA (lanes 3, 6, and 9) from a BALB/c mouse 3T3 cell line transformed by SV40. The results indicate that the Ad12-SV40–transformed human epithelial cell line had no detectable transcripts from the early 1 region of Ad12 but had substantial amounts of messenger RNA (mRNA) from the transforming region of SV40.

For confirmation that the early SV40 transcripts were translated in the Ad12-SV40–transformed cell line, immunoprecipitation was performed with a hamster SV40 tumor antiserum on an extract from cells that had been labeled with [^{35}S]methionine (Fig. 2B). Analysis of the resulting immunoprecipitates on a sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE) revealed both large T and small t antigens of SV40, showing that the transforming proteins of SV40 were expressed in this human epithelial cell line. Thus, only the SV40 T antigens of the transforming proteins coded for by Ad12-SV40 hybrid virus could be responsible for maintaining the growth properties of these established human keratinocyte cell lines.

The lack of tumorigenicity of Ad12-SV40 line 1 led us to inquire whether its growth properties might be altered further by addition of a virus containing a *ras* oncogene. Infection of the line at passage 10 with Ki-MSV (BaEV) (baboon endogenous virus) resulted in an alteration in cell morphology. As early as 5 to 6 days after infection, the cells began to pile up in focal areas, forming small projections and releasing round cells from the foci (Fig. 1D). The absence of any detectable alteration induced by helper virus alone implied that Ki-MSV was responsible for the rapid induction of transformed morphology. The Ki-MSV–altered cells released focus-forming virus and contained K-*ras* p21 protein (data not shown), confirming the presence and expression of the K-*ras* oncogene.

Analysis of the biologic properties of the doubly transformed cells revealed that their saturation density was approximately three times higher than that of Ad12-SV40 line 1 cells. Moreover, double transformants grew in soft agar with colony-forming efficiencies from 0.1 to 0.5 percent. When newborn and adult 129J nude mice were inoculated subcutaneously with as few as 10^6 Ki-MSV–transformed Ad12-SV40 line 1 cells (Table 1), the animals developed invasive, rapidly progressive tumors within 3 weeks. Such tumors were diagnosed as squamous cell carcinomas (Fig. 1E) with characteristic keratin pearls. Cell lines established from the tumors were readily transplantable and were confirmed as being of human origin.

Our results appear to represent the first induction of human epithelial cancer cells in culture. At least two and possibly more alterations in cellular growth properties seem to be required. One measurable event was the acquisition of apparently unlimited growth potential. This occurred at low frequency after infection with Ad12-SV40 hybrid virus and did not appear to be a function of productive or nonproductive interaction of the virus with the cells. In addition to their acquisition of continuous proliferative capacity, the Ad12-SV40–altered human epithelial cells showed an abnormal karyotype at the earliest measurable time. Nonetheless, despite their having properties characteristic of most malignant cells, the Ad12-SV40 continuous human epithelial cell lines lacked the ability to induce progressively growing tumors in nude mice. Thus, the alterations induced by Ad12-SV40 infection appeared to be necessary but by themselves not sufficient for complete progression to the neoplastic state.

Superinfection of early passage Ad12-SV40–altered epithelial cells with Ki-MSV, a virus with a *ras* oncogene, resulted in a further change in their growth properties. Morphologic alteration as well as the abilities to grow in soft agar and to form rapidly progressive squamous cell carcinomas in athymic mice appeared to be concomitantly acquired properties associated with the addition of this *ras* oncogene. The importance of the combined effects of Ad12-SV40 and Ki-MSV in the induction of neoplastic human epithelial cells is implied further by the inability of Ki-MSV to induce continued proliferation of primary epithelial cells under our assay conditions.

Analysis of the mechanisms taking part in transformation of rodent fibroblasts by adenoviruses and papovaviruses has revealed distinct immortalization and transforming functions of these viruses (*2*). Moreover, studies have shown that human *ras* oncogenes can complement the EIA gene of adenovirus (*3*) and cellular genes such as *myc* (*4*) in inducing the neoplastic phenotype in primary rat fibroblasts in tissue culture. Our results show that a multiple-step model for neoplastic conversion appears to be applicable to epithelial cells and to cells of human origin.

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