$8\pi = 1.4 \times 10^{-8}$ dyne cm^{-2} or 9×10^3 eV cm^{-3}. The ICE radio astronomy investigation derived a measure of electron density (670 cm^{-3}) and temperature (1.3×10^4 K) in the current sheet from the plasma noise spectrum (*13*). The corresponding electron pressure is 750 eV cm^{-3}, which is substantially less than is needed to balance the magnetic pressure. If the additional pressure is provided by ions, they must be significantly hotter than the electrons; that is, their pressure must be 11 times larger, so that the ion temperature, T_i, is 14.3×10^4 K or 12 eV. Alternatively, a small density (~10 cm^{-3}) of hot electrons (~1 keV) could supply the needed pressure.

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Neoplastic Conversion of Human Keratinocytes by Adenovirus 12–SV40 Virus and Chemical Carcinogens

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Efforts to investigate the progression of events that lead human cells of epithelial origin to become neoplastic in response to carcinogenic agents have been aided by the development of tissue culture systems for propagation of epithelial cells. In the present study, nontumorigenic human epidermal keratinocytes immortalized by adenovirus 12 and simian virus 40 (Ad 12–SV40) were transformed by treatment with the chemical carcinogens *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or 4-nitroquinoline-1-oxide. Such transformants showed morphological alterations and induced carcinomas when transplanted into nude mice, whereas primary human epidermal keratinocytes treated with these chemical carcinogens failed to show any evidence of transformation. This in vitro system may be useful in assessing environmental carcinogens for human epithelial cells and in detecting new human oncogenes.

IT IS NOW ACCEPTED WIDELY THAT cancer arises in a multistep fashion and that environmental exposures, particularly to chemical, physical, and biological agents, are major etiological factors (*1–3*). The malignant transformation of human cells in culture by carcinogenic agents permits analysis of alterations and evolution in cell properties that may be representative of cancer cells. Although most models of neoplastic transformation of human cells in vitro by carcinogenic agents were based on the use of fibroblasts, which are easy to culture (*4–11*), neoplastic transformation of human skin fibroblasts in culture has not been readily achieved. Infection by certain DNA tumor viruses and, rarely, by chemical carcinogens has led to the development of established, karyologically abnormal lines of fibroblasts that are tumorigenic in suitable experimental models. Since most human tumors are of epithelial origin, it is important to study such cell systems. However, because of the inability until recently to grow epithelial cells and subsequently transformed human epithelial cells in vitro, it has been difficult to study the genetic alterations involved in the process of malignant transformation of human epithelial cells. Although epithelial cell cultures derived from numerous organs of rodent model systems (*12–16*) have been neoplastically transformed by chemical carcinogens, there are few reports describing carcinogen-induced transformation of human epithelial cell cultures (*17–19*).

We recently developed an in vitro multistep model for human epithelial cell carcinogenesis (*20*). Primary human epidermal keratinocytes acquired an indefinite lifespan in culture but did not undergo malignant conversion in response to an adenovirus 12–simian virus 40 (AD 12-SV40) hybrid. The addition of Kirsten murine sarcoma virus (Ki-MSV), which contains a K-*ras* oncogene, to these cells induced striking morphological alterations and led to the acquisition of neoplastic properties (*20*). The availability of a human epithelial cell line that could undergo neoplastic conversion in response to a *ras* oncogene led us to inquire whether this system might be useful in detecting chemical carcinogens for human epithelial cells.

Primary human foreskin epithelial cells were initiated in NCTC 168 medium with 10 percent horse serum containing antibiotics (gentamicin, 100 μg/ml, and fungizone, 25 μg/ml) (*21*). The human epidermal keratinocyte line, designated RHEK-1, was established from primary human foreskin epidermal keratinocytes after they had been infected with Ad 12–SV40 virus (*20*). This line did not produce virus, had a "flat" epithelial morphology, and possessed a number of markers associated with epithelial cells. The line contained SV40 tumor antigens but was not tumorigenic in nude mice. Growth and maintenance medium consisted of Dulbecco's modified minimum essential medium (DMEM) with 15 percent fetal bovine serum (FBS), hydrocortisone (HC; 5 μg/ml), and antibiotics (gentamicin, 100 μg/ml, and fungizone, 25 μg/ml) (DMEM + 15 percent FBS + HC).

After exposure of primary human epidermal keratinocytes to various doses of the chemical carcinogens *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) or 4-nitroquinoline-1-oxide (4NQO), no morphological differences between treated and control untreated cultures could be seen. Neither control nor treated cultures were able to grow serially beyond two to three subcultures. The cells underwent progressive deterioration and were lost. In the RHEK-1 line exposed to MNNG at either 0.1 or 0.01 μg/ml, morphological alterations of cells and an abnormal pattern of growth were noted by the sixth subculture, 52 to 62 days after treatment; similar changes were not observed in the control RHEK-1 cells treated with dimethylsulfoxide (DMSO).

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Changes were first observed in the cells treated with MNNG at 0.1 μg/ml; however, alterations in the cells treated with MNNG at 0.01 μg/ml became more pronounced after further subcultivation.

When RHEK-1 cells were treated with 4NQO at 0.1 μg/ml, morphological alteration was observed in the seventh subculture, 62 days after treatment, but a dose of 4NQO at 1.0 μg/ml was lethal. The morphological changes observed in these cultures were similar to those observed with Ki-MSV (20)—namely, the transformed cells began to pile up in focal areas, formed small projections, and released round cells from the foci (Fig. 1, A–C). These foci grew in chains or as islets that stained heavily with Giemsa. In contrast, the cellular morphology remained unchanged in the untreated human RHEK-1 epithelial cell line, which continued to grow as nonoverlapping round to polygonal adherent cells that were flat and cobblestone-like in appearance (Fig. 1D).

Table 1. Biological properties of the RHEK-1 human epidermal line transformed by chemical carcinogens. One day after primary human epidermal keratinocytes or the 13th subculture of the RHEK-1 line were plated at 10^6 cells per 100 mm in Falcon plastic dish (*20*), the medium (DMEM ± 15% FBS + HC) was removed and replaced with medium containing MNNG at various concentrations (0.1 μg/ml to 0.01 μg/ml) or 4NQO (1.0 μg/ml and 0.1 μg/ml) in 0.5% dimethyl sulfoxide (DMSO). The control medium contained 0.5% DMSO. After 1 day of treatment with carcinogens, the cultures were washed, fed again with carcinogen-free growth medium, and subsequently passaged by trypsin treatment every 7 to 10 days. Cultures were observed biweekly for changes in morphology or growth pattern. At various times, cultures were fixed in alcohol and stained with Giemsa for further microscopy. Untreated RHEK-1 cells showed no alterations over more than 50 passages in culture. Similar results were obtained in repeated experiments. Saturation density was measured as the maximum number of cells obtained after initial planting with 5×10^3 cells/cm^2 and then incubating at 36°C, with growth medium changed every 3 days. Cell suspensions (1×10^5 cells/ml) were plated in 0.33% soft agar medium containing 10% fetal bovine serum. Visible colonies were scored at 21 days.

Cell line	Saturation density ($\times 10^5$/cm^2)	Soft agar colony formation (%)	No. with tumors per no. inoculated
DMSO (0.5%)	1.9	<0.01	0/19
MNNG (0.1 μg/ml)	5.8	0.6	16/16*
MNNG (0.01 μg/ml)	6.4	0.8	18/18*
4NQO (0.1 μg/ml)	8.2	0.7	16/17*

*Tumors were reestablished in tissue culture and confirmed as human; their resemblance to the cells of origin was determined by karyological analysis.

The chemically transformed cells were further characterized by quantitative differences in growth properties, such as saturation density and soft agar colony-forming efficiency associated with the neoplastic phenotype. The saturation densities of the chemical transformants were approximately three or four times higher than that of the untreated RHEK-1 cells (Table 1). Moreover, the chemical transformants grew in soft agar with colony-forming efficiencies of 0.6 to 0.8 percent, whereas the untreated cells did not grow in soft agar (Table 1).

Evidence of the human origin of all the cell lines was obtained by isoenzyme analysis and species-specific cell membrane immunofluorescence. The relatedness of the transformed cells to the parental RHEK-1 cells was further established by chromosome analysis. Untreated RHEK-1 cells and transformed cells had similar marker chromosomes, as detected by conventional staining (*20*). Moreover, there were no major changes in chromosome number. The cells for the most part were near diploid, like the RHEK-1 parent. A small fraction of polyploid cells was observed in both treated and untreated cultures.

When 129J nude mice were inoculated subcutaneously with 10^7 chemically transformed cells, the animals developed tumors within 3 to 4 weeks. Such tumors were diagnosed as squamous cell carcinomas (Fig. 1E). The cells were arranged in rows or columns, and individual cells often contained keratohyalin granules or prekeratin. Cultures established from the tumors resembled the carcinogen-treated cells (Fig. 1F), were confirmed as human, and resembled the cells of origin by karyological analysis. In contrast, subcutaneous inoculation of 10^7

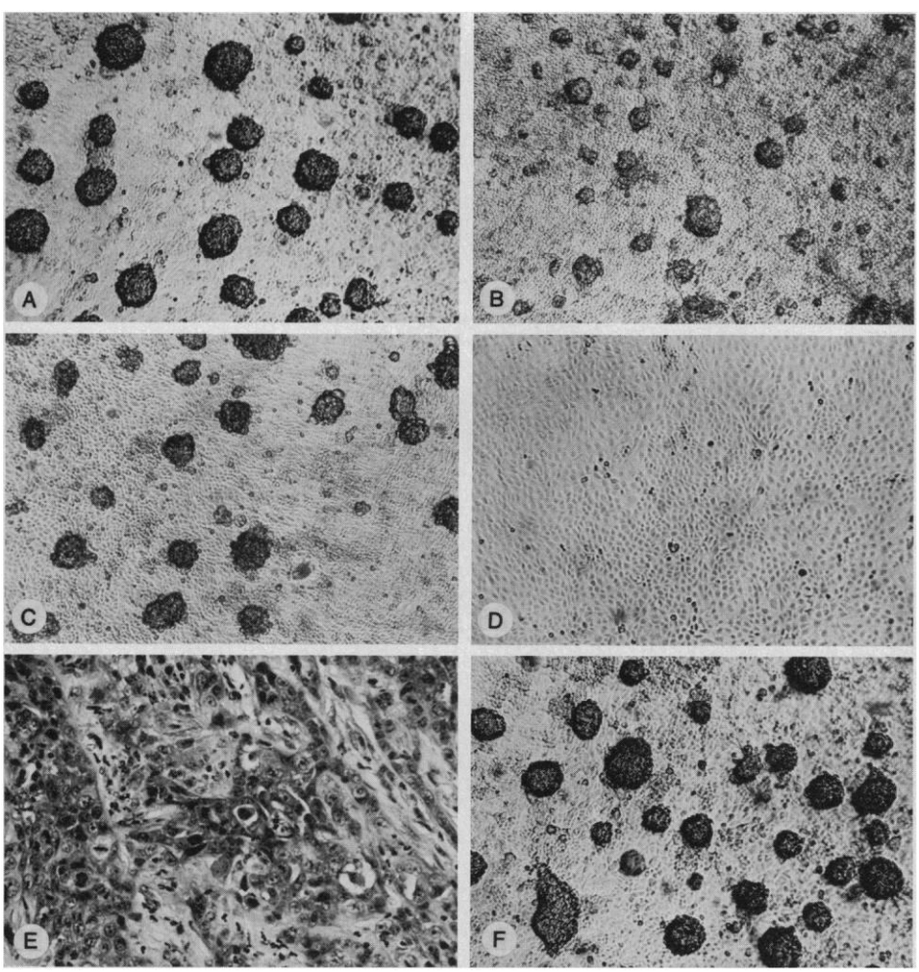

Fig. 1. Human epidermal keratinocyte cells (RHEK-1) treated with chemical carcinogens for 1 day, followed by seven subcultures in nutrient medium. (A) 0.01 μg/ml MNNG. (B) 0.1 μg/ml MNNG. (C) 0.1 μg/ml 4NQO. (D) Untreated (0.5% DMSO). (E) In vivo tumor induced by RHEK-1 cells treated with 4NQO (0.1 μg/ml). Moderately well differentiated squamous cell carcinoma. (F) Typical field of a culture originated from a primary tumor induced by RHEK-1 cells treated with 4NQO (0.1 μg/ml).

untreated RHEK-1 cells into nude mice produced only regressing cystic nodules that contained epidermoid cells (Table 1).

Since RHEK-1 cells can be transformed by Ki-MSV infection and become tumorigenic (*20*), we analyzed the *ras* oncogene p21 product in the chemically transformed as well as in the Ki-MSV–transformed RHEK-1 cells by using antibody to p21 and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (*22*). In contrast to the findings in the Ki-MSV–transformed cells, neither altered mobility nor increased expression of p21 was observed in the chemically transformed RHEK-1 cells (Fig. 2). Moreover, the DNA's from these chemically altered cells failed to induce detectable transformed foci upon transfection of NIH 3T3 cells. These results argue against activation of *ras* oncogenes in the chemically transformed human epithelial cell lines analyzed.

The present results appear to represent the first induction of human epithelial cancer cells in culture by the concerted action of a DNA tumor virus and chemical carcinogens. At least two and possibly more alterations in cell growth properties seem to be required. One measurable event was the acquisition of apparently unlimited growth potential as a result of Ad 12–SV40 infection (*20*). Treatment of nontumorigenic early-passage Ad 12–SV40 immortalized epithelial cells with chemical carcinogens resulted in further changes in their growth properties. Morphological alterations as well as the abilities to grow in soft agar and to form rapidly growing squamous cell carcinomas in athymic nude mice appeared to be concomitantly acquired properties of the chemically transformed cells.

The significance of the combined action of Ad 12–SV40 virus and chemical carcinogens in the induction of neoplastic human epithelial cells is emphasized by the inability of chemical carcinogens to induce continued proliferation of primary epithelial cells under our assay conditions. Thus, chemical carcinogens are similar to Ki-MSV in their ability to complement Ad 12–SV40 virus in fully transforming human epidermal cells. Unlike the rapid transformation of RHEK-1 cells observed after Ki-MSV infection (*20*), growth alterations associated with chemical carcinogen treatment were delayed in their appearance and required several subcultures for visualization. These findings suggest that multiple cell divisions are required for fixation and expression of the transformed phenotype in response to the carcinogen. It is possible that more than one genetic lesion may be required as well. Cooperating cellular or viral oncogenes have also been shown to induce malignant transformation of embryonic rodent fibroblasts (*23–27*). In addition, the combined action of tumor viruses and chemical carcinogens has been demonstrated to produce neoplastic transformation of rodent fibroblasts (*28–30*). Our ability to obtain malignant transformants as a result of chemical carcinogen treatment of Ad 12-SV40–altered human epidermal cells provides additional support for a multistep process of neoplastic conversion.

Earlier studies have shown that a continuous line derived from a human osteosarcoma (HOS) treated with MNNG acquired altered growth properties including tumorgenicity in nude mice (*31*). This altered phenotype was later shown to be associated with the activation of a previously uncharacterized cellular transforming gene, designated MET (*32*). The reproducible transformation of the RHEK-1 human epithelial line by chemical carcinogens suggests that cellular oncogenes may be activated as part of the process. Our evidence further indicates that *ras* oncogenes, which have been commonly implicated in chemical carcinogen–induced animal tumors and spontaneous human tumors (*33, 34*) were not activated in the transformants so far analyzed. Thus, this system may be useful in efforts to detect and characterize other cellular genes that can contribute to the neoplastic phenotype of human epithelial cells.

Certain carcinogenic polycyclic hydrocarbons have been identified in our environment, and some chemicals are known definitely to cause cancer in humans. Since it is estimated that 80 percent of human cancers are epithelial in origin, the human epithelial cell system described here may be a useful in vitro tool for screening potential carcinogenic agents.

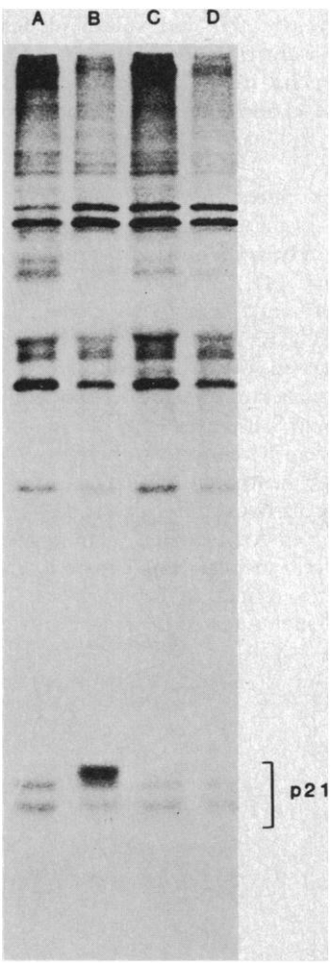

Fig. 2. Analysis of *ras* oncogene p21 product in RHEK-1 cells exposed to chemical carcinogens. [^{35}S]Methionine-labeled cell extracts from (A) untreated RHEK-1 cells, (B) Ki-MSV–transformed RHEK-1 cells, (C) MNNG (0.01 μg/ml)-transformed RHEK-1 cells, or (D) 4NQO (0.1 μg/ml)-transformed RHEK-1 cells were immunoprecipitated with anti-p21 monoclonal antibody Y13-259 (*35*) and analyzed by SDS-PAGE as described previously (*22*).

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Regulation of Erythrocyte Cation and Water Content in Sickle Cell Anemia

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The pathophysiological events in sickle cell disease are critically dependent on the intracellular concentration of hemoglobin S, which varies inversely with cell cation and water content. Erythrocytes of SS homozygotes exposed to oxygen or carbon monoxide decrease their potassium and water content through a pathway for potassium transport that is activated by both cell swelling and decrease in internal *p*H. This pathway is not inhibited by ouabain either with or without bumetanide. When SS erythrocytes were separated according to density, the *p*H- and volume-dependent potassium transport was greatest in the least dense fraction and was reduced in the densest cells. This pathway, which does not depend on polymerization of sickle hemoglobin, may be important in regulating the cation and water content of SS erythrocytes.

THE POLYMERIZATION OF DEOXYHEMOGLOBIN S is the proximate cause of the vaso-occlusive and hematologic manifestations of sickle cell disease. This concerted process is markedly dependent on the intracellular concentration of hemoglobin S (*1*). Therefore, alterations in cell volume would be expected to influence the pathogenesis of sickle cell disease. We recently reported that red cells of patients homozygous for hemoglobin C (CC) can regulate their volume through a pathway for K transport that is not active in normal AA erythrocytes (*2*). This pathway depends on cell volume and *p*H and is not inhibited by ouabain or bumetanide (inhibitors of the Na-K pump and of the Na-K-Cl cotransport). This pathway is probably responsible for the reduced K and water content observed in CC erythrocytes. We have tentatively attributed the activation of the volume-dependent K transport in CC erythrocytes, which does not occur in AA erythrocytes, to the presence of the more positively charged hemoglobin C, perhaps through an electrostatic interaction (*3*) between hemoglobin C and components of the red cell membrane.

Since hemoglobin S, like hemoglobin C, is less negatively charged than hemoglobin A [replacement of the glutamic acid at position 6 in the β chain of hemoglobin A by valine in hemoglobin S and lysine in hemoglobin C (*4*)] and since erythrocyte water content and hemoglobin concentration (MCHC) are important determinants in the pathogenesis of sickle cell disease, we investigated whether erythrocytes containing hemoglobin S also express a volume- and *p*H-dependent K transport pathway.

Experiments were carried out in oxygenated or carbon monoxide–treated erythrocytes from four patients homozygous for sickle cell anemia (SS). To overcome the problems related to heterogeneity of cell cation and water content (*4*, *5*), we used either the nystatin technique, to make cells homogeneous for cation and water content, or the Stractan method, to separate red cells according to density (*6*). In brief, the red cell membrane is made permeable to Na and K by exposure to nystatin; the Na, K, and water content are adjusted to desired values; and the nystatin is removed to restore Na and K permeabilities to normal.

When SS red cells were treated with nystatin to make their Na, K, and water contents similar to those of normal AA cells, the ouabain- and bumetanide-resistant K efflux showed a *p*H and volume dependence similar to those described for CC cells. The *p*H-dependence of K efflux gave a bell-shaped curve, with a *p*H optimum of 6.7 to 7.0 and inhibition at both acid and alkaline *p*H. Cell swelling induced by hyposmotic media (220 instead of 300 mos*M*) resulted in a four- to fivefold increase in K efflux.

Similar studies were performed in SS cells isolated with the Stractan density gradients. Three fractions are obtained: the top fraction contains the lightest cells, including reticulocytes; the middle fraction contains the mature discoid cells; and the bottom fraction contains the most dense cells, including irreversibly sickled cells (*6*).

The ouabain- and bumetanide-resistant K efflux was different in the three fractions of SS erythrocytes and was differently affected by changes in cell volume (obtained by altering the osmolarity of the media) (Table 1). In the top fraction, the K efflux was stimulated by hyposmolarity and reduced to almost normal values by hypertonicity. In contrast, the K efflux from the bottom fraction was small and showed much less volume dependence. However, the different hemoglobin concentrations and cation and water content of the three fractions could have been responsible for these differences. Therefore the cells were first separated with

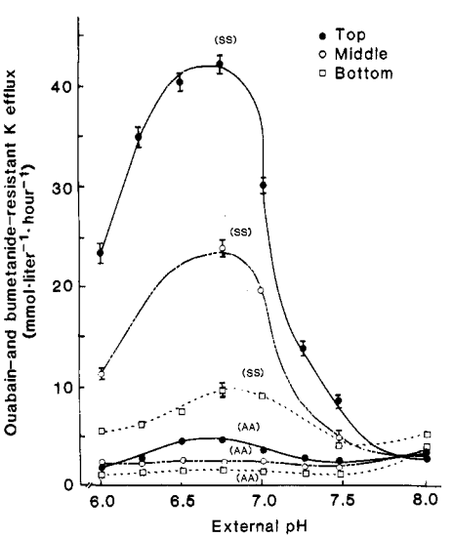

Fig. 1. Dependence on *p*H of the ouabain- and bumetanide-resistant K efflux from the top, middle, and bottom fractions of SS and AA erythrocytes separated on a discontinuous Stractan density gradient. The red cells had been incubated earlier with carbon monoxide. After separations, the fractions were treated with nystatin to obtain similar Na, K, and water contents. MCHC values before and after nystatin treatment for top, middle, and bottom fractions of SS cells were 32.6 and 31.1 g/dl, 37.8 and 32.1 g/dl, and 48.6 and 35.3 g/dl, respectively, and for the three fractions of AA cells were 31 and 31 g/dl, 32.6 and 32.2 g/dl, and 34.1 and 32.2 g/dl, respectively. K efflux was measured in room air with media containing 140 m*M* NaCl, 1 m*M* $MgCl_2$, 10 m*M* tris-MOPS (*p*H 6.0 to 8.0), 10 m*M* glucose, 0.1 m*M* ouabain, and 0.01 m*M* bumetanide. The incubation times were 5 and 25 minutes at 1% hematocrit (*6*). Results are means ± SEM of the fluxes. Similar results were obtained in two other patients and in one normal control.

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