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Enhanced Virus Transformation of Hamster Embryo Cells In Vitro[1]

JOSEPH H. COGGIN, JR.
Department of Microbiology, University of Tennessee, Knoxville, Tennessee 37916

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Since transformation of hamster cells in vitro by simian virus 40 (SV40) is a rare event in a homogeneously infected cell population, physiochemical studies of the events of virus transformation are difficult. Similarly, other deoxyribonucleic acid-containing oncogenic viruses produce transformed-cell foci in vitro with low efficiency. Sublethal doses of X-ray irradiation, as well as preincubation of hamster embryo cells with the radiomimetic analogue, 5-iododeoxyuridine, markedly sensitized hamster embryo cells to SV40 in vitro. Agents were used at dosages which neither produced lethality nor caused neoplastic transformation in the absence of virus. Embryo cells maintained in vitro for long periods of time became increasingly more sensitive to SV40 transformation. Radiation also stimulated transformation by adenovirus 31. Delay in the addition of virus to preirradiated cells reduced the susceptibility to transformation by SV40 which was observed for cells infected immediately after irradiation, suggesting that radiation repair mechanisms or, possibly, release from radiation-induced "mitotic delay" may interfere with the process of neoplastic conversion by SV40.

Production of tumors in hamsters by simian virus 40 (SV40) at an extended period after neonatal infection with the virus is well documented (4, 5, 6). Intracellular events leading to transformation by oncogenic viruses are necessarily obscured from direct biochemical study in vivo. In vitro transformation of primary cells by deoxyribonucleic acid (DNA) tumor viruses has been demonstrated by a number of investigators (1, 11, 13), and the results indicate that neoplastic conversion occurs, but with low frequency, in a uniformly infected population. Biochemical study of the events of transformation of primary cells in vitro has been limited because of this low frequency and also because of the poor plating efficiency of primary cells. Todaro and Green (16, 17) used a mouse cell line, 3T3, to measure early events in SV40-induced transformation in heteroploid cells. This cell line, although an established cell culture, retains the "normal" quality of contact inhibition, and the 3T3 cells plate with high efficiency. A reliable number of 3T3 cells lose the trait of contact inhibition when exposed to SV40, and this cellular change is used as an index of neoplastic conversion.

Stoker (14) observed that the frequency of neoplastic transformation by polyoma virus increased among BHK-21 hamster cells that survived X-ray irradiation. Exposure of mouse fibroblast cells to radiomimetic, thymidine analogues enhanced the efficiency of SV40 transformation fivefold (16), and Pollock and Todaro (10) reported recently that X-ray irradiation prior to and after infection stimulated SV40 transformation of 3T3 cells and human fibroblasts in culture sevenfold. X-ray irradiation and hydrocortisone treatments were reported to interfere with SV40 transformation of human amnion cells (3).

Results reported here establish the roles of X-ray irradiation, a thymidine analogue, and aging in sensitizing primary hamster embryo cells to SV40 transformation in vitro. Cultural procedures for conducting these studies with primary cells are described. Irradiation stimulated adenovirus 31 transformation of hamster cells also. Systems described afford a new in vitro working model which parallels the in vivo investigation of SV40 tumorigenesis in neonatal hamsters.

MATERIALS AND METHODS

Preparation of tissue. For primary tissues, kidneys and lungs were removed aseptically from 14- to 16-day embryos of Syrian Golden hamsters. Organs were freed of connective tissue, minced, trypsinized, and prepared for tissue culture as previously described (8). Cells were suspended for overlay culture or clon-

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ing in a medium of the following composition: Eagle's minimal medium (EMMS) containing a double complement of vitamins and amino acids, 45 μg of crystalline bovine serum albumin per ml, 0.5% lactalbumin hydrolysate, 15% fetal calf serum, antibiotics, and 0.3% algal hydrolysate. This medium was mixed at a ratio of 4:1 with "conditioned," spent medium, prepared by exposure of EMMS to primary embryo hamster fibroblasts for 4 days, and was subsequently filtered. Rapid lowering of the *p*H of the medium with 40% CO_2 tension immediately upon seeding of the cells (8) was essential for high cloning and plating efficiency of the primary tissue. Cells suspended in growth medium were seeded into 60-mm plastic culture dishes and maintained in a 5% CO_2 atmosphere upon completion of the attachment phase.

Cell treatment. After 5 to 7 days in culture, monolayers were treated with either irradiation (Maxitron 300 X-ray machine equipped with 0.5-mm aluminum and 0.42-mm copper filters delivering 549 r/min at a distance of 22 cm) or 5-iododeoxyuridine (IUDR), adsorbed with SV40 or adenovirus 31 for 3 hr, and subsequently detached by brief exposure to 0.1% trypsin. Treated cells and control cells were washed with fresh medium and seeded into culture dishes (60 by 15 mm), or single cells were captured by capillary force in acid-cleaned, sterile hematocrit tubes (microtubes) under microscopic observation. All cultures were incubated in a 5% CO_2 atmosphere at 37 C.

The prototype strain of SV40, VA 45-54, prepared in grivet monkey kidney cells, with a titer of $10^{7.8}$ $TCID_{50}$/0.2 ml [$10^{6.5}$ plaque-forming units (PFU) in BSC cells] was used throughout. Multiplicity of infection (MOI) was 600. Adenovirus 31 (Pereira) pools each possessing a titer of $10^{6.3}$ $TCID_{50}$/0.2 ml in human embryonic kidney cells were used at an MOI of 450. Microtube cultures derived from cloned cells were transferred into Leighton tubes at 6 days, and these cultures were examined periodically for signs of transformed progeny. Agar overlays were placed on dishes containing 200 (±20) isolated cell clusters each, and plates were observed daily for the presence of transformed foci. At the time of overlay, a representative number of dishes were used to determine the average number of viable, attached cells per dish per treatment. This number was taken as the initial number of clones and was used to determine cloning and transforming efficiency. The transforming efficiency, expressed as a percentage, is defined as the number of foci per dish divided by the average number of cells at the time of agar overlay.

Transformation assays. Individual cells reproduced rapidly with high cloning efficiency for 3 to 4 days in the capillary microtubes and could be removed to Leighton tubes after 6 days, after brief trypsinization. Complete monolayers were observed after 4 to 6 days, and transformed clones were easily distinguished from nontransformed clones by the "latticed" cell sheets and foci appearing by 16 to 20 days.

Sparse monolayers developed in 3 to 5 days when treated cells or control cells were seeded into culture dishes after infection with SV40. Double-strength EMMS medium was diluted 1:1 with 0.28% Special Agar (Noble; Difco) heated to 45 C, and the mixture was placed as overlay gel on the monolayers. Transformed cells produced foci of cell "mounds" after 15 days in the gel, whereas normal cells ceased to divide.

RESULTS

Cloning efficiency of hamster embryo cells. Cells, harvested from 10 to 15 embryos, were minced, trypsinized, and cultured in monolayer for 7 to 34 days. Some monolayers were passaged by 1:2 splits every 3 to 5 days. Cloning was performed by exposing the cells briefly to 0.1% trypsin, diluting the culture, then isolating individual cells or an occasional cell pair into 5-cm hematocrit tubes, and incubating them. Cloning efficiency is expressed as the percentage of the number of tubes producing viable cell populations upon transfer of cells from microtubes to Leighton tubes divided by the total number of tubes prepared initially.

Employing the microtube procedure, excellent cloning efficiencies were obtained with primary hamster cells in culture (Table 1). Increased cloning efficiency was observed as the explanted cells were subcultured through two passages in vitro and then tested for cloning efficiency.

Cloning efficiency of hamster embryo cells in 60-cm culture dishes for overlay experiments was somewhat dependent on the cell density, but generally for all experiments 2×10^4 cells were plated per dish, yielding an initial cloning efficiency of 2% which increased markedly with in vitro passage (Table 1). The overlay method was more convenient, but results reported in the following section were obtained employing both procedures.

Enhanced SV40 transformation. An experiment was conducted to determine whether preirradiation of hamster embryo cells altered the sensitivity of the cells to transformation by SV40 and adenovirus 31. Embryo cells were exposed, additionally, to IUDR prior to infection with SV40 to determine the effect of a radiomimetic chemical on SV40 transformation. Aged embryo cells possess-

TABLE 1. *Cloning efficiency of hamster embryo lung and kidney cells in culture*

No. of days post-explant into monolayer culture	Subculture no.	Cloning efficiency in	
		Microtubes	Culture dishes
		%	%
6	1	33	2.9
9	2	63	48.0
14	5	59	51.0
34	9	43	57.0

ing chromosomes with numerous "breaks" (8) associated with the senescent phase of growth were also tested for susceptibility to SV40 transformation and compared with cells from the same embryos in the early stages of culture (Table 2).

Data obtained when hamster embryo cells were infected with SV40 at various subcultures indicate that senescent cells (87 days old) were more than 20 times as sensitive to SV40 transformation than were young cells (9 days old) from the same cell harvest. Cells of intermediate age were found to show an intermediate sensitivity to SV40 transformation. Freezing and reintroduction of embryo cells into culture did not affect their sensitivity to SV40 or adenovirus 31 as compared to freshly explanted cells.

Pretreatment of embryo cells with X-ray at a sublethal (8) dose (150 r) sensitized the cells markedly to SV40 transformation (>50-fold). Previous studies (8) have established that radiation exposures to 250 r only temporarily delayed the normal proliferation rate of hamster embryo cells in culture and induced a 24-hr lag in DNA synthesis following irradiation. When virus infection was delayed for 10 hr after X-ray treatment, the transforming efficiency declined from 27.9 to 9.6%.

The thymidine analogue IUDR also increased the efficiency of SV40 transformation in vitro as shown in Table 2. However, the efficiency was only about 50% that observed for irradiated, SV40-infected cells. Addition of SV40 antiserum after virus adsorption did not affect the results.

Hamster embryo cells exposed to 150 r of X-ray irradiation and subsequently infected with adenovirus 31 were observed to produce increased numbers of transformed foci in vitro (Table 3).

DISCUSSION

Stoker (14) and Pollock and Todaro (10) reported that polyoma- or SV40-infected, established cells surviving X-ray irradiation showed an increased number of transformed colonies as compared with unirradiated controls. In their studies, the higher the levels of irradiation used, the greater the enhancement of transformation. Selection of a cell population which might be more susceptible to transformation or neoplasticity at the high levels of irradiation could not be eliminated. Radiation levels above 150 r did not significantly increase the transforming efficiency of SV40 in hamster embryonic cells in the system described here. This may be explained, in part, by the fact that embryo cells receiving irradiation in excess of 250 r fail to synthesize DNA at a normal rate for several days after irradiation, if ever, whereas normal rates of synthesis were achieved in cells receiving less than 250 r (8). Furthermore, radiation levels of 500 r of X ray and above inhibited 60 to 82% of normal DNA synthesis with the cells failing to recover a normal rate of synthesis and seriously reduced their differential growth potential (8). Todaro and Green suggested that active cell replication and DNA synthesis were essential to "fix" SV40 transformation of 3T3 cells in vitro (18), and radiation levels which permanently impair DNA synthesis might be expected to interfere with transformation by SV40 virus.

Borek and Sachs (2) observed that X-ray irradiation alone produced a low degree of trans-

TABLE 2. *Effects of X-ray irradiation, IUDR exposure, and aging on SV40 transformation of hamster embryo lung and kidney cells in the agar overlay assay*

Age and treatment of embryo cells	Day infected	Transforming-efficiency ratio[a]	Subculture number	Transforming efficiency (%)
Young cells + SV40	7	7/1,200	2	0.58
Young cells + SV40	9	3/623	3	0.48
Aged cells + SV40	40	21/1,255	8	1.67
Aged cells + SV40	63	82/1,178	12	6.96
Aged cells + SV40	87	104/1,857	20	5.60
Aged cells + CF[b]	87	0/1,205	20	0
X ray (150 r) + SV40	7	264/945	2	27.94
X ray (150 r) + CF	7	0/1,251	2	0
IUDR (25 μg/ml at 24 hr preinfection) + SV40	7	70/363	3	19.28
IUDR + CF[c]	7	0/227	3	0

[a] Transforming efficiency is defined as the percentage of the number of foci per dish divided by the average number of cell clones present at the time of agar overlay.
[b] Control fluid from noninfected cells which were used to produce the virus pools.
[c] IUDR reduced the cloning efficiency of embryo cells by 15.9%.

TABLE 3. *Effects of X-ray irradiation of hamster embryo cells on the transforming efficiency of adenovirus 31 in vitro*

Radiation treatment	Adenovirus-31 infected[a]	Ratio of no. of transformed foci to total cells cloned	Transforming efficiency[b]
			%
None	−	0/1,440	0
None	+	2/960	0.2
150 r	−	0/596	0
150 r	+	178/1,121	15.8
150 r	−	0/512	0
150 r	+	119/950	12.5

[a] Cells were infected with $10^{6.3}$ $TCID_{50}$ ($10^{5.8}$ PFU in human embryonic kidney cells) of adenovirus 31. Infected cells (MOI = 450) were inoculated at a density of 300 viable cells per dish. Radiation exposure was always given prior to infection. Virus was allowed to adsorb for 3 hr, at which time adenovirus 31 antiserum was added to the cell pellet prior to suspension of the cells for seeding.

[b] Transforming efficiency equals (number of transformed foci/total cells attached and viable 48 hr postseeding) × 100.

formation in hamster embryo cells and that the fixation of the transformed status required two cell generations after exposure. Employing my methods, I have never observed transformed foci among cells treated with X ray. Of 50 SV40 transformed clones derived from preirradiated SV40-infected cells examined by fluorescent staining, 47 contained SV40 specific T antigen. The possibility cannot be eliminated that the three clones containing no T antigen represent radiation-transformed cells; however, I have never observed transformed clones in irradiated, noninfected, cell cultures. Two randomly selected clones induced by SV40 after radiation pretreatment contained the surface, or S, antigen described by Tevethia et al. (15). These findings clearly suggest the intimate association between SV40 and the transformed cell, and it is unlikely that these cell clones resulted solely from radiation-induced transformation.

X-ray irradiation and IUDR (9) are known to induce lesions in chromosomes of cells, and results reported here and elsewhere (8, 14) show that these agents enhance SV40 or polyoma transformation of both established cells and primary hamster and human diploid-cell cultures. Radiation also sensitized hamster cells to the transforming influence of adenovirus 31. Jensen et al. (7) reported that senescent human cells in culture were more sensitive to rapid transformation by SV40 than were cells in early passages in cell culture. My results extend this observation to hamster embryo cells. Chromosome breaks are detected in increasing numbers as diploid human cells are subcultured in vitro (12).

The correlation between chromosome damage induced by X ray, IUDR, or aging and enhanced SV40 transformation in vitro suggests the possibility that the primary event in neoplastic transformation may involve DNA synthesis or DNA repair by the host cell. Perhaps during a period of repair of radiation-induced lesions, the virus is best able to become attached or integrated with the host genome and this new virus-host cell relationship ultimately leads to the neoplastic state.

In future work, it will now be possible to undertake pragmatic biochemical studies of the events of neoplastic transformation of primary cells using pretreatment with low level X-ray irradiation, since this procedure leads to the predictable transformation of 25% of the cell population to the neoplastic state. Most importantly, these studies can be performed with cells which are potential target cells in the much studied newborn hamster model system.

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