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Interactions between adenovirus, a tumor promoter, and chemical carcinogens in transformation of rat embryo cell cultures

(phorbol esters/cocarcinogenesis/polycyclic aromatic hydrocarbons/viral carcinogenesis)

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ABSTRACT **The tumor-promoting agent 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) caused a 2- to 3-fold enhancement of transformation of secondary rat embryo cells that had been infected with a temperature-sensitive mutant of adenovirus type 5 (H5ts125). In addition, transformed foci appeared earlier and were larger in cultures grown in the presence of TPA. The addition of TPA could be delayed until up to 7 days after viral infection and still enhancement was observed. Exposure of the cells to 7,12-dimethylbenz[*a*]anthracene or benzo[*a*]pyrene prior to H5ts125 infection also resulted in a 2- to 4-fold enhancement of transformation, and this enhancement was further augmented 2- to 3-fold when cells were grown in TPA after virus infection. Whereas TPA did not enhance the cloning efficiency of normal rat embryo cells, it did enhance the cloning efficiency of isolated colonies of adenovirus-transformed cells when these were grown alone or cocultured with a 100-fold excess of normal rat embryo cells. The enhancement of adenovirus transformation by TPA appears to be due to its ability to facilitate expression of the transformed state rather than an effect on virus uptake or integration.**

It is now well recognized that the carcinogenic process is often multifactor in terms of etiology and multistep in its evolution (1). One of the earliest demonstrations of this was the study by Rous and Kidd (2) demonstrating that wounding or chemical treatment led to the appearance of skin tumors on the ears of rabbits that had been infected with Shope papilloma virus. One of the most extensively studied multistep animal models is that of two-stage mouse skin carcinogenesis (3) in which application of a subcarcinogenic dose of an "initiating carcinogen" followed by repeated application of a "promoting agent" results in the induction of multiple skin tumors. The most potent promoting agents for mouse skin are 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and related plant diterpenes (4–6). Recent studies indicate that TPA enhances the *in vitro* transformation of fibroblast cell cultures previously exposed to either polycyclic aromatic hydrocarbons (7, 8) or ultraviolet light (9).

The purpose of the present study was to determine whether TPA might also enhance the *in vitro* transformation of cells previously infected with a transforming virus. The virus chosen for our studies is a temperature-sensitive mutant of adenovirus type 5 (H5ts125), which has a higher transforming efficiency than wild-type adenovirus type 5 (10–12). Since initiating carcinogens are known to act synergistically with certain viruses in tumor induction in the whole animal (13, 14) and during cell transformation *in vitro* (13–16), we have also examined the effects of exposing our cell cultures to polycyclic aromatic hydrocarbons prior to the addition of H5ts125 and TPA.

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MATERIALS AND METHODS

Virus Infection and Cells. Adenovirus H5ts125 was grown and titrated on KB cells, as described (10). Primary rat embryo cells were established from 14-day-old Sprague–Dawley rat embryos as described by Fisher and Goldstein (17), using Dulbecco's modified Eagle's minimum essential medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. Three cloned populations of H5ts125-transformed rat embryo cells designated A18 (isolated from cultures exposed only to virus), C5 [isolated from cultures treated with virus and 0.05 μg of dimethylbenz[*a*]anthracene (Me_2BzA) per ml], and E11 [isolated from cultures treated with virus and 0.05 μg of benzo[*a*]pyrene (BzP) per ml] were used for clonal and cocultivation studies. These clones were isolated as described below.

Viral Transformation Assays. Transformation assays were performed on secondary rat embryo cultures as described (18). Approximately 2×10^6 rat embryo cells were pretreated with 0.1–0.0125 μg of Me_2BzA per ml or 0.5–0.05 μg of BzP per ml for 18 hr. The carcinogen-containing medium was removed, the cells were washed twice with Ca^{2+}- and Mg^{2+} free phosphate-buffered saline, and the cells were then infected with 30 plaque-forming units of H5ts125 per cell (10–12) in 0.2 ml of phosphate-buffered saline for 3 hr at 37°. Control cultures were treated with either 0.5% acetone (carcinogen solvent) or growth medium (modified Eagle's medium) for 18 hr and then infected with H5ts125. The cultures were then trypsinized and 1×10^3 cells were plated in each of five 50-mm tissue culture plates for survival assays or 2×10^5 cells were plated in each of ten 50-mm tissue culture plates for transformation assays. Plates for survival assays were refed after 4 days with fresh growth medium and stained with Giemsa after 7–9 days. The number of colonies was then counted. Seventy-two hours after infection, transformation plates were transferred to low Ca^{2+} medium (19–21). Twenty-four hours, 72 hr, 1 week, 2 weeks, or 3 weeks after infection, 10 plates each were transferred to medium containing or lacking 0.1 μg of 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) per ml. Cultures were refed twice per week with the low Ca^{2+} medium with or without TPA and the number of transformed foci were counted after 6–7 weeks of growth. Transformed foci were detected by their epithelioid appearance, which is characteristic of adenovirus-transformed embryo cultures (18). Transformation was confirmed in 12 clonal isolates by growth in agarose, and the presence of integrated H5ts125 DNA sequences was confirmed by hybridization of cellular DNA with restriction cleaved DNA fragments of

Abbreviations: TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; Me_2BzA, dimethylbenz[*a*]anthracene; BzP, benzo[*a*]pyrene.

Table 1. Effects of TPA and Me_2BzA on adenovirus H5ts125 transformation of rat embryo cells

Me_2BzA, μg/ml	Plating efficiency[a]		Transformation without TPA[b]			Transformation with TPA[c]			TPA enhancement factor[d]
	Total colonies	Fraction of control	Transformed foci	Transformation frequency	Me_2BzA enhancement factor[e]	Transformed foci	Transformation frequency	Me_2BzA enhancement factor[f]	
0.1	245	0.52	10.0	96.2	3.70[g]	18.6	178.9	2.67[g]	1.86
0.05	371	0.79	12.6	79.8	3.07[g]	23.4	148.1	2.21[g]	1.86
0.025	406	0.86	15.4	89.5	3.44[g]	23.6	137.2	2.05[g]	1.53
0.0125	422	0.90	11.2	62.2	2.39[g]	26.4	146.7	2.19[g]	2.36[g]
Acetone	458	0.97	4.8	24.7	0.95	13.2	68.0	1.02	2.75[g]
Media	471	1.00	5.2	26.0	1.00	13.4	67.0	1.00	2.58[g]

[a] Five 50-mm tissue culture plates were seeded with 1000 cells each and colonies were counted after 7–9 days of growth at 37°. The fraction of cells surviving chemical treatment was obtained by dividing the total number of colonies in treated cultures by the total number in untreated cultures.

[b] Transformation efficiency was determined by plating 2×10^5 cells in each of five 50-mm plates and refeeding with reduced calcium medium (0.1 mM) (18–20) twice per week. The mean number of transformed foci per 50-mm plate was determined after 6–7 weeks of growth at 37°. The transformation frequency was calculated by dividing the number of transformed foci counted on plates receiving 2×10^5 cells by the fraction of cells surviving chemical treatment. Transformation frequency is expressed as the number of foci per 10^6 surviving cells. This calculation is based on the assumption that, under these conditions, cell survival is not a function of cell number.

[c] TPA at a final concentration of 0.1 μg/ml was first added to cultures 72 hr after infection with H5ts125. Cultures were subsequently fed with media containing reduced Ca^{2+} (18–20) and 0.1 μg of TPA per ml twice per week for 6–7 weeks.

[d] The enhancement factor is the transformation frequency in TPA-treated cultures relative to that in the corresponding cultures not treated with TPA.

[e] Me_2BzA enhancement factor is the transformation frequency in cells treated with Me_2BzA relative to untreated controls.

[f] Me_2BzA enhancement factor is the transformation frequency in cells treated with Me_2BzA relative to untreated cultures, both grown in the presence of TPA.

[g] Significant at the 1% confidence level using Casto values derived from Lorenz tables for determining statistical significance (13).

^{32}P-labeled adenovirus type 5 DNA (12). Individual clones of adenovirus-transformed cells were isolated, by the steel cloning cylinder technique, from H5ts125-infected rat embryo cells, and recloned by plating 100 cells per 50-mm tissue culture dish. Colonies were picked and maintained as clonal strains in low calcium medium (0.1 mM Ca^{2+}) (19–21) supplemented with 7.5% fetal bovine serum.

Effect of TPA on Cloning Efficiency of H5ts125-Transformed Rat Embryo Cells. One or two hundred adenovirus-transformed RE cells (clones A18, C5, or E11) or 1000 secondary rat embryo cells were seeded in 20 50-mm plates in low Ca^{2+} medium; 24 hr later fresh low Ca^{2+} medium with and without TPA (0.1 μg/ml) was added. The cultures were refed twice per week with and without TPA, and colonies >50 cells were enumerated after 2 weeks of growth.

Effect of TPA on Growth of H5ts125-Transformed Rat Embryo Cells in a Cocultivation Assay. One hundred adenovirus-transformed rat embryo cells (clone A18, C5, or E11) and 1×10^4 secondary rat embryo cells were seeded in 20 50-mm plates in low Ca^{2+} medium; 24 hr later fresh low Ca^{2+} medium with and without TPA (0.1 μmg/ml) was added. The cultures were refed twice per week with and without TPA and the number of transformed colonies (epithelioid morphology) against a background of normal rat embryo cells was determined after 2 weeks.

RESULTS

Tables 1 and 2 summarize the data obtained on plating efficiency and transformation when rat embryo cells were pretreated with various concentrations of Me_2BzA or BzP, infected with adenovirus H5ts125, and then grown in the presence or absence of TPA. Infection of rat embryo cultures with the virus alone (media control, Tables 1 and 2) resulted in a significant number of foci with the epithelioid morphology characteristic of adenovirus transformation (18–21). Treatment of the cultures with Me_2BzA (0.0125–0.1 μg/ml) or BzP (0.05–0.5 μg/ml), in the absence of virus, did not yield such foci. On the other hand, pretreatment of rat embryo cells with Me_2BzA (0.0125–0.1 μg/ml) or BzP (0.05–0.5 μg/ml) 18 hr prior to viral infection caused a 2- to 4-fold enhancement of the absolute number of transformed foci obtained and the transformation frequency (Tables 1 and 2). Appreciable toxicity by Me_2BzA and BzP was observed only at the higher concentrations and did not appear to be required for the enhancement of transformation.

The addition of TPA (0.1 μg/ml) to the culture medium 72

Table 2. Effects of TPA and BzP on adenovirus H5ts125 transformation of rat embryo cells

BzP, μg/ml	Plating efficiency[a]		Transformation without TPA[a]			Transformation with TPA[a]			TPA enhancement factor[a]
	Total colonies	Fraction of control	Transformed foci	Transformation frequency	BzP enhancement factor[b]	Transformed foci	Transformation frequency	BzP enhancement factor[c]	
0.5	302	0.49	9.4	95.9	4.57[a]	16.2	165.3	3.31[a]	1.72
0.05	598	0.98	8.6	43.9	2.09[a]	19.2	98.0	1.96[a]	2.23[a]
Acetone	621	1.01	4.4	21.8	1.04	9.6	47.5	0.95	2.18[a]
Media	613	1.00	4.2	21.0	1.00	10.0	50.0	1.00	2.38[a]

[a] See footnotes [a], [b], [c], [d], and [g] of Table 1.

[b] BzP enhancement factor is the transformation frequency in BzP-treated cells relative to untreated controls.

[c] BzP enhancement factor is the transformation frequency in BzP-treated cells relative to untreated cultures, both grown in the presence of TPA.

Table 3. Effect of time of addition of TPA on H5ts125 transformation of rat embryo cells

Time of initial addition of TPA,[a] days	Transformed foci[b]	Transformation frequency[b]	TPA enhancement factor[c]
1	7.2 ± 0.76	36 ± 3.80	2.40 ± 0.29[b]
3	7.0 ± 0.75	35 ± 3.75	2.33 ± 0.27[b]
7	6.6 ± 0.86	33 ± 4.30	2.20 ± 0.15[b]
14	3.2 ± 0.51	16 ± 2.55	1.07 ± 0.02
21	3.0 ± 0.37	15 ± 1.85	1.00 ± 0.08
Control	3.0 ± 0.49	15 ± 2.45	1.00

[a] All rat embryo cells were infected with H5ts125 at time zero and transferred to low Ca^{2+} medium, as described in Table 1 and *Materials and Methods*. Cultures were then exposed to TPA (0.1 μg/ml) at various time intervals, subsequently fed twice per week in media containing or lacking 0.1 μg of TPA per ml for 6–7 weeks, and then scored for epithelioid transformed foci. Data represent the mean ± SEM in 10 replicate cultures. Control cultures received media changes without TPA.

[b] See footnotes [b] and [g] of Table 1.

[c] See footnote [d] of Table 1.

hr after viral infection also resulted in enhancement of the absolute number of transformed foci and the transformation frequency when compared to controls not treated with TPA (Tables 1 and 2). Under the same conditions, rat embryo cells grown in TPA and not infected with virus gave no foci of transformation and no detectable toxicity. The enhancement effects of TPA are summarized in Tables 1 and 2. When cultures were infected with the virus and then grown in TPA (0.1 μg/ml, added at 72 hr), there was a 2- to 3-fold enhancement of transformation. As discussed above, pretreatment of rat embryo cells with Me_2BzA or BzP prior to virus infection produced a 2- to 4-fold enhancement of transformation. This effect was augmented about 2-fold when such cultures were also grown in the presence of TPA. The combined effects of Me_2BzA and TPA are illustrated by the fact that the number of H5ts125-transformed foci per plate rose from 4.8 for cells simply infected with the virus (acetone control) to 11.2 for cells pretreated with 0.0125 μg of Me_2BzA per ml before virus infection, to 26.4 for cells pretreated with 0.0125 μg of Me_2BzA per ml before virus infection and grown in the presence of TPA after virus infection (Table 1). Similarly, H5ts125-transformed foci per plate increased from 4.4 (acetone controls) to 8.6 for cells pretreated with 0.05 μg of BzP per ml to 19.2 for cells pretreated with 0.05 μg of BzP per ml prior to virus infection and grown for 6–7 weeks in the presence of TPA (Table 2). In addition to increasing the yield of transformed foci, the foci were considerably larger and they developed sooner (after 2–3 weeks as opposed to 4–5 weeks) when the cultures were grown in the presence of TPA. In all cases, the transformed foci had an epithelioid morphology similar to that of foci obtained with H5ts125 alone. This suggests that transformation was induced by the virus and that Me_2BzA, BzP, and TPA simply enhanced the process.

The ability of TPA to enhance H5ts125 transformation of rat embryo cells was dependent on the time that exposure of virally infected cells to TPA was initiated. An approximate 2-fold enhancement of viral transformation resulted when TPA was added 1, 3, or 7 days after infection. When TPA addition was delayed to 14 or 21 days after viral infection, no enhancement of transformation was found (Table 3). Additional studies indicated that TPA enhancement of adenovirus transformation could be obtained with as little as 30 ng/ml (data not shown).

Table 4. Effect of TPA on cloning efficiency and cocultivation assay of rat embryo cells and H5ts125-transformed clones

Cells plated	Media −TPA	Media +TPA
Cloning assay	No. of clones	
Rat embryo	3.06 ± 0.29	3.54 ± 0.30
A18	12.4 ± 2.17	25.3 ± 2.07
C5	9.8 ± 1.21	20.4 ± 2.52
E11	33.2 ± 2.87	51.5 ± 4.13
Cocultivation assay	No. of transformed foci	
Rat embryo + A18	13.2 ± 2.96	26.4 ± 1.87
Rat embryo + C5	10.2 ± 0.88	19.6 ± 1.04
Rat embryo + E11	30.2 ± 4.26	49.7 ± 3.92

Clonal assays were performed using 1000 secondary rat embryo cells or 100 H5ts125-transformed cells (clones A18, C5, or E11). Cocultivation assay initially contained 1×10^4 secondary rat embryo cells and 100 A18, C5, or E11 cells. Description of assays and cell types are given in *Materials and Methods*. Data represent the means ± SEM obtained with 10 replicate cultures.

On the other hand, phorbol, which is inactive as a tumor promoter (3–5), did not enhance viral transformation even when added at 0.2 μg/ml (data not shown).

The effects of TPA (0.1 μg/ml) on the cloning efficiencies of rat embryo cells and three H5ts125-transformed rat embryo clones (A18, C5, and E11) are presented in Table 4. The cloning efficiencies of all three transformants were enhanced 1.6- to 2-fold when cells were grown in the presence of TPA. In contrast, the cloning efficiency of rat embryo cells was not enhanced by growth in the presence of TPA. A similar increase in the number of transformed colonies resulted when 1×10^4 rat embryo and 100 A18, C5, or E11 cells were cocultivated in the presence of TPA (Table 4). In addition to an increase in number, the sizes of the transformed colonies were larger, in both clonal and cocultivation assays, when TPA was present in the growth medium.

DISCUSSION

The present studies demonstrate that TPA, a tumor-promoting agent on mouse skin, increases the yield of adenovirus-transformed foci. Our studies were performed with a temperature-sensitive adenovirus mutant, H5ts125, which at 37° is defective in a DNA binding protein and does not undergo DNA replication (10). This mutant is of particular interest for studies on cell transformation because it has a higher transformation efficiency than wild-type adenovirus type 5 and a greater extent of its genome is integrated into the transformed cells (10–12).

We have also found that pretreatment of rat embryo cells with the initiating carcinogens Me_2BzA or BzP prior to infection with H5ts125 further enhanced the yield of adenovirus-transformed foci. These findings are consistent with previous studies indicating that pretreatment of hamster or rat embryo cell cultures with chemical or physical (UV light or x-irradiation) carcinogens and mutagens enhances cell transformation by other strains of adenovirus (18, 22, 23). Our results indicate that this phenomenon extends to a mutant virus defective in DNA replication.

At the present time we can only speculate about the mechanism by which a promoting agent could enhance adenovirus transformation. Two alternative mechanisms can be postulated: (*i*) TPA might enhance virus uptake and/or integration of viral DNA; or (*ii*) TPA might selectively enhance gene expression

and growth in cells containing integrated viral DNA sequences. The first possibility seems unlikely since: (*i*) residual virus was removed from infected cells 3 hr after infection, and (*ii*) the addition of TPA could be delayed as long as 7 days after infection and still the enhancement was observed. Although it has not been possible to accurately quantitate DNA integration during the process of cell transformation, there is evidence that during abortive or productive infection integration of adenovirus DNA is largely completed by 63 hr after infection (24, 25). Hence, it is likely that the effect of TPA is exerted after viral DNA integration has occurred. Our finding that no enhancement was observed when the addition of TPA was delayed to 14 or 21 days after virus infection may merely reflect that by 14 days the cultures are nearly confluent and thus there may be an insufficient number of subsequent cell divisions for TPA to exert its effect.

The second postulated mechanism of TPA action is consistent with evidence that TPA induces changes in the morphology, cell surface, and growth properties of cells in culture that mimic those associated with cell transformation (26–28). A striking example is the ability of TPA to induce synthesis of plasminogen activator (a protease often associated with cell transformation) in certain normal cell cultures and to markedly enhance its synthesis in transformed cells (26, 27). TPA could, therefore, enhance the expression of genes related to transformation in rat embryo cells containing integrated copies of H5ts125. TPA did not enhance the cloning efficiency of normal rat embryo cells, yet it did produce about a 2-fold enhancement in the cloning efficiency of isolated H5ts125-transformed rat embryo cells (A18, C5, or E11). This was true when the transformed rat embryo cells were grown alone or cocultivated with an excess of normal rat embryo cells. The latter finding is similar to that of Sivak and Van Duuren (29), who found that TPA enhanced the cloning efficiency of simian virus 40-transformed 3T3 cells cocultivated with a 100-fold excess of normal 3T3 cells. We have found that although clones of H5ts125-transformed rat embryo cells isolated from TPA-treated cultures are larger and exhibit more piling up of cells than similar clones isolated from untreated cultures, both types of clones appear morphologically similar after growth in medium lacking TPA.

The ability of TPA to enhance transformation in cells previously exposed to a transforming virus provides evidence that tumor promoters do not act simply by influencing the metabolism or the DNA binding, damage, and repair associated with initiating chemical carcinogens. The ability to study in cell culture the interactions between viral agents, tumor promoters, and initiating chemical carcinogens should facilitate our understanding of the mechanisms by which multiple etiologic factors interact in a multistep carcinogenic process.

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