Enhancement by Estrogens of Adenovirus or Spontaneous Transformation of Hamster Cell Cultures

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SUMMARY

Small amounts of estrogens added to hamster cell cultures infected with human adenovirus type 12 produced a significant increase in rate of appearance and final numbers of viral transformed cell foci. A stimulation by estrogens of the formation of foci of morphologically transformed cells in uninfected control cultures was also observed. The transformation-enhancing effect of estrogens was expressed in cultures grown in fluid, as well as in agar-containing medium. In addition, an increase in rate of cell growth in both uninfected and infected cultures grown in the presence of estrogens was demonstrated.

INTRODUCTION

Female hamsters develop significantly more tumors than males given the same dose of human adenovirus type 12 at birth (12–14). The findings by Yohn et al. (13) that ovariectomy at 3 weeks of age reduces tumor incidence in female hamsters whereas castration of males at this age has no effect suggests that estrogens may play an important role in carcinogenesis. In support of this possibility, hamster tumors have been induced by administration of estrogens (4) or estrogen and androgen (5, 6). In the present study, the effect of estrogens on in vitro transformation of hamster cells by human adenovirus type 12 has been studied.

MATERIALS AND METHODS

Hamster cell cultures were prepared by trypsinization of 12- to 14-day-old whole embryos or trypsinization of kidneys removed from newborn animals (2). A stock of plaque-purified human adenovirus type 12 was grown and titrated in human embryonic kidney cell cultures (8). The virus stock had a titer of 3 × 10⁶ plaque-forming units/ml, and was oncogenic for newborn hamsters (12). The multiplicity of infection given refers to adsorbed multiplicity as determined by measuring the difference in plaque-forming units of a virus suspension before and after exposure to cells.

Conjugated estrogens with the trade name Premarin were purchased from Ayerst Laboratories, New York, N. Y. Premarin is a mixture of the sodium salts of the sulfate esters of the estrogenic substance, principally estrone and equilin, that are excreted by pregnant mares. It contains 50 to 65% sodium estrone sulfate, 20 to 35% sodium equilin sulfate, 2 to 5% δ-estradiol, and 15 to 20% α-dihydroequilin. Twenty mg Premarin were dissolved in 2 ml sterile distilled water in each vial. Further dilutions were made by culture medium.

The transformation procedure described by Casto (2) was used. Confluent or nearly confluent cultures of HmK or HmE, containing approximately 2 to 4 × 10⁶ cells/60-mm Petri dish, were washed twice and 0.2 to 0.4 ml virus suspension was added onto the cell layer. A multiplicity of approximately 20 to 50 was used. Virus adsorption was carried out for 4 hr at 37° in a humidified 5% CO₂-air mixture. The cultures were then washed once and 1.5 ml 0.25% trypsin were added to each plate. The resulting cell suspension was centrifuged and resuspended in Eagle’s minimal essential medium containing double the concentration of amino acids and vitamins and supplemented with 10% fetal calf serum. Approximately 2.5 to 4 × 10⁶ cells in 3 ml medium were placed in a new 60-mm plate. Control cultures were treated under the same conditions, but no virus was added. After 1 day, the culture medium was replaced with the above medium, now containing only 0.1 mM CaCl₂ and supplemented with various concentrations of estrogens. The medium was replaced with 3 ml fresh low calcium medium containing estrogens every 3 to 4 days. Plates were examined for transformed cell foci between 1 and 5 weeks after virus inoculation. Transformed foci were characterized by the appearance of dense areas of morphologically altered, epithelial-like cells as previously described (2, 9). The number of foci given represents the average number from 7 to 13 plates.

Cell counts were made at various times by detaching cells from replicate plates with 0.25% trypsin and counting the cells in a hemacytometer. The immunofluorescent antibody technique used for the detection of adenovirus 12 nonviral tumor antigen has been described (10).

For study of transformed foci production in agar
medium, at 12 to 13 days after virus inoculation 7 ml of an agar overlay consisting of the low calcium medium, 10% fetal calf serum, 0.5% agar, and various concentrations of estrogens were added to each dish. Final focus counts were made 10 to 18 days after the agar medium was added.

RESULTS AND DISCUSSION

Estrogen Effect on Cell Growth of Adenovirus 12-infected and Noninfected Cultures. Growth curves were obtained of adenovirus 12-infected HmE cell cultures grown in the presence of various concentrations of estrogens (Chart 1). Total cell counts were made at 3 to 9 day intervals. At 10 to 19 days after seeding, there were approximately twice as many cells in plates containing 10 to 50 µg estrogens/ml of culture medium as there were in the untreated controls. A concentration of 1 µg/ml gave a 1.2 to 1.5-fold increase in cell number at 7 to 19 days. An inhibitory effect on cell growth was obtained when 100 µg estrogens/ml were used. The reduction in cell counts, consistently observed within 1 to 2 days after plating, may be related to toxicity of the virus inoculum. The effect of estrogens on the growth rate of noninfected control HmE cell cultures was also tested. Estrogens stimulated the growth of noninfected cells, but mainly at a concentration of 50 µg/ml medium. At this concentration, an approximately 2-fold increase in cell number was found from 10 to 27 days after seeding.

Estrogen Effect on Adenovirus 12-induced and Spontaneous Cell Transformation. Foci, characterized by the appearance of multilayered dense colonies, were observed as early as 11 days after inoculation of HmE cell cultures with adenovirus 12, as has been reported by Casto (2). The number of foci increased thereafter to reach a maximum at approximately 5 weeks. Multilayered cell colonies also appeared in noninfected control hamster cells, but at a lower frequency. These apparently transformed foci were grossly indistinguishable from those observed in adenovirus-infected cultures. Spontaneous transformation of various cells in vitro has been observed (11). In HmK cell cultures, the first appearance of transformed foci was observed 16 days after virus inoculation.

Various concentrations of estrogens ranging from 1 to 100 µg/ml of culture medium were added to adenovirus 12-infected HmK or HmE cell cultures. Noninfected control cultures were similarly treated. Final numbers of transformed foci were counted in HmK cultures at 35 to 38 days after virus inoculation and transfer and in HmE cultures at 31 to 35 days.

Significant increases in final numbers of transformed foci were consistently observed in infected as well as control HmK cells over the range of estrogens tested from 1 to 100 µg/ml, as compared with untreated cultures (Table 1). There were approximately 3 to 6 times more foci in the virus-inoculated HmK cultures containing 50 to 100 µg estrogens/ml of medium, and 2 to 3 times more foci in cultures with 10 µg/ml, than in the untreated infected cultures (Table 2). Strikingly, in noninfected HmK cell cultures treated with 50 to 100 µg estrogens/ml of medium, there was an approximately 30- to 50-fold increase in foci number when compared with the untreated controls (Table 2).

The number of transformed cell foci was also increased in infected and control HmE cultures after estrogen treatment (Table 1). In infected HmE cell cultures treated with 1 to 10 µg estrogens/ml there were 2 to 30 times more foci than in the untreated infected cultures (Table 2). A concentration of 50 to 100 µg/ml apparently had a smaller stimulating effect (Tables 1 and 2).

The considerable variation in the frequency of adenovirus 12-induced transformation seen in different HmE and HmK cell cultures, particularly in the absence of estrogens (Table 1), may reflect a difference in cell susceptibility to adenovirus-induced transformation. Another factor which may play a role is the cell number at the time of planting. It was found that, in the absence of estrogens, approximately 5 times as many foci appeared in 5 weeks in plates seeded with 2.5 × 10^5 cells than occurred in plates seeded with 4 × 10^5 cells.

Both adenovirus 12-infected and spontaneously transformed HmE cells could be passaged at least 15 times in culture. One cell line, induced by adenovirus in HmE cells in the presence of 50 µg estrogens/ml culture medium, and passed 7 times in tissue culture, induced tumors in 2 out of 4 young adult hamsters. Immunofluorescent studies of a number of cell lines derived from adenovirus-transformed foci demonstrated the presence of adenovirus-specific tumor antigen. In contrast, spontaneously transformed cells passaged in culture did not exhibit the presence of this antigen.

Estrogen Effect on Time of Appearance of Transformed Foci. The number of transformed foci present in adenovirus 12-infected HmE cell cultures treated with 1 to 50 µg estrogens/ml medium was almost twice that

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**Chart 1.** Growth rates of adenovirus 12-infected HmE cell cultures in the presence and absence of estrogens. The estrogen concentrations refer to µg/ml culture medium.

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found in untreated control cultures at virtually every interval tested over a period of 31 days (Chart 2). At 11 days after virus inoculation, there were 3 times as many foci in cultures treated with 50 µg estrogens/ml as there were in the untreated cultures. Similar findings were obtained for control HmE cell cultures. An approximately 2- to 3-fold increase in spontaneously transformed foci was observed over a period of 11 to 31 days using 1 to 50 µg estrogens/ml (Chart 3).

**Estrogen Effect on Transformation Frequency in Agar Medium.** The adenovirus 12-infected and noninfected control HmE cells were also plated in agar medium in the absence or presence of estrogens (Chart 4). The number of foci increased with increasing concentrations of estrogens in both sets of cultures, so that 1.3- to 2-fold increases occurred with 1 µg estrogen/ml of agar medium, and approximately 3-fold increases occurred with 10 µg estrogens/ml. With 50 µg estrogens, a 4-fold increase in foci number was observed in infected cultures and a 10-fold increase was seen in noninfected cultures.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Effect of estrogens on maximum frequency of cell transformation</th>
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<tr>
<td></td>
<td>Final No. of transformed cell foci</td>
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<tr>
<td></td>
<td>HmK cell cultures&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Estrogen concentration (µg/ml)</td>
<td>Adenovirus 12-infected</td>
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<td>Experiment 1</td>
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<tr>
<td>0</td>
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<td>n.d.</td>
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<td>78</td>
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<sup>a</sup> Counts made at 35 to 38 days.
<sup>b</sup> Counts made at 31 to 35 days.
<sup>n.d.</sup> not done.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Effect of estrogens on maximum frequency of adenovirus-induced and spontaneous cell transformation</th>
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<td></td>
<td>Ratio of final foci No. in presence and absence of estrogens&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>HmK cell cultures</td>
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<td>Adenovirus 12-infected</td>
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<tr>
<td>50</td>
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<td>100</td>
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<sup>a</sup> Calculations were based on the data given in Table 1.
<sup>b</sup> n.d., not done.

Chart 2. Number of transformed foci found in adenovirus 12-infected HmE cell cultures grown in the presence and absence of estrogens. The estrogen concentrations refer to µg/ml culture medium.

Chart 3. Number of transformed foci found in noninfected HmE cell cultures grown in the presence and absence of estrogens. The estrogen concentrations refer to µg/ml culture medium.

Chart 4. Number of transformed foci counted in agar medium after plating adenovirus 12-infected or noninfected HmE cells in the presence and absence of estrogens. The estrogen concentrations refer to µg/ml culture medium.
The number of foci found in agar medium was less than in plates maintained in fluid medium (Charts 2 and 3). Such a difference may be due to several factors: the cell growth rate under agar is probably slower and produced fewer visible transformed foci, and transformed cells maintained in fluid medium may detach from the surface of plates and spread to other areas to give secondary transformed colonies.

The results presented above indicate that estrogens have a stimulating effect on growth of hamster cells in vitro and enhance cell transformation induced by adenovirus, as well as that occurring spontaneously. The enhancing effect of estrogens on adenovirus-induced transformation could be due to an increase in mitotic activity of cells altered by virus. Estrogens may also act directly as carcinogens to cells, since spontaneous cell transformation is enhanced by estrogens. Although conflicting results have sometimes been reported, several investigators have shown that estrogens stimulate cell growth in tissue culture. For example, estrone increases mitoses of mouse prostate glands in vitro (7), and several estrogens induce proliferation of vaginal epithelium in tissue culture (1, 3). The in vitro study of cell transformation may offer a system for the study of the mechanism of action of estrogens on cells.

ACKNOWLEDGMENTS

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REFERENCES


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