In Vitro Transformation of Hamster Cells by Herpes Simplex Virus Type 2 from Human Prostatic Cancer Cells[1]

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SUMMARY

A cell-associated herpes simplex virus type 2 found in a human prostatic carcinoma induced in vitro transformation of hamster embryo cells. The transformed cells (YW-74) have been shown to be hamster cells by karyotype analysis. Their epithelial morphology and growth pattern, which are different from the parental cell, have remained stable through cell passages. The presence of herpesvirus antigens in the transformed cells was determined by specific immunofluorescence and colony inhibition tests. Immunofluorescence staining with specific anti-herpes simplex virus type 2 serum showed an intense and distinctive nuclear and perinuclear fluorescence in about 95% of the transformed cells. In addition, exposure of these transformed cells to herpes simplex virus type 2-sensitized lymphocytes resulted in inhibition of growth and colony formation, while no effect was seen with nonsensitized lymphocytes. Both observations are consistent with the involvement of herpesvirus type 2 in the transformation event. This virus, which does not produce a lytic infection and is not found either in extracellular spaces or supernatant fluid of the transformed cell cultures, is unique in the fact that it is cell associated, noncytopathogenic, and capable of transforming cells in vitro, and its antigens are clearly demonstrated in the transformed cells.

INTRODUCTION

An association between herpesvirus genital infections and human cancer has been implied by epidemiological studies of patients with carcinoma of the cervix (15–17). This association has been strengthened by the finding that UV-irradiated herpesvirus type 1 and type 2 can transform cells in vitro and that such cells cause tumors in newborn hamsters (5–7). A strong implication of the oncogenicity of herpesvirus in its natural host, man, was provided by Frenkel et al. (9) who showed that human cervical tumor cells contained a fragment of herpesvirus DNA in their genome.

Our previous work on the incidence of herpesvirus in a random population of asymptomatic males has demon-strated by special cocultivation techniques that the male genitourinary tract serves as the reservoir for this virus (2). Further investigation on the presence of herpesvirus particles or its antigens in genitourinary tract tissues led to the discovery of a herpesvirus particle in cancer cells from an untreated carcinoma of the prostate (3). This virus particle, which was identified as a herpesvirus on the basis of specific immunofluorescence staining, morphology, and size, could not be recovered as infectious virus from the supernatant fluid of organ cultures of the original tumor. The presence of a nonlytic, cell-associated herpesvirus in human prostatic cancer cells led to this investigation, which sought to determine whether this particle was capable of transforming cells in vitro.

MATERIALS AND METHODS

Cells and Media. Primary cultures of Syrian hamster embryo cells, obtained from Microbiological Associates, Rockville, Md., were trypsinized, divided, and grown in 2-oz plastic tissue culture bottles. The SV40[2]-transformed WI 38 cells (diploid human embryonic lung) were kindly donated by Dr. G. Stein, Biochemistry Department, University of Florida. The growth medium for both cell lines consisted of Eagle's basal minimal media supplemented with 10% fetal calf serum, 1% glutamine, 0.075% $NaHCO_3$ and antibiotics. Rabbit kidney cells and human embryonic kidney cells in tube culture, obtained from Hem Research, Inc., Rockville, Md., were maintained in Eagle's medium supplemented with 2% fetal calf serum.

The technique used for the growth of cells in semisolid agar was similar to that of MacPherson and Montagnier (14). Briefly, 2-ml base layers of complete medium containing 0.5% agar were placed on 35-mm petri dishes and allowed to harden. Two ml of a single cell suspension in complete media containing 0.3% agar were placed over the base layer. The cultures were then incubated at 37° in a 5% CO_2 humidified atmosphere.

Virus. Herpesvirus type 1 (Shealy stain) and herpesvirus type 2 (Hicks strain) were originally obtained from A. Nahmias, Emory College of Medicine, Atlanta, Ga. Both strains had been propagated in human embryonic lung

[2] The abbreviations used are: SV40, simian virus 40; HSV-2, herpes simplex virus type 2; HSV, herpes simplex virus; EB, Epstein Barr; CMV, cytomegalovirus.

cultures and the virus stocks stored in small volumes at −70°. Portions of the human prostatic cancer tissue in which herpesvirus particles had been found (3) were used as a source of the HSV-2 tumor-associated virus. These tumor specimens had never been in tissue culture but were immediately frozen after surgery.

Virus Assay. To test for the presence of infectious virus in the transformed hamster embryo cultures, rabbit kidney cells, and human embryonic kidney, tube cultures were inoculated with 0.2 ml of supernatant fluid and examined daily for evidence of viral cytopathic effect.

Immunofluorescence Techniques. The presence of HSV-specific antigens was determined by indirect immunofluorescence techniques. The antiserum to herpesvirus was prepared in 3 New Zealand White rabbits. The animals were immunized to herpesvirus type 1 (Shealy strain) or herpesvirus type 2 (Hicks strain) by weekly i.m. and i.p. injections of 1 ml of virus (10^6 plaque-forming units/ml) for a period of 3 weeks. Sera, obtained from the animals 2 weeks after the last injection, were pooled and the neutralizing activity was tested in human embryonic kidney tube cultures with both the Shealy and Hicks strain (10^3 plaque-forming units) as the challenge virus. In this test the sera inhibited virus cytopathogenic effects at greater than a 1:64 dilution. The fluorescein-conjugated goat anti-rabbit IgG serum, specific for IgG heavy chain, was obtained from Cappel Laboratories, Inc., Downingtown, Pa. Transformed cell cultures grown on coverslips were washed twice with 0.1 M phosphate buffer (pH 7.2):0.07 M NaCl) and fixed in cold acetone for 20 min. Rabbit antiserum to herpesvirus type 1 or type 2 or normal rabbit serum at a 1:5 dilution was placed over the coverslip and incubated for 30 min at 37° in a moist chamber. The coverslip was then washed with an excess of phosphate-buffered saline (0.1 M phosphate buffer, pH 7.2: 0.07 M NaCl) and fluorescein-labeled goat anti-rabbit IgG antiserum was added. The coverslip was reincubated, washed again as described above, mounted in 90% glycerol solution, and examined in a Leitz fluorescence microscope. The specificity of the reagents was also tested with other members of the herpesvirus group such as EB and CMV. The EB-positive lymphoid cells as well as the EB-reference serum were kindly donated by Dr. R. Smith, Pathology Department, College of Medicine, University of Florida. The CMV-infected human lung diploid cells and reference serum were obtained from the Obstetrics and Gynecology Department, College of Medicine, University of Florida. These cells were processed in the manner described above using fluorescein-conjugated goat anti-human IgG serum from Cappel Laboratories, Inc.

Colony Inhibition Test. The Hellström colony inhibition technique (10, 11) was used to determine the ability of the transformed cells to form colonies in the presence of HSV-2-sensitized lymphocytes. For sensitization, inbred Hartley guinea pigs were inoculated with herpesvirus type 2 (Hicks strain) in each of 2 footpads and skin-tested with HSV-soluble antigen 14 days postinoculation (12), at which time positive skin reactions were elicited. Sensitized and nonsensitized splenic lymphocytes were obtained by the method of Waldman and Henney (18), and the final cell

population was tested for viability using trypan blue. Single-cell suspensions of the transformed hamster embryo cells were seeded in 2-oz bottles at a cell density of 6 × 10^3/bottle and incubated at 37° in a 5% CO_2 atmosphere for 24 hr. The cultures were then thoroughly washed and 1 ml of either sensitized or nonsensitized lymphocytes was added to the bottles at a ratio of 1000 viable lymphocytes per transformed cell. After 45 min incubation, fresh medium was added and the cultures were incubated for an additional 72 hr prior to staining with crystal violet for colony count. As an external control and also as a measure of specificity for this assay we included SV40-transformed WI 38 cells. Because of the difference in susceptibility of different target cells we determined the ratio of nonsensitized lymphocytes to SV40-WI 38 cells that produced the least amount of toxicity. For these experiments a ratio of lymphocyte to target cell of 200:1 was used. The results are quantitated by counting the number of target cells surviving in each well or bottle. The percentage of target cell survival is calculated as follows:

Av. no. of target cells in test assay unit/av.
no. of target cells in cell controls × 100

Electron Microscopy. Colonies of transformed cells on coverslips were fixed in 2.5% glutaraldehyde and postfixed with 1% osmium tetroxide. After removal from the coverslip, the cells were dehydrated in ethanol and embedded in Epon 812. Thin sections were cut, stained with uranyl acetate and lead citrate, and examined in a Zeiss 9S-2 electron microscope.

Chromosome Analysis. Chromosomal analysis of Syrian hamster (*Mesocricetus auratus*) cells and viral-transformed hamster cells were performed using standard techniques. Metaphase spreads were stained with Giemsa or by the quinacrine mustard banding technique (1).

RESULTS

Transformation of Hamster Embryo Cells. A small piece of the original prostatic cancer tissue was minced in Eagle's basal minimal media and the cells were disrupted with a Ten Broeck homogenizer. One ml of the resultant homogenate was inoculated onto each of 3 monolayer cultures of Syrian hamster embryo cells and incubated at 37°; uninfected cultures were also included as controls. The cultures were maintained with Eagle's basal minimal media supplemented with 2% fetal calf serum for 6 days, at which time each culture was trypsinized and seeded into 2 new bottles. These grew well to a confluent monolayer without any cytopathic effects or changes in cell morphology. Within 10 days the cultures entered into a growth crisis, that is, they began to degenerate and most of the cells died. The supernatant fluid of each culture was inoculated into tube cultures of primary rabbit kidney and human embryonic lung cells to test for the presence of infectious virus. None of the cultures, which were kept for 3 to 4 weeks, exhibited any evidence of cytopathic effect and were considered negative for infectious virus.

The hamster embryo cell cultures that had entered the

crisis phase were fed twice weekly in an effort to recover any viable cells. The media consisted of Eagle's basal minimal media supplemented with 20% fetal calf serum, 20% NCTC 109, 1% glutamine, and antibiotics. A few weeks later, approximately 40 days since the original inoculation of the hamster embryo cells, 2 foci of transformed cells appeared in 1 remaining bottle. These foci that developed into distinct colonies of identical cellular morphology were transferred to another flask and grown into a cell line designated YW-74. The colonies consisted of epithelial cells, which differed from that of the parental cell line and grew in a compact fashion. The center of the colony was 4 to 5 cells thick, from which clumps of cells peeled off and floated in the medium. When these floating cell clumps were transferred to another bottle, they attached and formed colonies with morphological characteristics similar to those of the original transformed colonies (Fig. 1). The cultures of transformed cells never grew into a monolayer as did the parental cells, but rather into tight, multilayered dense colonies. When the cell density per flask was such that the top of the colony started to peel off, the culture could be trypsinized and distributed into several bottles.

Growth was also obtained when a single-cell suspension in semisolid agar medium was plated on petri dishes with a hard agar base. Colony formation had begun and distinct colonies could be seen after 3 days. Initially, this ability of the transformed cell to grow in soft agar was used for cloning experiments to determine whether the colony morphology would remain stable. In all cases both cellular and colony morphology were the same.

Presence of HSV Antigens in the Transformed Hamster Embryo Cells. Indirect immunofluorescence methods were used to detect viral antigens in the transformed cells. Although weak diffuse cytoplasmic staining was observed with antisera to type 1, antisera to type 2 gave intense perinuclear fluorescence with less intense cytoplasmic staining (Fig. 2). Such a reaction was found in about 95% of the stained cells (Fig. 3). A dull nonspecific fluorescence was observed in all the cells of the control samples.

On the basis of the immunofluorescence staining this herpesvirus belongs to the type 2 group; however, it seemed advisable to test the specificity of our reagents. The possibility of any anti-hamster cell activity in the rabbit antisera was precluded since the herpesvirus stocks used in antibody production were produced in human embryonic lung cultures. When our reagents were tested on EB-positive cells with the appropriate reference antiserum to EB virus, specific fluorescent staining was seen with the reference EB-virus antiserum, while no staining was observed with the antiserum to herpesvirus type 2 or type 1. Similar results were obtained with CMV. Thus the specificity of our reagents was indicated.

Electron Microscopy. Thin sections of the transformed cells were examined by electron microscopy for the presence of viral particles. No viral particles were seen in the cytoplasm or extracellular spaces. Many virus-like particles were seen in the perinuclear region in most of the cells; these were similar to those reported by Duff and Rapp (8) in hamster embryo cells transformed by UV-irradiated herpes-

virus type 2. Although a few complete forms could occasionally be found in some of the cells (Fig. 4), the majority of the particles did not contain dense cores and they must represent incomplete particles, which may account for our failure to recover infectious virus from any of our transformed cell cultures.

Colony Inhibition Test. The involvement of this cell-associated herpesvirus in the transformation of hamster embryo cells was further defined by the colony inhibition test. The growth and colony formation of the transformed cells in the presence of HSV-sensitized and -nonsensitized lymphocytes were measured after 72 hr incubation. At the end of this period control bottles that received media only and those that received the nonsensitized lymphocytes had a comparable number of healthy distinct colonies. In contrast, cultures receiving the sensitized lymphocytes had no growth and colony formation was totally inhibited (Table 1). The fact that HSV-sensitized lymphocytes killed the transformed cells while no effect was seen with nonsensitized lymphocytes demonstrates the presence of HSV-antigenic determinants in these transformed cells. The specificity of this test for HSV-antigenic determinants is demonstrated by the fact that no effect was seen when SV40-transformed cells were used as the target cell.

Karyotype Analysis. Karyotypic analysis of the normal Syrian hamster cells demonstrated a modal number of 44 chromosomes and a normal male chromosome distribution. The banding characteristics were similar to those previously described for BHK21C13 Syrian hamster cells with the large submetacentric A4 and large acrocentric D16 chromosomes being quite distinctive (13). Transformed hamster cells, on the other hand, were found to have gross numerical alterations, being aneuploid and subtetraploid with the number of chromosomes per cell ranging between 69 and 71. The chromosome banding of transformed cells was not as distinctively demarcated as the normal hamster cells, and major alterations in chromosome morphology and banding were observed. All transformed cells contained 9 large acrocentric chromosomes and 1 very small acrocentric (marker) chromosome. The fluorescent banding pattern showed that the distinctive A4 and D16 hamster chromosomes were no longer identifiable. Although chromosomes could be observed with morphological and staining characteristics analogous to the human A1 and A3 chromosomes,

Table 1

Effect of splenic lymphocytes on the growth of YW-74 and SV40-WI 38 cells

Cell type	Source of lymphocytes	Lymphocyte: target cell ratio	% survival[a]
YW-74	HSV-2 sensitized[b]	1000:1	0
YW-74	Nonsensitized	1000:1	71
SV40-WI 38	HSV-2 sensitized	200:1	69
SV40-WI 38	Nonsensitized	200:1	66

[a] Percentage of survival was calculated after 48 hr incubation with either sensitized or nonsensitized lymphocytes.

[b] Sensitized lymphocytes were obtained from a group of 4 guinea pigs that were skin test positive 14 days after immunization with HSV-2.

the brightly fluorescent centrimeric region that is sometimes present in the human A3 chromosome was not found.

DISCUSSION

In these experiments we have described the transformation of hamster embryo cells by a herpesvirus particle found in human prostatic carcinoma cells. Although this virus particle has been identified as a herpesvirus, it does not fit the general characteristics of the known standard herpesvirus. It does not produce a lytic infection and is not found either in extracellular spaces or in supernatant fluid of the transformed cell cultures. It is unique in the fact that it is cell associated, noncytopathogenic, and capable of transforming cells *in vitro*, and its viral antigens persist in the transformed cells.

These transformed cells differ from the hamster embryo parental cell in morphology and growth pattern, and both characteristics are reproducible after repeated cell passages. Further evidence of transformation is the ability of these cells to grow in suspension and to form colonies in soft agar, which is the basis of a selective assay for *in vitro* transformation.

Specific immunofluorescence staining with herpesvirus antisera reveals herpes antigens in over 95% of the transformed cells in a given microscopic field. With antisera to herpesvirus type 2, this fluorescence is most evident and intense in the perinuclear region with little cytoplasmic staining. Since the specificity of our reagents ruled out cross-reactivity with CMV and EB virus, the specific staining is due to herpesvirus, specifically herpesvirus type 2.

That the transformed cells contain HSV-2 antigens is further documented by colony inhibition tests. In these tests, HSV-2-sensitized lymphocytes inhibited both growth and colony formation of these cells, while little effect was seen when the same cultures were exposed to nonsensitized lymphocytes at the same ratio. Nonrelated target cells (SV40-WI 38) that were included for specificity were not selectively inhibited by the HSV-2-sensitized lymphocytes, indicating that our system is specific. The ratio of lymphocyte to target cell is lower in the SV40-WI 38 system, but it is in accordance and within the range of those used for cells containing SV40 antigens.

The karyotype analysis of the transformed cells demonstrates that the transformed cell is indeed a hamster cell. Our findings of large acrocentric chromosomes and small acrocentric (marker) chromosomes are similar to those obtained in other transformed Syrian hamster cells (4). In this case, during the continuous *in vitro* growth of polyoma virus-transformed Syrian hamster cells, a near-tetraploid cell line emerged and a marker chromosome was noted in 1 instance. Furthermore, new chromosomes with an uncommon banding pattern have also been observed in hamster cells transformed with 4-nitroquinoline-*n*-oxide (16). The possibility of contamination with prostatic cancer cells is a very unlikely event for the following reasons. The prostate tissue was dry frozen for several months, the cells were thawed and subjected to extensive cell disruption, and,

lastly, carcinoma of the prostate cells are extremely difficult to grow and maintain in tissue culture.

The frequency of transformation of the hamster embryo cells by inoculation with the cell-associated herpesvirus found in prostatic carcinoma cells is not low when compared to other transformation studies dealing with herpesvirus type 2. In transformation experiments using hamster embryo cells infected with UV-irradiated herpesvirus type 2 (8), 2 out of 17 cultures exhibited transformed foci. These cultures also went through a growth crisis prior to the establishment of the cell line, which may indeed be a characteristic of herpesvirus oncogenesis.

Any attempt to link neoplasia with herpesvirus infections is hampered by the fact that in *in vitro* infection of human cell cultures, as well as in the acute phase of herpetic infections *in vivo*, rapid cell lysis occurs and the virus is easily recovered in an infectious form. In this situation transformation of the infected cells is an unlikely event, as compared to a more favorable condition such as infection with a nonlytic virus in which no cell death occurs and virus determinants can continually be present in the transformed cell.

In this study we provide evidence for the transformation of hamster embryo cells by tumor-associated herpesvirus type 2 from a human prostatic carcinoma. It is not our contention to interpret these results to indicate that all human prostatic carcinomas have a HSV-2 viral etiology but to report a new finding, that the unique herpesvirus type 2 isolated from an untreated human carcinoma is cell associated, is noncytopathogenic, and can transform cells *in vitro*. Studies on the oncogenicity of the transformed cells in syngeneic animals as well as the survey incidence of this particular virus in carcinoma of the prostate cases are now in progress.

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Fig. 1. Photomicrograph of a colony of transformed hamster embryo cells. The center of the colony is 4 to 5 cells thick and the center portion has peeled off. Note the compact cell arrangement of the edge of the colony.

Figs. 2 and 3. Fluorescent staining of transformed hamster embryo cells. Specific immunofluorescent staining for herpesvirus type 2 is seen in nuclear membrane of the cells.

Fig. 4. Complete herpesvirus particles in the HSV-transformed cells. *A*, particle at the edge of the nuclear membrane. × 71,600. *B*, virus particle in the cytoplasm of the cell. × 76,890.

4A

4B