New Properties of Mammalian Cells Transformed by Herpes Simplex and Cytomegaloviruses

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The relationship of herpes simplex viruses (HSV) and cytomegaloviruses (CMV) to human neoplasia has been difficult to assess. On the basis of seroepidemiological evidence, HSV type 2 has been associated with the etiology of squamous cervical carcinoma (1–5). Women with cervical carcinoma have increased frequency of antibodies to HSV-2 than do controls matched for race, age, and socioeconomic level. The percentages vary considerably from study to study, but this may be due to the use of different serological techniques (a very serious and confusing problem) in determining antibody titers.

Herpesvirus particles have been reported in one case of prostate carcinoma cells as demonstrated by electron microscopy (6). This observation is in need of confirmation and its value is questionable, since the same group of workers previously alleged that 15 percent of men from 15 years of age harbor HSV-2 without symptoms in the prostate and vas deferens and pass this virus venereally (7). Women do tend to experience acute infections with this virus, with recurrent infection an almost certain manifestation. Virus is not readily isolated, however, from the female genital tract during latent stages nor from biopsies of cervical carcinoma. Tumor biopsies have repeatedly failed to exhibit virus antigens, but HSV-2 antigens have reportedly been observed by immunofluorescence in the cytoplasm of exfoliated cervical carcinoma cells (8–10). Furthermore, one culture of tumor cells was induced to produce HSV-2 particles under the stress of high pH (11). As with many other such reports, this observation has not been extended by the investigators submitting the original work.

One cervical tumor was found by hybridization experiments to contain 39 % of HSV-2 DNA in up to 3.5 fragments per cell and these were linked to host cell DNA (12). The tumor cells also contained small amounts of RNA transcripts which were complementary to virus DNA. These results may point to the requirement of a repressed virus genome in the maintenance of malignant transformation, and clearly suggest that less than the total genome may be involved. However, at this point the evidence is insufficient for further discussion.

It therefore became obvious that additional reproducible evidence was needed to demonstrate the oncogenic potential of HSV. We thus turned to an examination of the ability of this virus to transform mammalian cells *in vitro*.

Transformation by Herpes Simplex Virus In Vitro

It was well known that direct inoculation of HSV into newborn animals (specifically rodents) leads to death of the animal in most cases. Attempts to transform cells *in vitro* were also hampered by the cytopathology induced by the virus. These problems have now been resolved by the use of various methods to inactivate the infectivity of the virus, while boosting its transforming ability by introducing defective particles into the virus population.

Ultraviolet (UV) light was the first method of inactivation employed; it had previously been used to destroy virus infectivity while augmenting transforming potential of known tumor viruses (12–15). UV-irradiation of HSV-1 and HSV-2 with subsequent inoculation onto mouse L cells deficient in the enzyme thymidine kinase (TK), biochemically transformed these cells into thymidine kinase positive cells (16, 17). This phenomenon, involving the new synthesis of a virus-specific enzyme, was demonstrated to be a heritable change of the cells. The significance of this experiment lies in the fact that this was the first evidence that genetic information of HSV could be maintained and expressed in established mammalian cells.

Duff and Rapp (18–20), in experiments carried out simultaneously, extended the usefulness of UV-irradiation to demonstrate transformation by numerous strains of HSV-1 and 2. Inactivated virus was able to transform hamster embryo cells *in vitro* into continuous cell lines with morphological and growth characteristics differing from those of the parental cells.

The continued presence of the virus in the transformed cells has been demonstrated by the presence of herpes-specific antigens in the cytoplasm of approximately 5–30 % of the cells and on the membranes of approximately 60 % of the cells (18–20). CP-1 antigen (a partially purified virus antigen of HSV) was found by immunological techniques on the membranes of the HSV-1-transformed cells (21). Furthermore, one of the original HSV-2 hamster cell lines (333–8–9) has been found to contain virus RNA transcripts hybridizing to both HSV-1 and HSV-2 DNA (22). This result is not surprising since HSV-1 and HSV-2 DNA share base sequences (23, 24). It is significant to note that only a small percentage of the viral genome is transcribed in the transformed cells (22). In more recent experiments with a variety of HSV-2-transformed hamster cells, approximately 8–38 % of the virus genome has been detected by reassociation kinetics (Frenkel, Roizman and Rapp, unpublished experiments).

The oncogenic potential of the transformed hamster cell lines was evaluated by inoculation into newborn hamsters (18, 20). Some of these cell lines have been shown to induce primary tumors with extensive metastases. Both fibrosarcomas and adenocarcinomas have been induced by the cells tested (the former by fibroblastoid cultures and the latter by epithelioid cultures). The tumor-bearing hamsters developed neutralizing antibody to HSV inversely proportional to the latent period and, therefore, to the oncogenicity of the cell line (20). Pre-immunization of these animals with HSV-1 led to increased metastases.

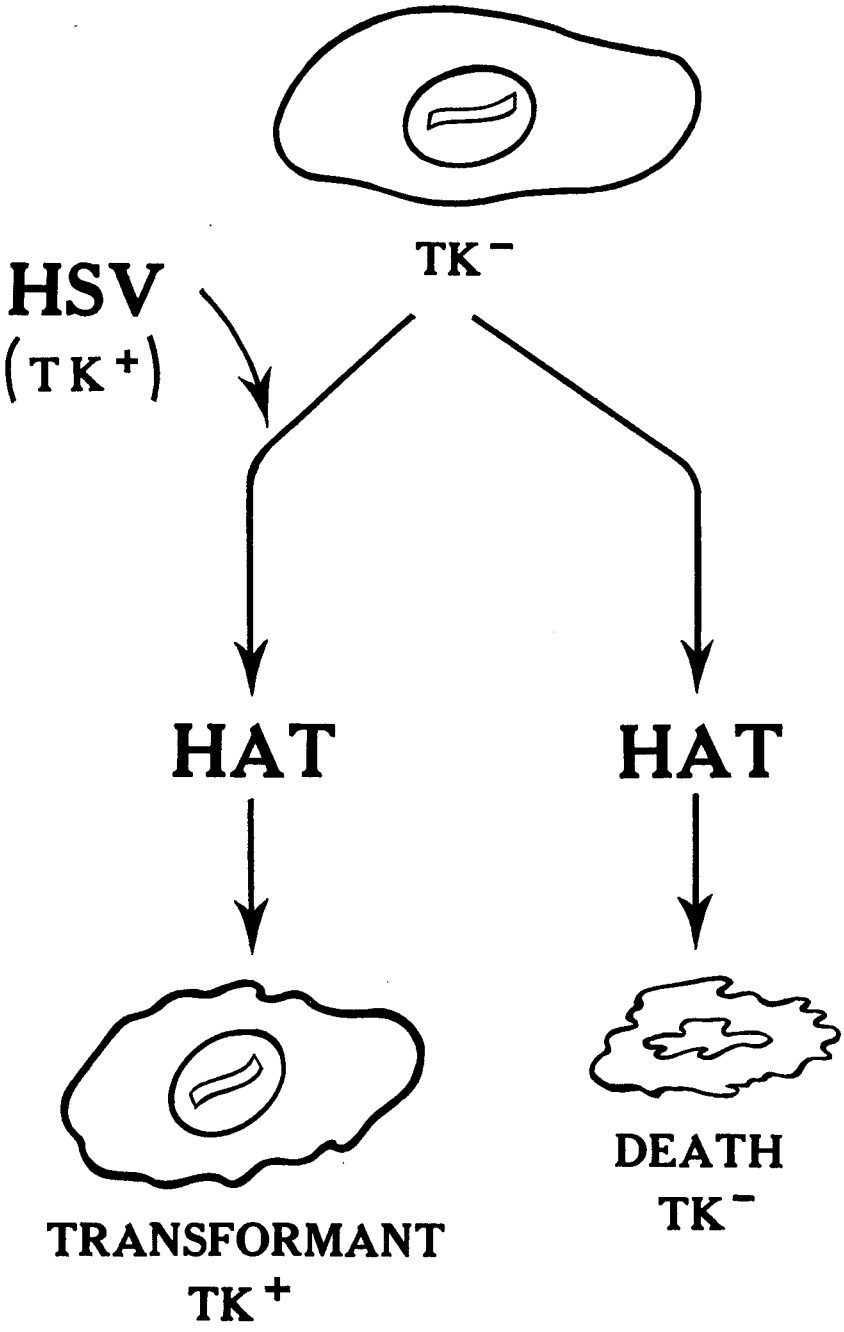

Fig. 1: Use of selective HAT medium to measure transformation of 3T3 cells by herpes simplex virus. Only those cells acquiring the ability to synthesize thymidine kinase can replicate.

replicate. The advantage of this system is the elimination of background foci which allows standardization of the technique. The foci observed (Fig. 2) can be counted and differences in virus isolates or strains quantitated (Fig. 3).

Photodynamic inactivation can also reduce infectivity of viruses (30, 31). Photodynamically inactivated HSV-1, HSV-2 and SV40 can transform hamster embryo fibroblasts (32) and the transformed clones exhibit loss of contact inhibition and morphological alterations. The SV 40-transformed cells consistently synthesized the T antigen, while only 8–10 % of the HSV-1 and HSV-2 cell lines showed diffuse cytoplasmic HSV antigens by immunofluorescence assays. No gs antigens of on-

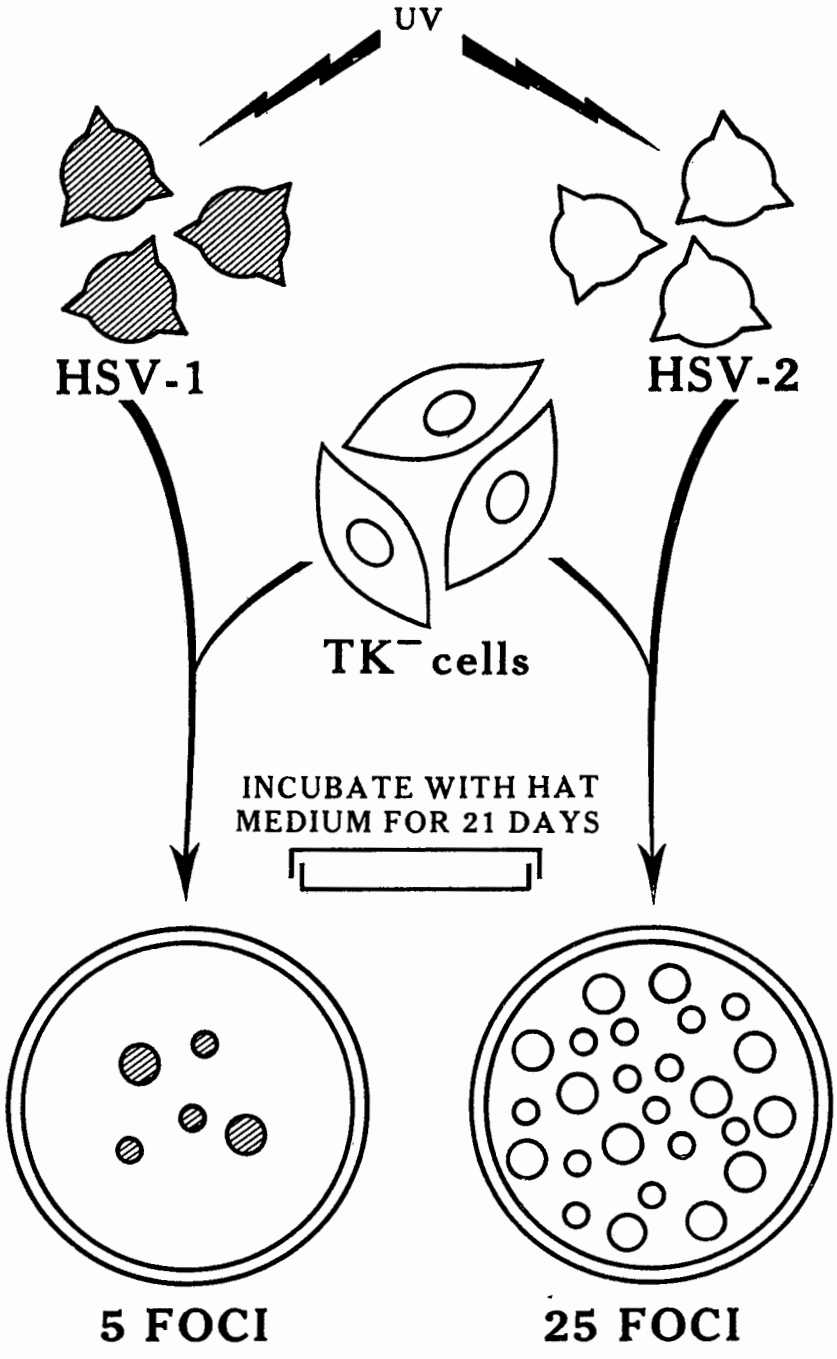

Fig. 3: Schematic representation of strain differences observed for HSV-1 and HSV-2 in ability to transform 3T3 cells.

cornaviruses were detected in these cell lines. The three transformed cell lines established have since been inoculated subcutaneously into syngeneic hosts, i. e. newborn hamsters, and though they differ in relative oncogenic potential, primary metastatic tumors have developed (33). Explanted tumor cells synthesized virus-specific cytoplasmic antigens. Tumor-bearing hamster sera also reacted with infected and transformed cells but no neutralizing activity could be detected in these sera. These data indicate that though photodynamic treatment, like UV, may inactivate viruses such as HSV, it may also potentiate their transforming ability.

In vitro transformation of cells by non-inactivated virus particles would be

useful because virus rescue would then be a theoretical possibility. Darai and Munk (34) exposed human embryonic lung cells to HSV-1 strains at high temperatures (42 °C) to inhibit the lytic infection. They observed transformed cells with epithelioid morphology, differing from the original fibroblastoid lung cells. Syncytia formation and cytoplasmic antigens were observed. Some resistance to superinfection was also noted. These data have not been confirmed and it is possible that the original lines were derived from HeLa cell contamination.

Macnab (35), using temperature-sensitive mutants of HSV-2 and wild-type HSV-1 and 2, transformed rat embryo cells. Transformed cell lines were either epithelioid or fibroblastoid initially, but occasionally changed to mixed morphological types upon passage. Indirect immunofluorescence tests revealed virus-specific antigens in the transformed cells, though no virus particles were seen by electron microscopy. Rescue of infectious herpesvirus by co-cultivation with susceptible cells proved futile and there was no evidence for the presence of RNA tumor viruses. This investigator also repeated the work of Duff and Rapp (18, 19) by transforming hamster embryo fibroblasts with UV-irradiated HSV-2. These cells were tumorigenic after inoculation into newborn hamsters, where fibrosarcomas were induced. The oncogenicity of the HSV-transformed rat cells has not yet been reported.

Takahashi and Yamanishi (36) transformed human embryo and hamster embryo cells by temperature-sensitive mutants of HSV type 2 at restrictive temperature (38.5 °C). Morphologically transformed foci appeared 3–4 weeks after virus inoculation. The transformed hamster cells induced the formation of tumors in newborn and adult hamsters. Both species of transformed cells contained HSV-specific antigens in the cytoplasm of 5–10 % of the cells, as detected by indirect immunofluorescence techniques. Again, no infectious virus has been isolated from either the transformed or tumor cells by co-cultivation with or inoculation onto permissive human embryo lung cells at 32 °C.

Use of restriction enzymes and fragments of HSV DNA in transforming experiments (37) have also yielded positive results. This type of experiment may soon reveal whether a specific fragment of HSV DNA is responsible for the transforming event.

Transformation by Cytomegalovirus In Vitro

Support for the theory that CMV may induce neoplasia is seen in the data presented by St. Jeor *et al.* (38, 39). These observations indicate that CMV can stimulate host cell DNA synthesis under permissive (human embryo lung cells) and restrictive (human embryonic kidney cells and monkey Vero cells) conditions. Heat-treated virus and UV-irradiated virus were unable to induce this stimulation. Therefore, it seems plausible that some virus function(s) is necessary for elevation of cell DNA synthesis.

Other investigators have subsequently reported the stimulation of host cell cytoplasmic RNA in human fibroblast cells (WI-38) after infection with CMV (40). This induction was postulated to require an early virus-specific protein. Stimulation of host cell DNA was not observed in this system. However, these same investigators subsequently reported the induction of both host-cell DNA and ribosomal-RNA synthesis in guinea pig cells abortively infected with CMV (41).

Again, virus inactivated by heat or UV-irradiation did not cause stimulation of macromolecular synthesis.

In general, these results are similar to those already obtained with SV40 (42, 43), polyoma (44, 45) and adenoviruses (46–48), all established tumor viruses. In addition, this stimulation of DNA synthesis was seen in human leukocytes infected with EBV (49).

Strengthening the case, Albrecht and Rapp (50) exposed hamster embryo fibroblasts to UV-irradiated CMV and obtained morphologically transformed foci. Virus-specific antigens were detected in the cytoplasm of 0.5 % of the cells while 47 % yielded bright membrane fluorescence. Mixed hemagglutination assays and ^{125}I-labeled anti-globulin tests revealed that the virus-specific membrane antigen(s) present in transformed cells are similar to antigens found in CMV-infected human cells (51).

The CMV-transformed cells were oncogenic on transplantation to animals, inducing fibrosarcomas. Tumor-bearing hamster sera did not contain neutralizing antibody to CMV, but did react with CMV-infected, transformed and tumor cell antigens in fluorescent antibody tests. Microcytotoxicity tests performed with spleen cells from tumor-bearing hosts revealed that these animals have a cell-mediated immune response to the homologous tumor cells (52).

Lang *et al.* (53) have also indicated that human diploid fibroblasts can grow for generations in agarose after infection with human CMV. Eventually, however, cytopathology and cell lysis resulted and cell lines could not be established.

The most recent finding concerns the possible transformation of human cells by CMV (54). Normal human prostate cells, obtained from a 3-year-old male donor, yielded infectious virus upon early passages of *in vitro* culture, revealing the carrier state of these cells. Subsequent passage of these prostate cells led to virus latency and persistence of the virus genome. Virus-specific antigens were observed both in the cytoplasm and on the membrane, and the frequency of virus positive cells increased with IUdR pretreatment. Other evidence for the presence of CMV genetic material was ascertained indirectly by the observation that splenic lymphocytes from hamsters with CMV-specific tumors were cytotoxic for the transformed human prostate cells and directly by molecular hybridization which demonstrated 10–15 genome equivalents of CMV per transformed cell. All attempts to rescue virus were unsuccessful. Long term cultures have now been established; these have maintained the diploid human karyotype and preliminary results suggest they may be oncogenic when transplanted into athymic (nude) mice. This observation of *in vitro* transformation of human cells by CMV requires extension but should open new pathways for further study of the interaction of CMV with mammalian cells.

Discussion

Herpesviruses, now including EBV, HSV and CMV, have clearly demonstrated the ability to induce transformation of a variety of cell types. Cells from tumors caused by herpesvirus-transformed cells have been grown in culture and yield cell types morphologically similar to the original transformed cells. Markers of virus presence are evident in both transformed and homologous tumor cells. Virus-

specific antigens (cytoplasmic, nuclear and membrane), virus-coded enzymes and glycoproteins and the virus genome have been detected in most of the transformants. However, infectious virus particles have been isolated only rarely and only from EBV-transformed lines.

It has been established that herpesviruses can induce chromosomal aberrations and breaks (55–58). It is possible that one of the early proteins specified by the virus aids in the association and, perhaps, integration of the virus genome into the cellular genetic material where breaks have occurred. This could occur during the repair process.

Following transformation, some of the virus-specific glycoproteins are incorporated into host cell membranes. This should affect cell-cell relationships, possibly leading to loss of contact inhibition. In addition, the immunogenicity of the new surface glycoproteins will greatly influence the oncogenic potential of these virus-transformed cells.

The data presented thus far clearly demonstrate the *in vitro* transforming ability of three human herpesviruses, EBV, HSV and CMV. The relationship of cell culture studies to naturally occurring neoplasias is obviously in need of further investigation.

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