Biochemical Approaches to Detection of Herpes Simplex Virus Type 2 in Cervical Carcinoma

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Summary

Nucleic acid hybridization techniques are highly sensitive methods for determining the presence of herpes simplex type 2 genomes. Measurement of the rate of reassociation between labeled, purified viral DNA and DNA extracted from the tumor cells may be necessary to achieve adequate sensitivity if only a portion of the viral genome is present and if tumors contain substantial proportions of cells other than tumor cells. A large number of cervical tumors in various stages, as well as many control tissues, need to be studied to permit a decision on the involvement of herpesvirus type 2 in causing cervical carcinoma.

Introduction

Numerous seroepidemiological surveys consistently have indicated higher incidences of antibody to HSV-2 in groups of women with cervical carcinoma than in matched groups of women without cervical carcinoma. Nahmias et al. (17) and Rawls et al. (21) first reported the correlation; since then, additional studies have used more carefully matched control groups and more sophisticated statistical analysis, and the correlation seems to persist. Thus, seroepidemiology has clearly indicated that HSV-2 is worth investigating intensively as a possible etiological factor in cervical carcinoma.

It is not possible, of course, to perform controlled inoculation experiments in humans with HSV-2 to test for a possible etiological role of that virus in cervical carcinoma. Because of the importance of the problem, however, it seems appropriate to use alternatives to seroepidemiological surveys in an effort to gain additional data on the possible relationship.

This brief review will examine the possibilities offered by biochemical means, especially hybridization procedures, to detect viral genomes in cervical cancers, and the significance of information derived by these methods in evaluating the etiological role of HSV-2 in cervical carcinoma.

Presence of HSV-2 Genome in Cells of Cervical Carcinomas

Current practice seems to be to define certain viruses in terms of biochemical tests. Some RNA C-type viruses are identified by whether 70 S RNA and reverse transcriptase are detected in a sucrose gradient at a density of 1.16 to 1.18 g/ml. Thus, it does not seem out of line to use biochemical means for detecting HSV-2 genomes in cervical carcinoma cells. Indeed, this seems a straightforward approach to the problem, especially since molecular hybridization methods can detect even partial genomes. Also, there is good precedent from other systems.

Soon after the discovery that polyoma virus, SV40, and certain adenoviruses induce tumor formation in appropriate animals, it was noted that the resulting tumor cells did not release infectious virus. Thus, it was apparent that the mechanism by which these viruses cause the malignant change differed from the mechanism of oncogenesis by RNA-containing viruses known at that time. Indirect evidence for the continued presence of the viral genome in the transformed cells was obtained from many observations of new, viral-specific antigens in the transformed cells.

Gerber and Kirschstein (11) and Sabin and Koch (23) demonstrated that some cells transformed by SV40 produce infectious virus, and therefore those cells must contain at least 1 complete viral genome. Neither polyoma nor adenovirus-transformed cells produced virus, however.

Except for the work cited above, the first convincing, direct evidence for the presence of all or part of the viral genome in cells transformed by DNA viruses was provided by Benjamin (3) for polyoma virus and by Fujinaga and Green (9) for certain adenoviruses. The experimental technique used in both cases was to extract radiolabeled mRNA from transformed cells and to test it for viral-specific base sequences by hybridization with known viral DNA. Thus, not only was at least some viral genetic material shown to be present, but it was being transcribed in the transformed cells.

Green (13) surveyed 200 human cancers that had arisen from 19 different organ types for presence of genetic information related to human adenovirus types 2, 7, and 12. In this survey, unlabeled RNA was extracted from the tumors; this RNA was tested for its ability to compete for hybridization with known, labeled mRNA and the homologous adenoviral DNA. No inhibition of hybridization was found; as Green (13) pointed out, however, the procedure probably was not sensitive enough to rule out adenoviruses.
as etiological agents of human cancers.

So far, only limited experiments have been performed to detect HSV-2 genomes in cervical carcinoma cells. During the discussion period at a recent meeting in Florida, zur Hausen (25) reported that an associate of his, Dr. Schulte-Holthausen, had tested for hybridization between HSV-2-labeled cRNA and DNA extracted from cervical carcinoma cells. All 13 of the carcinomas tested were negative for viral genomes by his test.

However, the sensitivity level was such that, at best, the test could have detected 1 to 3 genomes/cell. One important factor among several contributing to low sensitivity is that only 10 to 15% of the genome is transcribed into cRNA in the synthesis reaction. Since this small portion may not be random, it is possible that the part of the viral DNA transcribed \textit{in vitro} is not complementary to the portion that may be in the malignant cells. The negative result therefore does not rule out the possibility that all or part of the HSV-2 genome is present in transformed cells. As Dr. zur Hausen (25) pointed out, however, the negative result indicates that if HSV-2 is responsible for cervical carcinoma, there is a basic difference in the interaction of HSV-2 DNA and cervical cells, compared with the interaction between EBV and Burkitt lymphomas, in which there are many complete viral genomes per cell (18, 26).

Frenkel \textit{et al.} (8) and Roizman and Frenkel (22) have also used hybridization methods for detecting viral genomes in cervical cancer cells. Instead of measuring hybridization between HSV-2 cRNA and tumor cell DNA, they measured the rate at which known, labeled HSV-2 DNA hybridized with the tumor cell DNA. This procedure, the reassociation kinetics test, is the most sensitive method presently available for detecting viral DNA. It can detect less than 1 genome/cell, sometimes as little as 0.1 genome/cell.

In the 1 tumor reported so far, these workers found 1 to 3.5 copies/cell of a piece of the viral DNA that was equivalent to about 40% of the entire viral genome. This positive result is only a beginning. Only 1 tumor and no control cervical tissues were studied, so that conclusions should not yet be drawn about the association of HSV-2 and cervical carcinoma on the basis of these results.

Furthermore, these findings appear to differ in some respects from findings with other oncogenic herpesviruses and their transformed cells, especially with regard to the number of viral genomes per cell.

\textbf{Number of Viral Genomes in Transformed Cells and Their Relation to Cell DNA}

Population surveys for incidence of HSV-2 genetic material in cervical carcinomas by hybridization methods are certainly feasible now and are urgently needed. For an adequate study, carcinomas of different stages should be examined, and at least an equal number of controls. Control tissues should include human fetal tissues, cervical tissue from uteri removed for fibroids, cervical tissue from patients with genital herpes infection both presently active and active in the past, and uterine cancers other than cervical carcinomas. The antibody status with respect to both herpes simplex virus type 1 and HSV-2, as well as sexual history, should be recorded for each patient and control.

Such a survey, however, even if carefully and extensively done, still could at best satisfy only the first of Koch’s postulates. It is not possible, of course, to satisfy strictly the other postulates in the human situation.

In addition to showing an association between HSV-2 and cervical carcinoma, other evidence is needed to determine cause. Transformation of hamster embryo cells in culture by HSV-2, as described by Duff and Rapp (5), offers a model for comparison. Whether few or many genomes are present in transformed cells may be indicative of whether few or many are to be expected in tumor cells.

Some determinations have been made of the number of EB viral genome equivalents in cell lines derived from Burkitt’s lymphomas. By hybridizing radiolabeled cRNA synthesized with the use of EB viral DNA as template against DNA extracted from the Raji cell line (a line of Burkitt lymphoma cells that does not produce EBV), 40 to 100 genome equivalents were calculated per cell (18, 26). However, 2 to 5 genome equivalents per cell were estimated with data from DNA-DNA hybridization on nitrocellulose filters (27, 28). By reassociation kinetics with labeled DNA synthesized with EB viral DNA as template against DNA extracted from Raji cells, Nonoyama and Pagano (20) estimated about 50 genome equivalents per cell. This last method is probably the most sensitive and accurate, and the results agreed with the estimates made using labeled cRNA.

Not only the number of viral genome equivalents per cell, but also their relationship to the cellular DNA is important. Evidence with other DNA-containing tumor viruses—polyoma, SV40, and adenoviruses—indicates that the viral genome in those cases becomes integrated into the cellular DNA and covalently bonded.

The limited biological evidence available so far with oncogenic herpesviruses is suggestive of a different relationship between viral and host cell DNA. Numerous reports with various herpesviruses have revealed that transformed cells, such as circulating lymphocytes, carry the viral genome in an intact but repressed state. A general characteristic seems to be that the oncogenic herpesviruses become associated with circulating lymphocytes but are not detectable antigenically, by electron microscopy, or by infectivity assays for free virus. After being cultured or, in some cases, by cocultivation with susceptible cells of even the same species, the virus is expressed antigenically and infective virus can be recovered. This type of result, with some experimental variations from system to system, has been reported for EBV (6), Marek’s disease virus (4, 16, 24), \textit{Herpesvirus saimiri} (1, 7), guinea pig herpes virus (15), and cottontail rabbit lymphoma virus (14). Aurelian \textit{et al.} (2) have reported isolation of HSV-2 from cervical carcinoma cells after many passages \textit{in vitro}. Granoff (12) has reviewed the temperature dependence of the replication of the herpesvirus associated with Lucké adenocarcinomas of leopard frogs. This last instance is the only one in which there is some evidence concerning the mechanism controlling the repression of the viral genome. With the exception of hamster tumor cells induced by SV40, which release SV40 in low titer (10, 11, 23), tumors induced by polyoma...
and adenovirus do not release virus, even after being cul-
tured.

Thus, the result cited above (8, 22), if it is generally true
for cervical carcinomas, differs from what has been found
with all other oncocogenic herpesviruses. Furthermore, the
possibility that only a portion of the viral genome is present
conflicts with the isolation of virus from cervical tumor cells
by Aurelian et al. (2).

In our laboratory, Dr. B. C. Casto (unpublished data)
have obtained cells transformed by UV-irradiated HSV-2 by
following the procedure of Duff and Rapp (5); morpholog-
ically, the cells seem to be identical to those transformed
by Duff and Rapp (5). However, after several weeks in cul-
ture, many of the cells in the apparently transformed colo-
nies undergo a cytopathic effect such as is usually seen with
HSV-2 infection. It therefore seems that a complete ge-
nome is present, but temporarily repressed.

It should be emphasized, however, that the fact that only
40% of the genome was present in the tumor described
above (8, 22) is strong evidence against the possibility that
the virus was present only as a passenger or as an incidental
infection.

Frenkel et al. (8) and Roizman and Frenkel (22) also
concluded that the viral DNA is integrated into the cellular
DNA. This was determined by studying the rate of reas-
sociation of the DNA under conditions in which cell DNA,
because it contains repetitive sequences, reassociates rapidly
while viral DNA would reassociate slowly. It was found that
the viral DNA also reassociated rapidly, implying a coval-
ent linkage to cell DNA. However, HSV-2 DNA also
contains repetitive sequences (8, 22), so that it might also
reassociate rapidly under the same conditions.

In contrast, by centrifuging DNA extracted from Raji
cells through alkaline sucrose gradients and testing each
fraction for viral genomes by hybridization, Nonoyama and
Pagano (19) found that the viral sequences banded at a
location identical to the location of the viral DNA band
when the viral DNA was simply mixed with human DNA. It
was not associated with the cell DNA and therefore was not
covalently bonded to it. The viral-specific DNA was shown
to be associated with the chromosomes of the cells, however.
Unfortunately, these experiments are also not definitive,
since alkali-labile bonds in the viral DNA could break and
yield the result observed; it need not be due to lack of
covalent linkage. It is also possible that most of the genomes
present in the EB cells are not integrated into the cell DNA
and that only a portion of genome is integrated. This last
portion could be responsible for the malignant change, yet
would not have been detected in the experiments described.

There is no reason to believe in advance that, in inducing
transformation, the genome of oncocogenic herpesviruses
should be integrated by covalent bonds into the cellular
DNA, and the available evidence is inconclusive. It will be
necessary to study in detail the lines of hamster and human
cells, if possible, transformed in culture by HSV-2 to
determine the relationship between the viral and cellular
 genomes. This type of study will give a basis for compari-
sion of cervical carcinoma cells that contain viral genomes.
Such evidence will bear on whether the viral genome in
cervical carcinoma cells is associated in a way that is con-
sistent with its possible etiological role. For instance, if the
HSV-2 genome is present in the tumor only as a passenger
or as a secondary invader, it may very well have a different
relationship to the cellular DNA than it would have if it
were etiologically responsible for the tumor.

Function of the Viral Genome in Transformed and Malignant
Cells

Whether mRNA molecules specific for viral DNA are
transcribed in cervical carcinoma cells that contain viral
genetic material is another parameter that needs to be
measured. HSV-2 genetic material could be present in
cervical carcinoma cells as a passenger or secondary
invader, as a causal agent, or as a latent agent. (The
relationship of viral and cellular genomes in the last 2
possibilities could be the same.) A determination of how
many and which species of mRNA are transcribed may
permit one to distinguish among the possibilities and draw
conclusions regarding the role of HSV-2 in the etiology of
cervical carcinoma.

Conclusions

In this brief review, I have discussed molecular hybridiza-
tion methods that are available for use with herpesviruses. It
seems technically possible now to make a survey of
populations of women with cervical carcinoma to determine
the incidence of viral genetic material in the tumors.
Additional data on how many genomes are contained in the
malignant cells, the relationship of the viral genetic material
to the cellular DNA, and the function of the viral genetic
materials in terms of the mRNA molecules transcribed are
needed to evaluate the role of the genome in the malignant
process. For a complete evaluation, parallel studies with in
vitro transformation are also required.

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