A Search for Herpes Simplex Virus Type 2 Markers in Cervical Carcinoma

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Summary

Carcinoma of the uterine cervix is now clearly recognized as the second most common malignant disease of women in the United States. Epidemiological studies provided the first suggestive evidence that an infectious, venereally transmitted agent was involved in this disease in either a causal or a casual fashion. Later cytohistopathological, virological, and seroepidemiological studies confirmed this observation and identified the suspect agent as herpes simplex virus type 2. Recently, several laboratories have directed their energies towards establishing lines of direct evidence linking herpes simplex virus type 2 etiologically with human cervical carcinoma. Some of these approaches have involved attempts to detect infectious virus, viral components, or virus-specific modifications in neoplastic cervical tissues. Results obtained utilizing human tissues will be reviewed and discussed.

The 2nd most common malignant disease of women in the United States is now clearly recognized as carcinoma of the uterine cervix. Although there are approximately 35,000 new cases of cervical carcinoma and 10,000 deaths reported annually, the incidence of anaplastic cervical pathology is surely much higher when one considers the myriad cases of cervical dysplasia that may or may not progress to frank carcinoma. The mortality statistics from this disease would be far more depressing were it not for the test developed in 1943 by Papanicolaou and Traut (53) correlating the cytopathology of exfoliated cervical cells with the histological diagnosis of cancer. In a historical sense, the Papanicolaou (Pap) test was the sine qua non for much of our present understanding of cervical carcinoma. In addition to providing an invaluable tool for early diagnosis and posttreatment monitoring, this test furnished the 1st practical means of studying the pathogenesis, epidemiology, and etiology of cervical carcinoma on a large scale.

Fortunately, the epidemiology of cervical carcinoma has received considerable attention for over 30 years. An excellent perspective on the key findings in this area has been presented by Rotkin (61). In spite of the lack of total agreement among various investigators regarding individual aspects of these studies, certain trends emerged that eventually facilitated the identification of several cultural and sexual factors associated with increased risk (35, 61). Thus, it became clear that the pivotal demographic and epidemiological characteristics that distinguished women at greater risk to developing cervical cancer were low socioeconomic status, early age of 1st coitus, and number of sex partners. The suggested etiological involvement of a venereally transmitted agent in cervical carcinoma was a natural extension of these studies and it was not surprising that such speculation included the possible involvement of a DNA virus (15, 60). During the last decade, a virtual plethora of evidence has emerged which unequivocally associates HSV-2 with human cervical carcinoma. These studies have utilized the disciplines of cytohistopathology, virology, seroepidemiology, immunology, and molecular biology. The seroepidemiological studies have been extensively reviewed (35, 43, 55) and will be discussed elsewhere in this Symposium. This discussion will focus on those studies which have dealt with the detection of infectious HSV, subviral components, or virus-specific modifications in anaplastic human cervical tissues.

The isolation of HSV from the primary lesions of 3 cases of acute vulvovaginitis by Slavin and Gavett (64) in 1946 provided the 1st evidence for herpesvirus infection of the human female genitalia. Neutralizing antibody was detected in the convalescent, but not acute, sera of the 3 patients, and evidence suggesting a venereal mode of transmission was detected in 1 case. This report had signal importance in explaining cervicovaginal cytological findings reported later, since it clearly described the ability of HSV to initiate infection and induce pathological lesions on the female genitalia. The genital cytological findings were heralded by the demonstration of characteristic HSV-induced sequential cytomorphologic changes in cells of primary or recurrent nongenital lesions (10). Although similar changes were reported in cervicovaginal smears (36, 69), convincing evidence relevant to the involvement of HSV in their etiology was not presented until 1963. It was at that time that Stern and Longo (65) described characteristic HSV cytological features in vaginal and cervical Pap smears from a case of acute vulvovaginitis. More importantly, the cytological diagnosis was supported by virus isolation and by the demonstration of an elevated titer of neutralizing antibody in the patients’ convalescent phase serum. The efficacy of cytolog-

1 The abbreviations used are: HSV-2, herpes simplex virus type 2; HSV, herpes simplex virus; HSV-1, herpes simplex virus type 1; cRNA, complementary RNA; EBV, Epstein-Barr virus.
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ichological techniques in detecting genital herpes infections was later corroborated in similar, although more extensive, human (49) and murine (44) studies. Successful viral isolations were nearly always (>90%) paralleled by positive cytopathological findings, while there was a 65 to 75% correlation by the reverse comparison (44, 47–49). However, infectious virus (3) and HSV structural antigens (2, 7) have not been identified in cervical specimens taken between recurrences of cytopathologically detectable herpetic cervicitis. Thus, genital HSV mimics the classic picture of viral latency displayed by HSV-1 in nongenital tissues (17, 66).

It is now known that herpes genitalis is a common venereal disease (41, 42, 49, 56) that shares several epidemiological characteristics with cervical carcinoma (30, 31). The clinical features and epidemiological patterns of the infection have been reviewed recently (34, 43). The observation that HSV isolates of facial-oral (HSV-1) and genital (HSV-2) origin possessed different serological, biological, and physical properties was particularly significant (20, 40, 54, 63). An overwhelming majority of the cases of herpes genitalis, including herpetic cervicitis, have been shown to be caused by HSV-2 (20, 32, 40).

The first evidence associating HSV with genital cancer came from simultaneous screens of Papanicolaou smears for anaplastic and herpetic cytopathological markers (47). In this and later studies (1, 9, 29, 48–50, 52, 70), an increased incidence of cervical anaplasia was noted in women with cytopathologically detectable genital herpes virus infections. These observations stimulated extensive seroepidemiological studies that largely confirmed the associative relationship between HSV and cervical anaplasia (35, 43, 55). In spite of inherent difficulties related to the serological specificity and cytohistopathological interpretation of the tests, women with invasive cervical carcinoma, carcinoma in situ, or cervical dysplasia tended to have higher titers of HSV-2-neutralizing antibodies than controls. In addition, women with cervical anaplasia showed an increased frequency of genital herpes-virus infection as determined by serological, rather than cytopathological, examination. This finding is consistent with the ability of serological analyses to detect evidence of both past and recurrent infections and with the often latent nature of the infection when the patient is clinically and cytopathologically asymptomatic (30, 32).

Utilization of cytopathological techniques to obtain evidence for an etiological relationship between genital HSV and cervical anaplasia has led to an awareness of the advantages and limitations of this approach. Detection of cytopathological changes induced by genital HSV is entirely dependent on the ability of the observer to recognize and differentiate specific HSV-induced changes from nonspecific cytopathomorphic alterations. Nonspecific changes include multinucleated endocervical repair, trophoblastic, anaplastic, and foreign-body giant cells (49) and genital cells infected with lymphohgranuloma venereum (37), condyloma acuminatum (24, 46), and cytomegalovirus (45). Although HSV and varicella-zoster virus induce practically identical cytopathological changes (10), the latter virus has not been isolated from the cervix. Other limitations relate to the difficulty of detecting infection between periods of recrudescence, to differentiating between HSV-1- and HSV-2-induced cytopathological changes, and to distinguishing between primary and recurrent infections (45, 49). In this respect, it is pertinent that approximately 5 to 14% of genital HSV isolates were typed as HSV-1 (34, 39, 40). A comparison of the data from previously published studies indicates that procedures for detecting genital HSV infections decrease in sensitivity according to the order serological > virological > cytopathological > clinical examination. Histological diagnosis of cervical HSV infection has been reported to be more difficult than cytopathological diagnosis since it appears to depend on the time at which specimens are obtained, the origin and size of the specimens, and the thoroughness with which the tissues are examined (49). However, differentiation of herpetic from anaplastic cytomorphic modifications was sometimes easier to resolve by histological rather than cytopathological examination when both types of changes appeared in the same specimen (47, 49). Review of biopsy or hysterectomy specimens, as opposed to cervicovaginal smears, also permitted localization of the respective changes relative to particular cell types and associations within tissues.

Taken together, these facts point to a rather restricted use of cytohistopathological techniques for detecting genital HSV-2 and for studying its relationship to cervical carcinoma. Cytohistopathological techniques, however, are extremely useful as diagnostic tools to identify individuals with an increased risk of developing cervical cancer. Pap smears offer the simplest, least expensive method currently available to screen large numbers of women for evidence of cervical neoplasia. It would seem a pity to exclude simultaneous examination of smears or tissue sections for HSV markers since HSV-2 infection has been highly associated with cervical cancer.

The search for HSV markers in cervical tumor cells has been extensive. Infectious virus, viral structural antigens, and HSV-specific cytoplasmic changes have not been detected directly in cervical cancer biopsies (2, 5, 7). However, virion structural antigens were observed in exfoliated tumor cells and tumor cells on the periphery of neoplastic lesions (2, 7, 62). The observed serological reactivity seemed to be directed against virion proteins made relatively late in the HSV-2 growth cycle. Fluorescence-positive anaplastic cells contained no virus particles but did exhibit cytopathological changes similar to those induced by HSV. There was no correlation between the stage of the disease and the number of HSV antigen-positive cells. Extensive control studies pointed to the specificity of the immunofluorescent techniques used for both HSV-2 and cervical carcinoma cells.

Further evidence for the persistence of the HSV genome in cervical tumor cells was garnered from in vitro experiments in which infectious virus, viral antigens, and characteristic HSV nuclear alteration were detected in spontaneously degenerating cell cultures derived from a carcinoma in situ (3, 8). Changes similar to those appearing spontaneously could be induced by exposure of the cells to medium of high pH, but they were undetected in cultures showing no signs of degeneration. A culture derived from an invasive cervical carcinoma behaved quite differently (3). Virus expression was limited to 2% of the cells and appeared as a membrane fluorescence that was detected with human sera containing antibody to either HSV-2 or HSV-1.
Spontaneous or high pH-induced cell degeneration failed to augment virus expression in this culture.

In yet another approach, soluble membrane antigens were extracted from cervical, vaginal, and vulvar carcinomas and were shown to react in complement fixation tests with an antiserum prepared against partially purified HSV-2 (27). Interpretation of these results is difficult since data relevant to the recovery of infectious HSV from the specimens prior to extraction or to the genital HSV status of the patients at the time of tumor removal was not reported. In later studies, the nature of this serological reactivity was defined by showing that soluble membrane antigens from cervical carcinomas fixed complement in the presence of antibody directed against HSV nonviral antigens (28). The nonviral antigens used in these tests were prepared from HSV-infected cultured mammalian cells and were shown to contain no immunologically demonstrable virus structural components. In addition, sera from cervical cancer patients with no anti-HSV structural antigen activity were reported to react in complement fixation tests with partially purified HSV nonviral antigens. Antibodies to HSV nonviral antigens were also detected in a large percentage of patients with squamous cell carcinomas of the head and neck (26).

In similar, although more extensive studies, human sera from cervical cancer cases and matched controls were screened for reactivity with antigens that appeared in cultured human cells 4 hr after infection with HSV-2 (4, 6). This antigen, called AG-4, appeared to be distinct from those viral structural proteins involved in neutralization. Antibody to AG-4 was detected in women with cervical neoplasia and displayed a pattern of increased prevalence in patients with more advanced stages of the disease. Antibody to AG-4 was not detected in the majority of matched controls, in patients who had undergone successful therapy for cervical carcinoma, and in women with carcinomas at other sites. AG-4 reactivity was also shown to be present in extracts of cervical cancer biopsies and in cell cultures derived from 2 different cervical carcinomas.

By use of molecular hybridization techniques, Frenkel et al. (23), and Roizman and Frenkel (59) have obtained evidence for the presence of HSV nucleic acids in 1 cervical carcinoma biopsy. Analysis of the kinetics of hybridization in liquid of purified radiolabeled HSV DNA with excess unlabeled RNA from the tumor yielded evidence that the tumor contained RNA transcripts complementary to only 5% of HSV-2 DNA. Preliminary results further indicated that both early and late HSV-2 functions were expressed by the 5% of the genome transcribed and that about one-half of the sequences transcribed were shared in common by HSV-1 and HSV-2 DNA.

The same cervical tumor biopsy was also examined for DNA sequences homologous to those found in HSV-1 and HSV-2 DNA (23, 59). Evidence obtained from the rate of reassociation of labeled HSV-2 DNA in the presence of unlabeled DNA from the tumor indicated that only a fragment consisting of about 39% of HSV-2 DNA was present in tumor cells. Because of the uncertainty of the exact ploidy of cells from this tumor, the DNA fragment was calculated to be present at from 1 copy/heteroploid cell to 3.5 copies/diploid cell. At least a portion of the HSV-2 DNA present in this tumor was determined to be covalently linked to host cell DNA.

In contrast to the above results, other investigators have failed to demonstrate HSV-2 DNA in cervical carcinoma biopsies. zur Hausen et al. (73) found no HSV DNA in 10 cervical carcinomas examined with cRNA (probes synthesized from HSV-1 or HSV-2 DNA with E. coli RNA polymerase). It was also reported (73) that both Schulte-Holthausen and Petersen, also using HSV-2 cRNA as probe, failed to find HSV-2 DNA in approximately 30 cervical tumor biopsies. As acknowledged by zur Hausen et al. (73), results obtained with cRNA do not negate results obtained by the more sensitive assays used by Frenkel et al. (23) since E. coli RNA polymerase may not synthesize RNA transcripts of the complete HSV-2 genome. Thus, if only fractional HSV-2 genomes are present in tumor cells, they will go undetected unless RNA transcripts complementary to these sequences are present in the probe. cRNA probes have been used successfully to detect EBV DNA in lymphoblastoid cell lines, in Burkitt’s and nasopharyngeal tumors, and in cells from infectious mononucleosis patients (51, 71–73). The sensitivity of the cRNA hybridization assay for detecting EBV DNA has been estimated to be between 2 and 3 genome equivalents/cell (71). However, it should be kept in mind that the complete EBV genome is present in cells of EBV-associated diseases (Burkitt’s lymphoma, nasopharyngeal carcinoma, and infectious mononucleosis) or in cells transformed in vitro by EBV, a situation not found for HSV in cervical tumors and in vitro-transformed cells. Using the more sensitive DNA-DNA renaturation kinetics technique, it has been reported (73) that Pagano was also unable to detect evidence for HSV DNA in biopsies of 5 cervical tumors. Hence, to date, only 1 cervical carcinoma biopsy has been reported to contain nucleic acids homologous to those of HSV-2.

**Discussion**

In this communication a large body of information has been reviewed concerning the relationship of HSV-2 to squamous cell carcinoma of the human uterine cervix. A definite pattern of association between HSV-2 and cervical carcinoma has emerged from these studies, but etiological involvement has not been proven. Seroepidemiological studies in which higher titers of neutralizing antibody to HSV-2 have been found in sera of cancer patients compared to matched controls have provided the strongest associative evidence. More direct approaches, however, have met with only qualified success.

A major question that needs clarification is whether HSV-2 genetic information is present in all cervical tumor cells and, if so, in what state. As discussed previously, infectious HSV-2 was isolated from a cell culture derived from a carcinoma in situ (3, 8) and HSV-2 membrane antigens were detected in a small percentage of cells from a culture, not yielding infectious virus, originating from an invasive cervical carcinoma (3). The observed variations in virus expression in these cultures may simply reflect the selection in vitro of cell populations not representative of the respective tumors. Nevertheless, the isolation of infectious HSV-2 from cultured cells of a carcinoma in situ certainly suggests that
the complete viral genome persisted in these cells. Two possibilities could explain the failure to isolate infectious virus from the invasive cancer: (a) a more stringent control of virus expression at the transcriptional or translational level; or (b) a defective viral genome. It is unfortunate that cell cloning and molecular hybridization analyses were not used to quantitate and identify the HSV-2 genetic information in these cell cultures.

The relative ease with which HSV-2 structural and nonvirus antigens have been detected in exfoliated tumor cells and in tumor specimens, respectively, stands in sharp contrast to the limited success enjoyed in detecting HSV-2 genetic information in cervical tumors. It is presently unknown whether the appearance of these antigens reflects the release from repression of endogenous HSV-2 genetic information or exogenous infection of susceptible tumor cells by genital HSV. The failure to detect signs of active genital herpetic infection in cervical cancer patients expressing HSV-2 antigens in tumor cells would not unequivocally rule out the latter possibility since infectious HSV-2 has been shown to produce an abortive infection in certain classes of transformed cells (12, 18, 19, 68).

There is a good rationale for proposing that defective, noninfectious herpes virions have greater oncogenicity than fully infectious virions. Productive infection with herpesviruses invariably leads to cell death due to inhibition of host cell DNA, RNA, and protein syntheses (33, 58). Hence, cell transformation and production of progeny herpesviruses are mutually exclusive processes. The oncogenic potential of herpesviruses has been postulated to reside in the expression of early viral functions exclusive of those responsible for inhibition of host cell macromolecular synthesis (57).

The molecular hybridization results of Frenkel et al. (23), and Roizman and Frenkel (59) discussed previously provide evidence for the association of defective HSV-2 with cervical carcinoma. Support for such an association also comes from in vitro transformation studies. In vitro transformation with HSV-1 and HSV-2 has been accomplished only with inactivated virus or under conditions that restrict the complete HSV replicative cycle (16, 21, 22, 38, 67). Although infectious HSV has never been recovered from any of the transformed cell lines, HSV products have been demonstrated in these lines by immunofluorescent, enzymatic, and molecular hybridization techniques (14, 21, 22, 38). Clearly, additional molecular hybridization studies, using the most sensitive techniques, are indicated to determine whether HSV-2 nucleic acids are present in human cervical carcinomas. Since there is a strong possibility that only fractional HSV-2 genomes are associated with tumor cells, primary emphasis obviously should be placed on the development of more sensitive nucleic acid probes. Bacterial restriction endonucleases appear to show particular promise in this regard. Specific HSV DNA fragments generated with these enzymes could be used to examine cervical tumors and HSV-transformed cell lines for virus-specific information. Restriction fragments of HSV DNA could also be used in in vitro transformation experiments similar to those used to identify the transforming portion of the adenovirus genome (25). Additional studies concerning the presence of HSV-2 RNA transcripts in cervical tumors and in vitro transformed cells also appear warranted.

The soluble nonvirus antigens which have been extracted from HSV-2-infected cell cultures and cervical tumors must be characterized more fully. It is unknown whether these antigens are viral-coded products or cellular products produced as a consequence of HSV-2 infection in a manner analogous to fetal antigen and fetal thymidine kinase induction in SV40-infected cells (11–13). It would be interesting to know whether HSV nonvirus antigens appear in cultured cells infected with other oncogenic and nononcogenic viruses or in chemically transformed cells. Regardless of whether herpes nonvirus antigens are virus or cell coded, continued study appears warranted based on their potential diagnostic and prognostic significance in cervical carcinoma.

References

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