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Cervical Cancer: Is Herpes Simplex Virus Type II a Cofactor?

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CERVICAL CANCER

Approximately 1.6% of all women will develop cancer of the cervix during their lifetime (4). Cervical cancer is the second leading type of cancer in women and accounts for approximately one-sixth of all cancer deaths in females. In many ways, cervical cancer behaves as a sexually transmitted disease. The major risk factors associated with cervical cancer are the early onset of sexual intercourse, multiple sexual partners, and/or sexual contact with promiscuous partners (66, 94). The disease is composed of several pathological stages ranging from cervical intraepithelial neoplasia (CIN) to invasive squamous carcinoma. Substantial evidence suggests that CIN is a precursor of invasive cervical carcinoma (76, 88). Cytological and colposcopic studies have determined that most initial neoplastic events occur within the cervical squamous epithelium adjacent to the endocervical epithelium (the squamocolumnar junction or transformation zone [reviewed in reference 108]). At the junction where columnar epithelium becomes squamous epithelium, there is a temporary state of genetic imbalance that renders the immature cells susceptible to neoplastic events. It is believed that unique developmental aspects of the squamocolumnar junction as well as exogenous factors are necessary for induction of cervical carcinoma.

INVOLVEMENT OF HPV WITH CERVICAL CANCER

Certain human papillomavirus (HPV) types that are transmitted primarily by sexual contact are commonly detected in CIN and invasive carcinoma. Papillomaviruses are members of the family *Papovaviridae* and contain a genome that is composed of double-stranded DNA approximately 8 kb long (54) (Fig. 1). HPV early (E) genes (E1 to E7) encode proteins which regulate transcription, replication, or episomal maintenance. The late (L) genes (L1 and L2) encode structural proteins which are required to form a viral particle. More than 70 different types of HPV exist, with each type infecting either cutaneous or squamous epithelium at defined anatomical locations. HPVs can be classified with respect to oncogenic potential as either low, intermediate, or high risk. HPV-6 and

HPV-11 are found primarily in genital condylomata (44, 45) and are rarely detected in cervical carcinomas; therefore, they are regarded as low-risk viruses. HPV-31, HPV-33, and HPV-35 are found in approximately 10% of cervical dysplasias, primarily premalignant lesions (37), and are classified as intermediate-risk types. HPV-16 and HPV-18 are found in biopsy specimens of most invasive carcinomas (9, 20a, 28, 36, 44, 60), and are considered high-risk types (9, 44, 75, 120, 122). In cervical cancer cells, HPV DNA is frequently integrated with cellular DNA, while benign lesions contain episomal viral DNA (33). At the site of integration, cellular sequences are frequently deleted and/or rearranged, but integration does not appear to occur at specific cellular loci (85, 112). However, integration generally disrupts two early viral genes, E1 and E2.

The E6 and E7 genes of high-risk HPVs are almost always expressed in cervical cancer cells (74, 85) and can immortalize primary human keratinocytes (33, 34, 65, 116; reviewed in reference 28) or fibroblasts (23, 91). The E7 proteins of high-risk HPVs bind to the retinoblastoma protein (RB), a tumor suppressor gene product, with 10-fold-higher efficiency than do E7 proteins of low-risk HPVs (99, 107). Deletion or inactivation of the gene encoding RB plays a crucial role in the development of retinoblastoma (31). RB participates in regulating the G₁ phase of the cell cycle, presumably by interacting with other regulatory proteins (71). For example, RB interacts with (i) transcription factor E2F, thus inhibiting E2F-dependent transcription (12, 52); (ii) transcription factors involved with SP-1-dependent transcription, thus activating SP-1-dependent transcription (67); (iii) transcription factor ATF-2, thus activating tumor growth factor β 2 transcription (68); (iv) D cyclins (30); and (v) c-Abl, thus blocking its tyrosine kinase activity (113). In addition to binding to RB, E7 can *trans*-activate gene expression (89). The E6 protein of high-risk HPVs binds to the cellular tumor suppressor protein, p53, induces degradation of p53 (96), and can *trans*-activate the adenovirus E2 promoter (99). In response to DNA damage, p53 activates transcription of a cyclin-dependent kinase inhibitor (Waf-1/CIP-1), which blocks entry into S phase, thus allowing repair of DNA damage (64, 87). In summary, the interactions between HPV oncogene products E6 and E7 and tumor suppressor gene products p53

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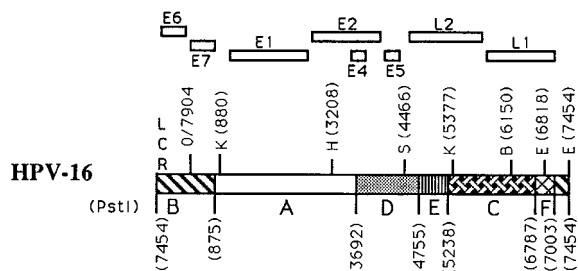


FIG. 1. Schematic of the HPV-16 genome, showing a restriction enzyme map of the prototype HPV-16 genome and the location of open reading frames. The organization of open reading frames in all high-risk-type HPV strains is similar.

and RB, respectively, are essential for immortalization of human cells and development of cervical carcinoma.

RELATIONSHIP OF HSV-2 WITH CERVICAL CANCER

In addition to HPV, other factors are postulated to play a role in the development of cervical cancer (reviewed in reference 21). These include smoking (8), human cytomegalovirus, human herpesvirus 6 (27, 28), human immunodeficiency virus (111), and HSV-2. HSV-2 is spread primarily by sexual contact, and therefore the risk factors are similar to those of other sexually transmitted diseases (105). A recent epidemiological study suggested that a biological interaction between HSV-2 and HPV-16 or HPV-18 occurs during the development of cervical carcinoma (53). This study also suggested that infection with both viruses is a significant risk but that infection with one or the other is not nearly as significant. Several independent epidemiological studies have also linked HSV-2 infections

to an increased incidence of cervical carcinoma (19, 25, 26, 66, 80, 81, 93; reviewed in references 21 and 36). Unfortunately, these studies do not include the contribution by the risk associated with HPV infection. In contrast, other studies found no correlation between HSV-2 and cervical cancer (see, for example, references 8, 20a, 24, 47, 60, 70, and 86). Some of the negative studies may have minimized the risk factor because women used as case controls were overmatched for sexual activity. Furthermore, the use of immunoglobulin A rather than immunoglobulin G as a marker for the presence of HSV-2 correlates with various stages of cervical cancer (18). In studies that found no correlation of HSV-2 with cervical cancer, the risk may have been underestimated if immunoglobulin G levels were used to measure HSV infection. Although the epidemiological studies do not conclusively demonstrate that HSV-2 plays a role in cervical cancer, there is evidence that HSV-2 has the potential to be a risk factor associated with a subset of cervical cancer cases.

Six of eight cervical cancer biopsy specimens that contained HSV-2 DNA sequences also contained HPV DNA (25, 26). Approximately 30% of genital tumors have DNA sequences homologous to HSV-2 DNA, which can transform rodent or human cells (25, 26). HSV-2 DNA sequences which have transforming potential are contained in a *Bgl*II N or *Bam*HI E fragment (Fig. 2). In 29 of 55 neoplastic genital tissues, the HSV-2 large subunit of ribonucleotide reductase (RRA), which is encoded by the *Bam*HI E fragment, was detected by immunochemistry (25). However, this viral antigen was not detected in normal tissue or in tumors at other sites. An independent study also demonstrated that RRA is detectable in more than 50% of cervical cancer biopsy specimens (3). HSV-2 RNA can be detected in a higher proportion of CIN than of squamous cell carcinoma, supporting the hypothesis

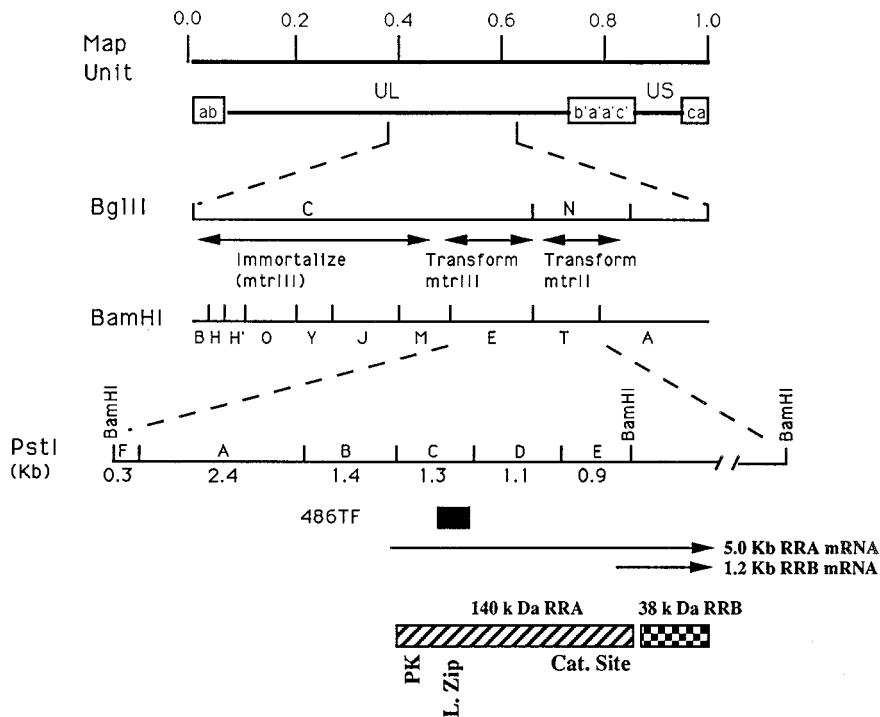


FIG. 2. Location of *mtr*II and *mtr*III within the HSV-2 genome. The positions of 486TF, mRNAs encoding RRA and RRB, RRA protein (140 kDa), and RRB protein (38 kDa) are presented. Within the RRA protein, the location of the protein kinase domain (PK) is between amino acids 1 and 411 (14). The position of the leucine zipper (L. Zip) is between amino acid 409 and 437 (72, 103). The catalytic site (Cat. Site) is in the C terminus of the protein (83).

that HSV-2 is linked to the initiation of cervical carcinoma (35). In contrast, HSV-2 RNA or DNA was not detected in normal cervical tissue. Although HSV-2 may not be associated with all cases of cervical cancer, there is molecular evidence indicating that HSV-2 and HPV coinfections occur in cervical cells and that the infected cells survive and may become neoplastic.

TRANSFORMING DOMAINS IN THE HSV-2 GENOME

Inactivated HSV-2 will alter the growth properties of rodent or human cells, and after passage in culture, these cells can form tumors in rodents (32, 73). Early experiments suggested a "hit-and-run" mechanism since the continued presence of virus is not required for transformation (38, 100). When inactivated HSV-2 was topically applied to the mouse cervix, lesions similar to those seen in women with cervical cancer were detected in approximately 60% of the animals (2, 115). Approximately 21% of these animals developed invasive cervical carcinoma. No cancers were detected in control animals treated with human or calf thymus DNA. Preimmunization of animals with HSV-2 prevented the progression to carcinomas (114). Two distinct and separate morphological transforming regions (designated mtrII and mtrIII) have been identified in the HSV-2 genome (38–41, 58, 59, 62) (Fig. 2). mtrII is contained within the *Bgl*II N fragment, and mtrIII is contained within the *Bgl*II C fragment. mtrII and mtrIII contain the genes for the RR (77) small subunit (RRB) and large subunit (RRA), respectively, suggesting that inappropriate expression of this enzyme may play a role in transformation by altering deoxynucleoside triphosphate pools.

mtrII

The *Bgl*II N fragment (mtrII) will induce neoplastic transformation of established rodent cells (38, 39, 41) and human keratinocytes immortalized with HPV-16 (29). The minimal transforming fragment of mtrII is a 737-bp fragment that does not contain the intact coding sequences for RRB. Furthermore, retention of mtrII is not consistently observed, implying that it initiates transformation but is not required for maintenance of the transformed phenotype. A stem-loop structure is present within the minimal transforming fragment of mtrII, and this structure has been implicated in the transformation process (38). Although the stem-loop structure resembles transposons of *Drosophila melanogaster* (P elements), the viral stem-loop structure is not large enough to encode a transposition function, nor does it appear to be a classical insertion sequence. mtrII can act as a mutagen (39), suggesting that it can activate proto-oncogenes or inactivate tumor suppressor genes. A plasmid rescued from rat cells transformed by mtrII contains rat DNA flanked by mtrII DNA sequences (5), suggesting that mtrII is capable of transducing rat DNA sequences. If the transduced rat DNA is hybridized to HSV DNA, specific hybridization occurs with the HSV-2 *Bam*HI E fragment (contained within mtrIII [Fig. 2]). Interestingly, the transduced rat DNA is frequently rearranged after transformation by mtrII and the transduced rat DNA induces neoplastic transformation, suggesting that it contains a cellular oncogene. In summary, the ability of mtrII to induce mutations or transduce cellular DNA sequences is required for initiating neoplastic transformation but not maintaining the transformed phenotype.

mtrIII

The *Bgl*II C fragment of HSV-2 (mtrIII) will induce multi-step transformation of primary hamster cells (58). The left-hand two-thirds of *Bgl*II-C can immortalize primary hamster cells but does not immortalize primary human fibroblasts (23, 59), suggesting that the requirements for immortalizing hamster cells are less stringent. The right-hand one-third, *Bam*HI E fragment, induces tumorigenic conversion of established cell lines (59, 121). The minimal transforming region of *Bam*HI-E maps to a 486-bp *Pst*I-*Sal*I fragment (designated 486TF) or a 1.3-kb *Pst*I fragment (*Pst*I-C), which contains 486TF (Fig. 2) (1, 62). Plasmids containing mtrIII can cooperate with HPV-16 to morphologically transform human cells (fibroblasts or keratinocytes), and HSV-2 DNA sequences are retained in some transformed cells (22, 23).

The only intact gene within the *Bam*HI E fragment is RRA (109) (Fig. 2). Deletion of the RRA gene prevents virus replication in animal models and restricts viral growth in arrested cells (7, 10, 46, 57). Although RR enzymatic activity is expressed as an early enzyme, the RRA promoter has a *cis*-acting motif (TAATGARAT), which is found in all immediate-early genes of HSV (119). However, RRA RNA expression does not readily occur in the absence of protein synthesis, suggesting that it is not regulated as a typical immediate-early gene in infected cells (50, 110). Interestingly, the RRA promoter can be *trans*-activated by cellular proto-oncogenes (*jun*, *fos*, and *c-myc*), the tumor suppressor gene RB, and the HPV E2 gene (50, 117, 118). The RRA promoter contains two AP-1-binding sites, which can be activated by *jun*-*fos* heterodimers (92), two RB control elements, and a consensus E2-binding site (50, 92, 117). The RRA protein consists of at least three functional domains (Fig. 2). Unlike any known mammalian RRA, the N-terminal domain (amino acids 1 to 411) has serine/threonine-specific protein kinase activity (14). Apparently, the protein kinase domain is a transduced cellular gene fragment fused to the amino terminus of RRA (103). As with other mammalian RRs, catalytic activity is contained at the C terminus of RRA (83). Between the protein kinase domain and the catalytic domain, a novel leucine zipper-like motif which facilitates subunit association and enzymatic activity is found (72, 102).

Does RRA play a role in neoplastic transformation? Inappropriate expression of RRA may lead to higher mutation frequencies or unscheduled cell division. Although transient-transfection assays suggest that HSV-2 RRA and RRB are not mutagens (15), stable cell lines expressing mutant forms of mammalian RRA have increased mutation frequency (11). The HSV-2 RRA protein has been reported to have *trans*-phosphorylating activity (14, 102), but HSV-1 RRA apparently does not (17). A fraction of HSV-2-encoded RRA localizes to plasma membranes, suggesting that it can function as a growth factor receptor (14, 72). A recent report demonstrated that the protein kinase domain of RRA phosphorylates and activates *ras* (102), suggesting that this activity is important for neoplastic transformation. If *Pst*I-C (which contains 486TF and the protein kinase domain of RRA) is placed in a mammalian expression vector and transformation ability in rodent cells is compared with that of *Pst*I-C in a plasmid lacking mammalian regulatory signals, the efficiency of transformation is increased (1). Furthermore, an RRA temperature-sensitive mutant of HSV-2, containing mutations in the protein kinase domain of RRA, does not morphologically transform established rodent cells as efficiently as wild-type HSV-2 does (101), suggesting that the protein kinase domain enhances transformation. The amino-terminal domain of HSV-2 has only 38% similarity to

that of HSV-1, but the remainder of the protein has approximately 93% similarity (83), adding credence to the importance of the protein kinase domain in neoplastic transformation. Given the importance of *ras* in signal transduction and oncogenic transformation (79), the finding that HSV-2 RRA activates *ras* is an attractive model to explain the mechanism by which *mtrIII* induces neoplastic transformation.

Other studies have indicated that 486TF alone (which does not contain the protein kinase domain of RRA) can induce tumorigenic transformation of Rat-2 cells or induce altered growth of human fibroblasts or keratinocytes (22, 23, 62). In transient-transfection assays, 486TF is a complex transcriptional regulatory element composed of two distinct promoters, a transcriptional silencer, and a distal activating element (42, 61, 63). Novel proteins in HSV-2-transformed cells specifically bind to 486TF, suggesting that transformed cells contain novel transcription factors. Transcriptional regulatory motifs within 486TF may alter cellular gene expression following integration and may consequently induce transformation. Whether promoter elements in 486TF are utilized to synthesize novel viral transcripts which are necessary for morphological transformation is currently not known. Like *mtrII*, 486TF contains repetitive DNA sequences which can be folded into relatively stable hairpin structures (62, 63). Nuclear proteins specifically bind to sequences within these structures (63), suggesting that the structures are stabilized by cellular proteins. Perhaps stem-loop structures in 486TF mediate neoplastic transformation by facilitating recombination or altering expression of growth-regulatory genes. Although the protein kinase activity of RRA enhances neoplastic transformation, the fact that 486TF has transforming activity suggests that two transforming domains are present in *BamHI*-E.

INTERPLAY BETWEEN HSV-2 AND HIGH-RISK HPV

Two studies have examined the ability of *mtrIII* to cooperate with HPV-16 or HPV-18 to transform human fibroblasts or keratinocytes (22, 23). If primary human fibroblasts are transfected with a retrovirus shuttle vector containing HPV-16 and fragments derived from *mtrIII*, aneuploidy occurs in nearly 90% of the transformed cell lines and the cells exhibit anchorage-independent growth but do not form tumors in *nu/nu* mice (22). Transformed cells consistently retain HSV-2 DNA sequences as judged by PCR analysis. As expected, immortalized cell lines were established after primary human fibroblasts were transfected with HPV-16 alone. Aneuploidy was detected in only 10% of the cell lines transfected with HPV-16, and these cells do not grow efficiently in soft agar. For these studies, primary human cells were obtained from two patients: gingival fibroblasts from a "normal" adult (N16), and gingival fibroblasts from an epileptic patient treated with an anticonvulsant drug, phenytoin (R30). One side effect of phenytoin is that it induces gingival overgrowth, thus making it necessary to surgically remove the overgrown tissue. Karyotype analysis revealed that R30 cells contained a stable translocation between chromosomes 8 and 18. Since the human proto-oncogene *c-myc* is present on chromosome 8 (82), the status of *c-myc* in R30 cells was examined. Higher steady-state levels of *c-myc* RNA were observed in R30 cells than in N16 cells, but the genomic organization appeared normal. Stable cell lines of R30 but not N16 were efficiently established when transfected with HPV-16 or HPV-16/*mtrIII*, implying that *c-myc* expression facilitated establishment of these cell lines. Amplification of *c-myc* has also been suggested to play a role in cervical cancer (84).

When human keratinocytes immortalized with HPV-16 (FEPL) or HPV-18 (FEA) were transfected with plasmids containing *mtrIII*, the resulting cell lines grew to higher saturation densities (22). FEPL but not FEA cells transfected with *mtrIII* developed into benign lesions in *nu/nu* mice. These lesions were macroscopic and consisted of squamous epithelial cells secreting keratin. Although the organization of *c-Myc* and *p53* was not altered after introduction of *mtrIII* into FEA and FEPL cells, rearrangements of HPV-18 DNA were frequently observed in FEA cells. FEA and FEPL cells transfected with *mtrIII* did not express higher steady-state levels of HPV mRNAs which encode E6 or E7 relative to their parental counterparts, suggesting that changes in growth properties were not merely the result of higher levels of E6 or E7 expression.

A plasmid containing *mtrII* can convert genital epithelial cells containing integrated HPV-16 sequences into tumorigenic squamous cell carcinomas (29). The tumors were aneuploid with a near triploid number and had five copies of chromosomes 2 and 9. Furthermore, several marker chromosomes were derived from chromosomes 5, 7, and 16. Upon prolonged passage, the HSV-2 DNA sequences were "lost" as judged by Southern blotting studies. However, oncogenic potential was not lost, thus supporting the role that *mtrII* is required for initiating oncogenic transformation but not for maintaining the transformed phenotype. The notion that high-risk HPV types are necessary but not sufficient for cervical cancer is supported by these studies. Although these studies demonstrate that HSV-2 transforming fragments (*mtrII* and *mtrIII*) can cooperate with HPV to transform human cells, it will be necessary to corroborate these findings with studies that compare the risk of cervical cancer or invasiveness of disease in women exposed to both viruses with the risk in women exposed to one virus.

ABILITY OF HSV TO INDUCE MUTATIONS, GENOMIC REARRANGEMENTS, OR ACTIVATION OF ONCOGENES AS A MECHANISM FOR INITIATION OF TUMORIGENESIS

HSV infection can induce chromosomal breaks (48, 106), amplification of chromosomal DNA (97), and formation of double minute chromosomes (13, 40, 51). Furthermore, infection of a simian virus 40-transformed hamster cell line induces up to 500-fold amplification of integrated simian virus 40 DNA sequences (43). Flanking cellular sequences are also amplified, and simian virus 40-encoded T antigen is necessary for amplification. Amplification of HPV-18 DNA sequences also occurs in HeLa cells after infection by HSV-2 (6), suggesting that HSV infections induce cellular DNA synthesis. The ability of HSV-2 to induce unscheduled DNA synthesis in several cervical cancer cell lines (HeLa, CaSki, C-33A, and SiHa) was examined (69). Flow-cytometric analysis in all of the cell lines indicated increases in the proportion of G₁- and S-phase cells which were synthesizing DNA. In HeLa cells, this was an early event after infection, but in C-33A and SiHa cells, it was a late event. If HSV-2 replication is blocked with phosphonoformate, virus-induced changes are abolished in HeLa cells but not in C-33A or SiHa cells, suggesting that unscheduled DNA replication is cell type dependent. Replication of episomal DNA or unscheduled DNA synthesis (repair replication of chromosomal DNA) is necessary for DNA amplification to occur (104), suggesting that these two processes are linked. Six HSV-encoded genes are involved in amplification of episomal DNA (51): UL5, UL8, UL52 (components of the viral helicase-primase complex which is essential for viral DNA replication), UL29 (single-stranded DNA-binding protein necessary for

DNA replication), UL30 (DNA polymerase), and UL42 (subunit of DNA polymerase). In summary, these studies indicated that an HSV infection induces cell cycle progression which ultimately leads to amplification of chromosomes.

Infection of permissive cells with UV-irradiated HSV-1 (98) or nonpermissive cells with HSV-2 (90) increases the mutation frequency of the hypoxanthine-guanine phosphoribosyltransferase gene. Mutation frequencies are similar to those produced by the chemical carcinogen 4-nitroquinoline-1-oxide, and DNA damage is followed by cellular DNA repair (98). The mutagenic properties of HSV were extended by using a plasmid-based gene (*supF*), which is located on the shuttle vector pZ189. This system allows one to identify where specific mutations are introduced into the *supF* gene. Although the mutagenic effects of HSV were primarily point mutations typically induced by chemical carcinogens, deletion and insertion mutations were also observed (56). Increases in mutagenesis of pZ189 occur early in infection but do not require functional RRA activity, RRB activity, or viral gene expression (15). Penetration of virus into the cell is required, suggesting that a viral component mediates mutagenesis. Although the viral gene(s) necessary for mutagenesis has not been identified, it is clear that entry of a noninfectious HSV particle into a cell could lead to mutagenesis but not death of the cell. The ability of HSV to act as a potent mutagen and induce DNA amplification may have important ramifications with respect to its role in cervical cancer, because cervical cancer biopsy specimens frequently have abnormalities in chromosome 11 (49) or chromosome 17 (95).

POTENTIAL INTERACTIONS BETWEEN HSV-2 AND HPV DURING INDUCTION OF CERVICAL CANCER

It would be easy to dismiss HSV-2 as a cofactor in the development of cervical cancer, because it is not a prototype DNA tumor virus. Unlike simian virus 40, HPV, or adenoviruses, HSV-2 does not apparently encode a viral oncogene which disarms p53 and/or RB. If HSV-2 plays a direct role in cervical cancer, it is reasonable to hypothesize that some infected cells survive and that a subset of viral genes are expressed during a persistent or abortive infection. Alternate sites of latent or persistent infections do occur for HSV-2 in addition to latent infections in sensory neurons (16; reviewed in reference 20), supporting the concept that persistent or latent infections occur in cervical tissue. A potential model for the involvement of HSV-2 in cervical carcinoma is outlined in Fig. 3. HSV-2 infection of cervical epithelial cells (normal or HPV⁺) leads to mutations and/or rearrangements of cellular genes which have growth regulatory function. The neoplastic properties of mtrII and mtrIII may also contribute to altered growth properties. If cells are HPV⁺ prior to HSV-2 infection, mutagenesis would be more detrimental, because p53 is sequestered and degraded by E6 (96). Consequently, p53 would not restrict cell cycle progression after DNA damage (64, 87), suggesting that HPV⁺ cells that are mutagenized by HSV-2 continue to proliferate. Since HPV infections are frequently associated with CIN and cervical cancer (9, 20a, 28, 36, 44, 60), it does not seem likely that HSV-2 frequently induces cervical cancer by itself. However, it is possible that novel growth factors are produced after cervical cells (HPV⁺ or HPV⁻) are infected by HSV-2, resulting in proliferation of adjacent cells latently infected with HPV. Considering that HPV morphogenesis is coupled to epithelial cell differentiation and that HPV oncogene expression (E6 and E7) is higher in undifferentiated cells (55, 78), development of cervical pathologic changes would be favored after HSV-2-induced mutagenesis.

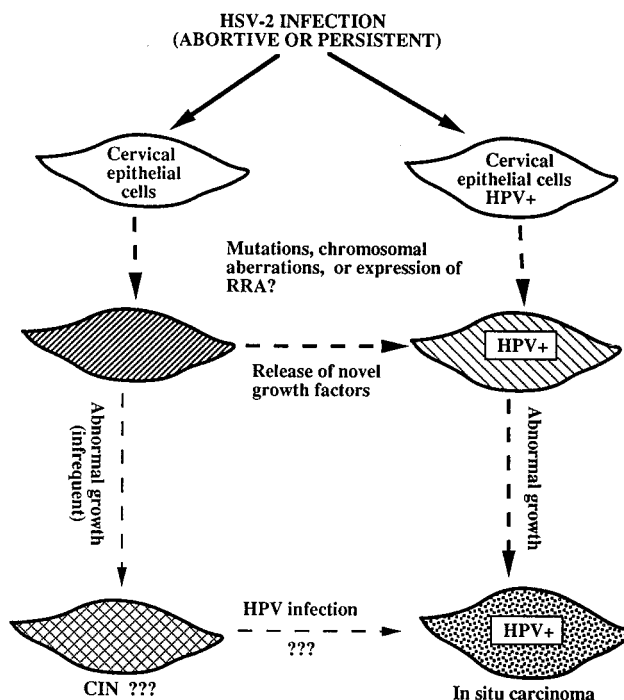


FIG. 3. Model by which HSV-2 can function as a cofactor in cervical cancer.

There is no doubt that cervical cells can survive infection by HPV plus HSV-2 and then progress to cervical cancer, because DNA from both viruses has been detected in cervical cancer biopsy specimens (25, 26). The observation that HSV mutagenesis is an early event during a lytic infection cycle (15, 56) suggests that cervical cells can be mutated during a nonproductive infection or even by a defective virus genome which is packaged. In summary, the most logical way that HSV-2 participates as a cofactor in progression of CIN to cervical carcinoma is to induce DNA damage or chromosomal abnormalities in cervical epithelial cells that are latently infected with HPV.

ACKNOWLEDGMENTS

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