Epidemiology of Genital Papillomaviruses and Cervical Cancer

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Cervical cancer is an extremely common disease. Its natural history has been well described, and individual risk factors have been defined. It is clear from the epidemiologic evidence that cervical cancer has a multifactorial etiology involving infection with sexually transmitted agents such as genital papillomaviruses and cofactors such as pregnancy, smoking, use of hormonal contraceptives, and diet. The evidence implicating papillomavirus as an etiologic agent of cervical cancer has come from a variety of observational laboratory studies. Genital papillomaviruses induce dysplastic lesions. Most invasive cervical cancers contain papillomavirus DNA, as do cell lines derived from cervical cancers. Viral DNA appears to be integrated into cellular DNA, and integration involves highly conserved, transcriptionally active regions of the viral DNA.

On a global basis cervical cancer is an extremely important preventable disease. It is estimated that ~400,000 women worldwide develop cervical cancer yearly; cervical cancer ranks second only to breast cancer as a documented cause of death from cancer in women [1]. Squamous cancers of the uterine cervix appear to evolve from mild to severe cervical intraepithelial neoplasia (CIN) and then to invasive disease over a variably prolonged period. Because of this local progression over time and because the cervix is readily sampled cytologically, cervical cancer is amenable to secondary prevention by screening and early treatment. Cervical cytology screening programs and other factors are believed to have lowered the incidence of invasive cervical cancer, particularly in North America and Western Europe. Yet even in low-risk areas, the prevalence of cervical cancer remains high in certain population groups, including primarily lower socioeconomic classes, blacks, and Hispanics [1].

Individual risk factors for cervical cancer are well known, and models describing their interactions ultimately must be applied if successful control programs are to be implemented. Key risk factors for both CIN and invasive cancer are related to socioeconomic variables, sexual behavior, infection with sexually transmitted agents, smoking, diet, reproductive history, and use of hormonal contraceptives. Human genital papillomaviruses (HPVs) are currently regarded as the most likely sexually transmitted agents involved in cervical oncogenesis. However, the mechanisms by which HPV infection and behavioral and other risk factors interact in the natural history of cervical cancer have not been addressed by well-designed population-based studies.

Our intent in this review is to evaluate current knowledge concerning risk factors for cervical cancer, with special emphasis on possible interactions. A major portion of the review will be devoted to a discussion of data associating HPVs with cervical cancer—in particular, the possible molecular mechanisms of association.

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Incidence of Cervical Cancer in Different Populations

Geographic Occurrence

The incidence of cervical cancer varies dramatically throughout the world. Particularly high rates are found in Latin America and the Caribbean, where Panama, Jamaica, Colombia, Aruba, Brazil, Peru, Mexico, El Salvador, and Costa Rica have documented annual incidences in excess of 20 cases per 100,000 women [2–6]. The risk of cervical cancer is

also high in Hong Kong, Poona (India), Denmark, Rumania, and Canada's Northwest Territories [2]. It is not clear to what extent these geographic clusters represent simple deficiencies in screening-control programs or other aspects of public health care delivery, specific clusters of causal risk factors, or artifact. It is important to define incidence in other parts of Latin America as well as in Africa and Asia. Etiologic factors for cervical cancer may operate differently and result in differences in incidence in different populations.

Age-Specific Incidence

The age-specific incidence of invasive cervical cancer differs significantly between populations with high and low incidences. In low-incidence populations (age-adjusted rates, <15/100,000), risk increases beginning at ~20 y and plateaus by 35–40 y. In high-risk populations (age-adjusted rates, >25/100,000), incidence increases rapidly between 20 y and 39 y (with the annual incidence in this age range usually >1/1,000 women) and does not plateau until ~50–55 y. Variations in the age-specific incidence of cervical cancer likely reflect different types of interactions between risk factors.

Risk Factors for Cervical Cancer

Female Sexual Activity

As early as 1842 Rigoni-Stern recognized the importance of sexual activity as a risk factor for cervical cancer, noting that the disease occurs more frequently among married than unmarried women. Early studies revealed that women who report more than one sexual partner are at greater risk than sexually monogamous women, and risk appears to increase directly with the number of lifetime partners [7]. In addition, women who have sexual relations at an early age are at higher risk than either virgins or women whose sexual experiences begin later in life [8, 9]. Although few studies have attempted to assess the independence of the effects of early age at first intercourse and number of sexual partners, both factors apparently contribute independently to risk, and there is some evidence that number of partners may play a more important role [10]. This evidence suggests that exposure to an agent transmitted through multiple partners may be more significant than periods of enhanced susceptibility.

Male-Associated Risk Factors

Although most studies have focused on the role of female behavior in the etiology of cervical cancer, recent studies suggest that male-associated factors should also be considered. Cervical and penile cancers tend to cluster geographically [11–13], and several studies have found that wives of men with penile cancer have significantly elevated rates of cervical cancer [14–16]. One recent study showed a significantly higher prevalence of papillomavirus-associated preinvasive lesions in male sexual partners of women with CIN than in male sexual partners of women selected as controls [17]. Further support for a male-associated factor derives from a study in which wives of men previously married to patients with cervical cancer were found to have considerably higher rates of cervical cancer than control wives [18]. Finally, in studies comparing sexual histories of husbands of cervical cancer patients with those of control husbands, the former reported significantly more sexual partners than the latter [19–21] and the former were more likely than the latter to have had sexually transmitted diseases, early sexual experiences, extramarital sexual relations, and visits to prostitutes [20]. Indeed, patterns of male sexual behavior may account for some of the features of the epidemiology of cervical cancer, especially the high incidence in Latin America [22].

Sexually Transmitted Diseases

Infection with herpes simplex virus type 2. Although the relation of cervical cancer risk to sexual behavior has been recognized for many years, attempts to relate this association to an infectious agent have only recently been made. In the early 1970s, interest centered on the relation of herpes simplex virus type 2 (HSV-2) to cervical cancer risk, with numerous case-control studies showing a significantly higher prevalence of antibody to HSV-2 among cases than among controls [23]. Interpretation of these studies is complicated by cross-reactivity between HSV-1 and HSV-2 and variability in assays between laboratories. In addition, the case-control approach could not resolve whether or not HSV-2 infection preceded the development of cervical cancer. Although two prospective studies [24, 25] provided evidence that HSV-2 predisposes to cervical abnormalities, neither was able to adjust for the confounding effects of sexual activity variables. The

most informative prospective investigation suggested no relation between HSV-2 and subsequent cervical neoplasia among a cohort of women matched for sexual activity [26]. However, this study utilized a case-control design, and the possibility of overmatching could have negated an effective risk. Although HSV-2 infection covaries with cervical cancer risk, it seems most logical that seropositivity is a surrogate for sexual activity [27].

Infection with HPVs. Current interest concerning risk factors for sexually transmitted cervical cancer centers on HPVs. Early epidemiologic support for an association between HPVs and cervical neoplasia was provided by Franceschi et al., who found that patients with genital warts were more likely to have cervical dyskaryosis than were women with trichomoniasis or gonorrhea [28]. Use of new laboratory methods defining papillomaviruses in future epidemiologic studies should permit results that are considerably more informative with regard to the influence of HPVs on the risk of cervical cancer. A later section in this review will discuss HPVs as a risk factor.

Other Risk Factors

Smoking. Recently, a variety of nonvenereal risk factors for cervical cancer have been hypothesized, including smoking, oral contraceptives, and diet. Probably the best established of these associations is smoking, which numerous studies have shown to relate directly to the risk of both preinvasive and invasive cervical abnormalities [29–34]. Although it was originally suggested that this association merely reflected confounding by sexual or other variables, the link persisted after adjustment for a variety of factors. Support for causality is strengthened by findings that cervical cancer risk is increased for long-term smokers, high-frequency smokers, and users of nonfilter cigarettes [34].

Oral contraceptives. The possible relation between oral contraceptive use and cervical cancer has been examined in many studies, and the results have been contradictory. Case-control and cohort studies conducted in the early 1970s generally failed to identify an association. However, similarly designed studies published in the late 1970s found an increased risk of preinvasive cervical disease in long-term pill users. For the most part these early studies did not adequately control for sexual behavior or other sources of confounding [35]. Recent well-controlled studies have indicated that oral contraceptive use is an independent risk factor and that long-term users are at highest risk [36–38].

Dietary factors. The possible role of dietary factors in the etiology of cervical cancer is just beginning to emerge and could be particularly important with respect to regional differences in cancer incidence. Several case-control studies indicate a high risk for women with low dietary intake of either vitamin A or vitamin C [39–43]. Folic acid deficiency has also been implicated as a risk factor on the basis of studies among oral contraceptive users [44, 45].

Genital HPV Infection and Cervical Cancer

Detection of HPV Infections

Assay systems. HPV infection of the cervix can cause a variety of easily recognized clinical or subclinical lesions and can also be diagnosed by the presence of koilocytes or the detection of HPV antigens [46]. However, these methods do not distinguish between genetically different types of HPV and do not detect latent infections. The first HPV was formally characterized ~10 y ago, and there are now more than 50 published "types." A new type must share <50% DNA homology with other known types, as defined in a liquid hybridization assay [47]. HPVs cannot be grown readily in tissue culture, and there is extensive immunologic cross-reaction among the various types. Classification depends on DNA composition rather than antigenic analysis. Although a variety of HPV types have been described, most have not been defined by DNA reassociation kinetics and there is no standardized routine laboratory test to define new types or strains. Three basic DNA hybridization assays have been most commonly used to identify HPVs: Southern blot, filter in situ or dot blot, and tissue in situ. The relative sensitivity, specificity, and reliability of the methods have not been extensively studied [48, 49].

The Southern blot assay has been most commonly employed in studies to date. Material is usually obtained by biopsy, although scrape, swab, or cervicovaginal lavage can also be used. After extraction viral DNA is separated from cellular DNA by gel electrophoresis, and a positive reaction with viral DNA probes produces characteristic patterns, with implied specificity [50]. The method is labor inten-

sive and not well suited to large-scale studies. In addition, it is impossible to obtain cervical biopsy specimens from a representative sample of healthy women.

In the filter in situ hybridization method, exfoliated cells are obtained by scraping or cervicovaginal lavage; the sample is placed directly onto filter paper, lysed, and then hybridized against viral DNA probes [51]. Because this method uses significantly smaller amounts of material than the Southern blot, biopsy is not necessary; in addition, the labor-intensive step of DNA extraction prior to testing is obviated. A disadvantage of the filter in situ assay is the possible nonspecific trapping of viral probe, with consequent false-positive reactions.

The tissue in situ hybridization test directly measures HPV DNA in either fixed or frozen tissue sections. In condylomata, the unique distribution of reactive cells permits specific interpretation of the patterns observed in the sections [48].

Limitations of hybridization assays. These assays are limited primarily by adequacy of the sampling method, undefined sensitivity and specificity, and problems with interpretation. Sampling errors are difficult to define. In most lesions, HPV infection involves the upper cell layers of the epithelium; thus scraping or lavage techniques should be more likely to capture positive cells than biopsy. Most early studies with Southern blot or tissue in situ techniques used biopsy material, while studies measuring infection by filter in situ tests used samples taken by swabbing the surface of lesions (or the entire cervix). In situ hybridization using tissue sections is the method most vulnerable to sampling variation because foci of infection can be missed by biopsy or subsequent sectioning.

Most investigators agree that both Southern blot and filter in situ hybridization can detect amounts of HPV DNA corresponding to one or fewer viral genome copies per cell. The limit of detection of HPV genomes by the tissue in situ hybridization method is considerably higher: 20–100 copies of HPV genome may be required to produce a reaction [48, 49]. Data directly comparing the methods (in the same and different laboratories) are not yet available. Indeed, there is no agreement as to standardization of probes. Interpretive errors arise, in part, from the limits of detection of the hybridization methods (sensitivity). There is also an undefined degree of subjectivity in interpreting the results of any laboratory test. To date, no major publications have described comparisons of coded specimens in different laboratories; issues of interpretation therefore are not yet defined.

Natural History of Genital HPV Infections

In spite of technical problems in defining HPV infection, there is a general consensus that HPV types 6, 11, 16, 18, 31, 33, and 35 are most consistently associated with genital infection and disease. These types of virus appear to be sexually transmitted [17], but the natural history of genital HPV infection with different types is not well understood. Follow-up of individuals exposed to sexual partners with condylomata acuminata has shown that warts may appear from six weeks to three months after exposure [52]. Genital warts are normally caused by HPV type 6 or 11 [53]. Although other HPV types infecting the genital tract presumably have similar incubation periods, no specific information is available on this point.

Infections of the cervix with HPV seldom give rise to typical exophytic condylomata but rather are manifested as flat or inverted condylomata [54]. These characteristically contain koilocytes, which are pathognomonic of HPV infections. Certain epithelial abnormalities found in flat condylomata are similar to those belonging to the spectrum of CIN [55]. A number of studies have shown that 50%–70% of condylomatous lesions of the cervix are associated with CIN. In women followed prospectively ~10% of lesions diagnosed by the presence of koilocytes or viral antigen progressed to more severe stages of CIN, while the remainder regressed or persisted at the same stage [56–58]. In the largest study 343 patients were followed over a mean period of 18.7 mo; 25% of lesions regressed, while 61% persisted and 14% progressed to carcinoma [58].

Gissmann and co-workers were the first to identify unique HPV types associated with condylomata acuminata [53]. They found HPV type 6 or 11 DNA in ~95% of such lesions; subsequent studies have confirmed this observation [59–62]. These virus types are also associated with CIN, and their occurrence appears to be inversely related to the severity of such lesions [51, 63, 64]. In a recent report, for example, HPV type 6 or 11 sequences were found in 33% of CIN I lesions (the least severe form), 15% of CIN II lesions, and 4.5% of CIN III lesions [60].

In contrast, the other types of HPV infecting the genital tract seldom produce exophytic growths on the external genitalia but are associated with epithelial lesions of mucosal surfaces. The occurrence of HPV type 16 DNA varies directly with the severity of CIN. HPV type 16 DNA has been found in 10%–25% of CIN I lesions, in 25%–40% of CIN II lesions, and in 50%–75% of CIN III lesions [60, 65, 66].

In spite of these well-recognized associations, it is clear that not all HPV infections are associated with pathology. A variety of studies have shown that 10%–50% of normal women are infected with HPVs. For example, Cox and collaborators found HPV type 16 in nine normal cervical biopsy specimens from 26 patients; 13 of the women had had consistently normal cervical cytologies within the previous five years, and five (38%) of this group were infected [67]. Burk and associates studied patients from a New York City colposcopy clinic and found that 10 (29%) of 34 women with normal colposcopic findings and normal cervical cytologies were infected with HPVs [68]. Toorn and collaborators reported results from a British clinic for benign gynecologic conditions, where 12 (12%) of 104 women were infected with HPVs [69]. In our studies in Latin America, we have found ~50% of women from the general population with normal Papanicolaou smears or with cervicitis to be infected with HPVs [70].

HPV DNA in Cervical Cancers

The observed increasing prevalence of HPV type 16 with more severe grades of CIN and the finding of abnormal mitotic figures associated with lesions containing HPV type 16 suggest that certain types of HPV may function as carcinogens [71]. This concept is strengthened by the detection of HPV DNA sequences in invasive cervical cancers and in cell lines derived from these cancers.

Although DNA from HPV types 16, 18, 31, 33, and 35 has been detected in invasive cancers [60, 72–75], the majority of the DNA sequences found in these cancers are homologous to type 16. The results of a number of studies of HPV DNA frequency are summarized in table 1. The Southern blot method was used to identify viral DNA sequences in all but two of these studies, and the DNA-DNA hybridization reactions were conducted under either stringent or nonstringent conditions, with subsequent washing of the blots under stringent conditions. The latter method appears to have yielded slightly higher rates of positivity than the former. In total, HPV type 16 sequences were found in 116 (50%) of 231 tumors. However, the positivity rates varied from 14% to 92%; with the number studied it is not possible to conclude that the variations observed represent statistically significant geographic differences. Clearly not all cervical cancers contain HPV type 16 DNA; this finding is to be expected if other causal factors or other types of HPV are involved or may be due simply to inadequate sampling [84].

Table 1. Summary of prevalence of HPV type 16 DNA sequences in cervical cancers from different case series.

Assay*	Hybridization conditions†	Sample size	Proportion positive (%)	95% CI‡	Reference
SB	NS	18	61	0.35–0.82	73
SB	NS	9	89	0.16–0.82	73
DB	S	30	53	0.34–0.71	76
DB	S	6	50	0.11–0.88	66
SB	NS	13	92	0.63–0.99	77
SB	S	50	36	0.22–0.50	59
SB	S	13	46	0.19–0.74	78
SB	S	9	33	0.07–0.70	79
SB	NS	11	73	0.39–0.93	80
SB	NS	20	60	0.36–0.80	81
SB	S	11	45	0.16–0.76	82
SB	S	7	14	0.00–0.57	83
SB	NS	11	45	0.16–0.76	60

* SB = Southern blot; DB = dot blot.
† S = stringent; NS = nonstringent.
‡ CI = confidence interval.

The association of HPV type 16 with invasive cervical cancer was examined by means of the Southern blot method and a case-control design; viral sequences were detected in biopsy specimens from 31 (66%) of 47 patients with cancer and in cervical epithelial samples from nine (35%) of 26 controls [85]. The consecutively enrolled control group consisted of women treated at the same hospital at which the cases were treated. The controls were admitted for treatment of benign gynecologic disorders and differed significantly from the cervical cancer patients in age and marital status. After adjustment for age, the difference between cases and controls in the occurrence of HPV type 16 DNA was not significant.

Filter in situ hybridization was used in a second case-control study in which controls were randomly selected from women in the same neighborhood as cervical cancer patients and from patients hospital-

ized with nongynecologic diseases and were matched on the basis of age. HPV type 16 DNA was found in 31 (67%) of 46 cervical cancer patients and in 22 (43%) of 51 controls [70].

The prevalences of HPV type 16 among the cervical cancer patients in the two case-control studies were similar to those of the case series reports (table 1). However, the rates of HPV in control women were higher than those noted by most investigators studying women without condylomatous or neoplastic disease of the cervix. These differences could represent variations in enrollment criteria (particularly clinical criteria and age), methods of obtaining specimens, or assay systems used to detect HPV DNA. Through analysis of exfoliated cervical epithelial cells by the filter in situ method, HPV DNA was detected among 2%–11% of women with normal cytologic smears [49, 51, 63]. Southern blot analysis of exfoliated cells revealed viral DNA sequences in 11% of cytologically normal women in one study [86], while 12% of nonpregnant and 28% of pregnant women were positive in another study [87]. Southern blot assays of biopsies from normal cervices revealed HPV DNA in ~10% of women sampled [53, 88].

The rates obtained in these other studies of normal women are quite similar despite the use of exfoliated cells in some studies and biopsies in others and the use of different hybridization methods for detecting viral DNA. Thus the high rates among control women from the two case-control studies may reflect the process of selection of controls. In both case-control studies, the prevalence of HPV type 16 DNA increased with age, an observation at variance with the recent report of De Villiers and co-workers [89]. What is most important is that all studies of "normal" women have indicated that HPV type 16 may persist in histologically normal epithelium; this concept is supported by analysis of tissue adjacent to neoplastic or condylomatous lesions of the cervix [90, 91].

Data on the association of HPV types other than type 16 with cervical cancer are limited. HPV type 18 DNA has been detected in 15% of cervical cancers from German women and in 25% of cancers from African and Brazilian women [73]. Two reports from Japanese investigators indicated the presence of HPV type 18 DNA in 5% and 22% of the invasive cancers examined [59, 92]. In contrast to HPV type 16, HPV type 18 sequences in CIN lesions seem to occur independent of the severity of disease. HPV type 18 DNA has been found in 5%, 2%, and 4% of CIN I, CIN II, and CIN III lesions, respectively [60], in one study and in 23%, 20%, and 26% of these lesion types in another investigation [65]. In a third study, 25% of CIN I and CIN II lesions contained viral DNA and no CIN III lesions were positive [64].

HPV types 31, 33, and 35 have only recently been described. HPV type 31 was noted in 20% of mild and moderate dysplasia cases and in 6% of invasive cancer cases in the initial report [93]. A subsequent study revealed HPV type 31 DNA in 11%, 13%, 13%, and 9% of CIN I lesions, CIN II lesions, CIN III lesions, and invasive cancers of the cervix, respectively [60]. In a single case series, HPV type 33 DNA was found in 4%–8% of CIN lesions and invasive cancers [74]. Thus, while HPV types other than type 16 have been associated with cervical neoplasia, data presently available do not reveal an increasing prevalence of these agents with increasing severity of cervical disease, as has been noted for HPV type 16. Clearly, additional information is needed concerning the distribution of other HPV types in cervical neoplasias and normal populations.

The association of HPV type 16 with neoplastic disease is not restricted to the uterine cervix. Between 60% and 80% of samples from vulvar precancerous lesions (such as bowenoid disease) or from vulvar intraepithelial neoplasias have been found to contain HPV type 16 DNA sequences [60, 94]. HPV type 16 DNA has been detected in vulvar carcinomas [60], anal cancers [95], penile cancers [96], oral cancers [97, 98], tongue cancers [99], and head and neck cancers [100]; moreover, samples from bronchial esophageal cancers have been reported to react with a mixed probe containing DNA from HPV types 16, 18, 11, and 30 [101, 102]. The presence of HPV type 16 DNA in nongenital cancers may reflect a general lack of specificity for cervical cancers. Alternatively, HPV type 16 could be etiologically involved in the formation of squamous cell tumors at multiple sites.

Molecular Biology of HPV and Cervical Cancer

Integration of HPV DNA

As has been noted, many investigators have found HPV in cervical cancers; thus HPV infection may play a causal role in oncogenesis. In addition, independent evidence indicates the molecular mechanisms involved. Experimentally induced malignant transformation by DNA viruses generally involves

integration of viral DNA into the cellular genome as an essential step in oncogenesis. Early studies suggested that HPV DNA exists as free episomes in benign lesions and as integrated sequences in malignant lesions [72, 73, 103]. If integration is a necessary event in the oncogenic progression of lesions, the demonstration of integrated HPV DNA sequences would strongly support a causal role for the viruses, and it would be important to identify factors leading to integration.

In vitro models. Studies of the state of HPV DNA in relation to genital cancers were conducted with fresh biopsy material and established cell lines derived from neoplastic lesions. Cell lines from 12 patients with cervical cancer were examined; HPV DNA sequences were detected in 83% [72, 79, 104, 105], a prevalence generally similar to that of HPV DNA in biopsied invasive cancers. However, HPV type 18 DNA was found more commonly than HPV type 16 DNA in established cell lines (seven of 10 vs. three of 10), while the reverse distribution was found in biopsied invasive cancers. These findings suggest a possible selective advantage in culture for cells containing HPV type 18 sequences.

In all established cell lines studied to date, viral DNA appears to be integrated into host cell DNA [103, 106–110]. In most instances integration has occurred within the HPV E1 or E2 open reading frames (ORFs). Cloned cellular sequences at the integration sites have been used to identify the chromosomes in which viral DNA integration occurs. Chromosome 12 is involved in the SW756 cell line [103, 106, 107], chromosome 13 in SiHa cell lines [107], and chromosome 8 in both HeLa and C4-I cell lines [106]. In situ DNA-DNA hybridization analysis has confirmed the chromosome 12 location of HPV DNA sequences in the SW756 cell line [111] and the chromosome 8 location in HeLa cells [112]. Popescu et al. [112] also localized HPV type 18 DNA sequences in HeLa cells to a normal chromosome 9 and to two abnormal chromosomes derived from chromosomes 5 and 2, respectively. Viral upstream regulatory regions, as well as the E6 and E7 ORFs, have been found intact and transcriptionally active [107, 110, 113, 114]. Transcripts have been identified that include E6 and E7 ORF products and mRNAs representing spliced products of E6 and the 3′ cellular sequences at the integration site. In addition, proteins encoded by the E6 and E7 ORFs have been detected in cell lines [79].

Thus the data suggest that integration involves a specific region of the viral genome but occurs randomly within the DNA of the cell. If this is true, there are three possible mechanisms by which papillomaviruses might induce malignant transformation of cervical epithelial cells. First, the products of the early ORFs, such as E6 or E7, might function as transactivating molecules that alter expression of cellular growth control genes. The ability of the E6, E7, and E5 regions of the bovine papillomavirus genome to induce transformation in culture supports this hypothesis [115–118]. Second, the integrated viral transcriptional regulatory region could alter the transcription of cellular oncogenes. The chromosomal location of the integration sites in cell lines has varied and is not consistently in the regions of known oncogenes. However, elevated levels of *c-myc* mRNA have been found in two cell lines with HPV type 18 DNA sequences in the chromosome containing this oncogene [106]. Amplification of *c-myc* and *c-Ha-ras*, as well as increased levels of mRNA expression of these oncogenes, have been reported from studies of biopsy material [119]. Finally, fusion products created at the site of integration of the viral DNA might serve to alter the phenotype of the cell [110]. The apparent nonspecificity of the integration sites in the human genome and the variation in expression of the putative fusion mRNAs make this explanation unlikely, however [114].

Animal models. Although studies of established human cervical cancer cell lines have provided important clues to possible oncogenic mechanisms, these investigations have many inherent limitations. Indeed, evidence from animal models suggests that integration is not required for oncogenic transformation. Papillomavirus DNA usually exists as free episomes that can be identified by the unique properties of supercoiled-circle (form I) and open-circle (form II) DNA molecules. Shope papillomavirus induces tumors in domestic rabbits, and some of these papillomas undergo malignant conversion. In the benign papilloma viral DNA exists as form I and form II molecules, while in primary and metastatic carcinomas a large portion of the viral DNA exists as covalently or nicked oligomeric circles [120]. Similarly, most of the DNA in bovine papillomavirus–induced tumors persists in an extrachromosomal state [109]. Thus, while integration of rabbit papillomavirus DNA as an oligomer consisting of head-to-tail tandem repeats has been noted in carcinoma-derived

cell lines [121], integration of viral DNA does not appear to be a necessary event for tumor induction in the rabbit and bovine models of papillomavirus infection.

Studies of human clinical material. Although studies of tissue culture and animal models may help to clarify mechanisms, the results cannot be generalized a priori to human populations. Several techniques have been used to examine the physical state of HPV in human genital lesions; the conclusions have varied. Cesium chloride/ethidium bromide gradient centrifugation and Southern blot analysis of undigested DNA readily detect form I and form II DNA molecules—the only forms found in some cancers. However, some lesions contain viral sequences that migrate more slowly than form II molecules, and these could represent viral DNA integrated into large fragments of cellular DNA or multimeric forms of nonintegrated viral DNA. In order to distinguish between these possibilities, it is necessary to use appropriate restriction enzymes. If the DNA is episomal, fragments of genome length or smaller should be produced; the finding of viral DNA sequences in molecules larger than unit size would reflect integration of viral DNA (provided that the enzyme digestion is complete). Greater confidence regarding the physical state of the viral DNA can be gained from two-dimensional gel electrophoresis [120], and unequivocal evidence is provided by cloning and sequencing of the virus-cell DNA integration site [103].

The episomal state of HPV type 16 DNA in benign tumors and in CIN lesions, as determined by Southern blot analysis of uncut and restriction enzyme-cut DNA, has been confirmed by most investigators [53, 65, 77, 86, 122, 123]. Nevertheless, there are reports to the contrary. For example, DiLuca and co-workers [78] suggested that integration of viral DNA occurred in both invasive and CIN lesions. However, this conclusion was based on minor high-molecular-weight bands found on Southern blot analysis after restriction enzyme digestion, and these bands could have resulted from incomplete digestion. Similarly, in a second report suggesting integration of viral DNA in CIN, the submolar fragments taken as evidence of virus-cell junctions may also have represented products of incomplete restriction endonuclease digestion or differences in restriction enzyme sites between the sequences present in the tumors and the prototype HPV type 16 DNA [66].

The presence of HPV type 16 DNA sequences integrated with cellular DNA in cervical cancer tissue has been clearly demonstrated by Durst and coworkers, who cloned two distinct integration sites in DNA obtained from a cervical cancer [103] and subsequently localized the sites to chromosomes 3 and 20 [106]. However, the frequency with which integration occurs is less clear. In the initial study [103] cesium chloride/ethidium bromide gradient centrifugation analysis was used to examine the state of the HPV type 16 genome in DNA from six benign tumors, including two cases of mild dysplasia. Viral DNA existed as 8-kilobase circles in these benign lesions. A similar analysis of DNA from three cervical carcinomas and a specimen from a case of Bowen's disease yielded evidence of a head-to-tail genome arrangement of high-molecular-weight episomal HPV type 16 DNA as well as integrated viral DNA in two specimens and of integrated DNA alone in two additional specimens. The integrated state in these instances was confirmed by two-dimensional gel electrophoresis [103].

Other investigators have also found evidence for both integrated and episomal HPV DNA sequences in invasive cervical cancers. Reports on several series of cases suggested that integration occurs regularly [72, 73, 77, 79, 101, 103]. However, only seven of 31 HPV-positive cases studied by Meanwell and co-workers [85] were thought to involve integrated viral sequences, and Yoshikawa and associates [59] found viral DNA existing as form I and form II molecules in 11 of 19 cases. Choo et al. [76] found only free episomal viral DNA in six of 16 invasive cancers containing HPV type 16 sequences; another six cancers had only integrated sequences, and four had both. Integration sites present in relatively minor amounts could have been missed in the latter studies.

In summary, the results of these reports suggest that integration is not a constant feature of the association between HPV and the malignant state. This conclusion is supported by the observation of the episomal state of HPV type 6 DNA in Buschke-Lowenstein tumors [124], of the apparent episomal state of HPV type 18 DNA in both cervical and penile cancers [76, 96], and in the existence of nonintegrated HPV type 33 DNA in a case of invasive cervical cancer [74]. Thus, while integration of HPV type 16 DNA clearly occurs, the importance of this phenomenon in the genesis of the cancer remains to be clarified.

Stability of HPV DNA in Cancer Cells

In vitro studies. Finally, in addition to the conflicting evidence concerning integration of HPV DNA in transformed cells, data have been obtained on the stability of viral DNA in transformed cells. As with other molecular studies, the most consistent evidence comes from in vitro experiments, while results from in vivo protocols are less conclusive. HPV type 16 has been shown to induce malignant transformation of NIH 3T3 cells [92, 125] and to immortalize primary human keratinocytes [126]. In addition, viral DNA has been found to transform primary rat kidney cells when transfected with the *EJ-ras* oncogene [127], and the viral DNA in these in vitro transformed cells is stably integrated. The HPV DNA sequences in cervical cancer cell lines also appear to remain stable. This point has been demonstrated for HeLa cells, where lines maintained in different laboratories have been found to contain the same genome copy numbers of HPV type 18 [104]. Similarly, sublines of cells derived from the same cervical cancers, but differing in morphology, have been found to contain similar copy numbers of HPV sequences with identical restriction enzyme patterns. These findings have been reported for cell lines C4-I and C4-II, which contain HPV type 18 sequences [104, 105]; for cell lines SKG IIIa and SKG IIIb, which contain HPV type 16 sequences [92]; and for cell lines C33A and C33IV ([105] and W. Rawls, unpublished observation).

In vivo studies. A similar stable relation in vivo is suggested by the detection of HPV DNA in metastatic lesions originating from primary cervical cancers. DNA of HPV types 16 and 18 has been found in metastases to the peritoneal cavity [128], the liver and subclavicular lymph nodes [59], and the pelvic lymph nodes draining the uterine cervix [80]. In the latter study, cervical cancers and draining lymph nodes from 13 patients were studied. All cervical lesions contained HPV sequences, and the same type of HPV DNA was found in nodes from seven patients. Histologic examination of frozen sections revealed cancer cells in five of the seven HPV-positive nodes but not in the six HPV-negative nodes. These results support the concept of a stable interaction between the viral genome and the malignant cell.

This conclusion is tempered somewhat by an apparent heterogeneity of HPV DNA distribution in some primary cancers. HPV DNA was found to be focal when biopsies of invasive cancers were examined by in situ hybridization techniques [49, 129]. As noted previously, the tissue in situ assay is relatively insensitive, and the focal distribution observed could represent regions of the tumor where subsets of cancer cells have undergone differentiation associated with replication of endogenous viral DNA. However, heterogeneity of viral DNA in cancers has also been noted with the Southern blot technique. In a study of duplicate tissue samples from penile carcinomas, only 33% of 18 positive lesions contained detectable viral DNA in both samples [96]. A second study of multiple samples from 12 cases of cervical carcinoma revealed three instances of positivity for HPV DNA in one specimen and HPV DNA negativity in a second specimen obtained from an adjacent area of the tumor [85]. While the comparability of tumor cell content in the paired samples was not determined in these studies, the data argue against a stable relation between the viral genome and the cancer cell in primary cancers.

Conclusion

The hypothesis that HPV is an etiologic agent of cervical cancer derives from four major laboratory-based arguments. First, infection with genital HPVs induces dysplastic lesions that are colposcopically and histologically similar to preinvasive cervical neoplasia. Second, most invasive cervical cancers contain HPV DNA; preinvasive cervical lesions contain HPV DNA, proteins, and complete virions, and infection rates increase with the grade of the lesion. Third, cell lines derived from cervical cancers, such as HeLa, SiHa, and CaSki, contain integrated HPV DNA; although integration sites vary, integration generally occurs within E1 or E2 ORFs, and E6 and E7 ORFs are intact and transcriptionally active. Fourth, HPV DNA sequences appear to be stable in both established transformed cell lines and fresh biopsy material.

In spite of the fact that molecular biology studies are incriminating, the evidence that HPV causes cervical cancer is largely circumstantial. The investigators in most studies have enrolled small numbers of selected patients rather than sampling defined populations and generally have not reported details concerning basic parameters such as age or clinical stage; thus it is impossible to compare the results of various studies. In addition, because most studies have

been limited to cases of HPV infection and have not included controls, relative risks cannot be estimated. Some reports include data on "control" subjects, but these women have usually been enrolled at sexually transmitted disease clinics or in Papanicolaou smear screening programs and have not been selected from defined populations. In addition, controls have generally not been comparable to cervical cancer patients with regard to important attributes such as age. The few good epidemiologic studies that have been published or reported at meetings have indicated that HPV is less of a risk factor than was predicted from laboratory data; i.e., relative risk estimates are in the range of 2–4.

Other important basic laboratory issues must be resolved by hypotheses that HPV is a causal agent of cervical cancer. HPV infection is assessed by assays for DNA. A variety of laboratory methods, probes, and criteria for positivity have been used. Each assay is limited by sampling errors and its own intrinsic flaws. To date, no major published studies have compared assays either within or between laboratories. Test variability, sensitivity, and specificity remain undefined. Similarly, issues of viral persistence, latency, and reactivation have not been critically addressed. For example, 10%–50% of healthy women are infected with genital HPVs; the presence of viral DNA in anatomically normal tissue could result from sampling during the incubation period following primary infection or from persistence of virus in an occult form not associated with a proliferative response of infected epithelial cells.

It is clear that HPV infection alone is neither sufficient nor necessary for the development of cervical cancer. The diversity of known risk factors for cervical cancer indicates a multifactorial etiology, and the interrelations of the various factors are not well established. Two general classes of cofactors must be considered: fixed cofactors that are present at the time of infection (e.g., demography, genetics, diet) and variable cofactors that are introduced following infection (pregnancy, hormone exposure, diet, smoking, other sexually transmitted diseases) [130, 131]. None of these interactions has been evaluated epidemiologically, however.

Future analytic studies of HPV infection and cervical cancer must include cases from defined populations and appropriately matched controls (female and male) and must measure other risk (or confounding) factors (such as sexual habits, smoking, other sexually transmitted diseases, hormones, and dietary factors) in order to define a multifactorial model. These studies must also pay particular attention to well-standardized laboratory methods so that HPV infection is assessed with defined levels of precision.

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