Molecular Virology and Epidemiology of Human Papillomavirus and Cervical Cancer

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Introduction

In 1994, an estimated 55,000 women in the United States will be diagnosed with carcinoma in situ of the cervix, with an additional 15,000 invasive cases expected. An estimated 4600 women died from this disease in 1994, despite remarkable progress made in screening (1). Regarding extent of disease among women with invasive cancer in the United States from 1983 to 1987, 48% of tumors were localized, 34% were regional, and 10% had metastasized to distant sites (1). Although major progress has been made to control cervical cancer in the United States and Europe, it remains a major cause of morbidity and mortality in the developing world. Current understanding of the pathogenesis of cervical cancer reflects a complicated interplay between epidemiology and basic molecular biology. Historically, the observation that cervical cancer was a sexually transmitted disease predated our understanding that HPV3 were capable of infecting the anogenital tract, and only with the advent of DNA probes for HPV infection was the connection between HPV infection and cervical cancer made evident. Subsequent efforts focused on HPV infection and other recognized epidemiological risk factors in the pathogenesis of invasive cervical cancer and its precursor lesion CIN. Armed with a possible role for HPV in a clinically important malignancy, efforts to understand the molecular biology of HPV were expanded greatly. The last 10 years have seen an explosion in our understanding of the functions of HPV genes, patterns of HPV gene expression in cervical tissues, and how these genes may contribute to the development of malignancy. New forms of molecular probes were developed based on this knowledge, and new methods such as PCR were applied to the study of cervical tissues. These advances further refined our understanding of the epidemiology of HPV and its role in the pathogenesis of CIN and cervical cancer.

HPV is an important pathogen for two reasons: (a) it is one of the most common of sexually transmitted agents; and (b) it has been associated strongly with a variety of squamous cell cancers; the most common is squamous cell cancer of the cervix. The list of human malignancies associated with infectious agents other than HPV is relatively limited; examples of others are: hepatic cell carcinoma with hepatitis B virus; adult T-cell leukemia with human T-cell leukemia virus 1; nasopharyngeal cancer and Burkitt’s lymphoma with Epstein-Barr virus; stomach cancer with Helicobacter pylori; and cancers of the bladder and bile duct with Schistosoma haematobium and Clonorchis sinensis. Each of these organisms contributes to the development of malignancy through different pathways, and in most instances these remain poorly understood. In the case of cervical cancer and HPV, a great deal of progress has been made in recent years in both the epidemiology and molecular biology of the infection and its associated disease. In this paper, we will describe the histopathological changes induced by HPV, introduce some basic concepts of molecular biology, and discuss the salient features of the molecular biology specific to HPV. We will then discuss recent developments in epidemiological studies that link HPV to CIN and invasive cervical cancer, and examples will be provided of how the epidemiology of HPV infection intersects with the molecular biology to advance our understanding of the mechanisms that link HPV to the development of cervical cancer.

Histopathology of HPV-associated Disease

When HPV infection is detected in the epithelium, it may span a spectrum ranging from normal to condyloma planum (flat warts) or acuminatum (acuminate warts), to intraepithelial neoplasia and invasive cancer. Disease may occur at a wide variety of sites, including the conjunctiva, oral cavity, keratinized skin, and anogenital mucosa. HPV infection occurs in the basal cell layer of the epithelium, which constitutes the dividing cell population (Fig. 1; Refs. 2, 3). The importance of this observation is that the basal cells may then constitute a continuous reservoir of HPV DNA, and the viral genome is then partitioned to all daughter cells when the cells divide. HPV gene expression appears to be limited in the basal cell layer (4). However, as the cells begin to mature and rise through the epithelium, the viral DNA molecules continue to reproduce themselves in the absence of basal cell division, resulting in a higher HPV DNA copy number per cell. This process continues such that the highest DNA copy numbers per cell are found in the most mature cell layers, as indicated by in situ hybridization of HPV-infected tissues (Ref. 4; Fig. 2). As a result of increased viral copy number and/or alterations in keratinocyte factors that modulate HPV gene expression within mature keratinocytes, viral activity increases as the cells mature. Thus, the control of HPV appears to be linked closely to epithelial differentiation and is the result of a highly complex interaction between epithelial cell and viral regulatory elements as discussed below.

At the level of the individual keratinocyte the impact of HPV gene expression may be manifest in one of two ways: koilocytosis or dysplasia. Koilocytosis is considered to be pathognomonic of HPV infection when stringent diagnostic criteria are used. These include a well demarcated perinuclear...
SEVERE IN SITU DYSPLASIA

MICROINVASIVE CARCINOMA

CERVICAL INTRAEPITHELIAL NEOPLASIA

Fig. 1. Representation of different grades of cervical intraepithelial neoplasia. Histologically normal tissue is presented on the left. Typically, as the normal epithelium matures, the keratinocytes flatten out with smaller nuclear:cytoplasmic ratios and ultimately slough off the surface. HPV infection is believed to occur in the basal cell layer, which constitutes the dividing cell layer of the epithelium. As a consequence of HPV gene expression, in combination with other factors, CIN may develop. CIN grade 1 is characterized by 20-25% replacement of the epithelium with immature cells with high nucleus:cytoplasm ratios. CIN grade 2 is characterized by approximately 50% replacement with immature cells and grade 3 is characterized by complete or nearly complete replacement. Microinvasion (bottom) occurs when the cells traverse the basement membrane. Occasionally, microinvasion can be found with overlying CIN 1, as depicted by the small number of cells in the lower left. However, almost all invasive cancer occurs in conjunction with high grade CIN, as depicted by the larger number of cells below the basement membrane in the lower right.

Fig. 2. In situ hybridization of an intraepithelial neoplasia lesion with a probe mixture consisting of HPV 16 and 18 DNA. Dark signals in the nuclei (arrows) indicating HPV DNA positivity are found primarily in the more mature cell layers. These results indicate a higher copy number of HPV genomes per cell in the differentiating epithelium than in the basal layers, where signal is not detected (reprinted with permission from J. M. Palefsky et al., J. Clin. Invest., 87: 2136, 1991).

cavity or halo, as well as nuclear abnormalities consisting of irregular or enlarged nuclei (5). In contrast, a dysplastic epithelial cell demonstrates an increased nuclear:cytoplasmic ratio, with the nucleus having an irregular shape, hyperchromasia, and coarse chromatin. These cellular changes are manifest widely at the tissue level and span a spectrum consisting of: (a) nearly normal or “atypical,” with presence of some but not all of the features diagnostic of HPV infection; (b) condyloma, with the predominant cellular changes consisting of koilocytosis in the absence of marked nuclear atypia; (c) CIN, with the
predominant cellular changes consisting of cells with dysplastic morphology; and (d) invasive cancer, in which epithelial cells have traversed the basement membrane and are invading the underlying stroma (Fig. 1). Many lesions demonstrate features of both condyloma and CIN, and recent classification systems such as the Bethesda system recognize that the clinical differences between these two manifestations of HPV infection may be minimal. The Bethesda classification system thus places low grade CIN and condyloma into one group (i.e., low grade squamous intraepithelial lesion) and CIN grade 2 and 3 into another (i.e., HGSIL). Similarly, invasive cancer tissues often demonstrate features of condyloma and/or CIN in overlying areas above the basement membrane (Fig. 1).

The histopathological appearance of CIN 1 consists of modest nuclear atypia in the lower 20–25% of the epithelium (Fig. 1). There are few mitoses and no bizarre mitoses. In CIN 2, the more superficial layers of the epithelium may contain cells with abnormal nuclei, and immature cells with a high nuclear:cytoplasmic ratio may comprise the lower one-half of the epithelium. In CIN 3, cells with a high nuclear:cytoplasmic ratio are found throughout the epithelium, and dividing cells may be found in all cell layers. The clinical importance of CIN is that it may be a precursor to invasive cervical cancer, and as the degree of CIN increases, the risk of development of invasive cancer increases as well (6, 7).

Basic Concepts in Molecular Biology

This section explains some basic concepts and terminology in molecular biology that underlie fundamental processes in the biology of the cell and the viruses that may infect it. These include the process by which cellular and HPV proteins are expressed and the effects that these proteins may have on the function and level of expression of other proteins. As described below, the cellular abnormalities induced by HPV reflect complex interactions among viral proteins, among cellular proteins, and between cellular and viral proteins. These interactions occur at many different levels. For example, binding of one protein to another may alter profoundly the function of one or both of these proteins, and interactions between proteins and DNA may alter the level of expression of the proteins.

The amino acid sequences of HPV and cellular proteins are specified by the nucleotide sequences of the viral and cellular DNA that encode these proteins. DNA consists of a series of nucleotides consisting of adenine, guanine, cytosine, or thymidine. The DNA molecule consists of a double helix of two complementary strands of nucleotides bound to each other in opposite orientations, in which the adenine nucleotides are bound to the thymidine nucleotides and the cytidine nucleotides are bound to the guanine nucleotides. The beginning part of each strand is known as the 5’ end (upstream end), and the opposite end is known as the 3’ end (Fig. 3A; downstream end). The process by which the genetic information contained in DNA sequences is converted into the amino acid sequence of the protein that it encodes is complex and includes several intermediate steps. The DNA is first copied in the cell nucleus into mRNA by enzymes known as RNA polymerases in a process known as “transcription.” During transcription, the DNA strands separate and the mRNA is copied from one of the strands in the 5’ to 3’ direction. The mRNA is then transported to the cell cytoplasm where it becomes physically associated with ribosomes and tRNA. In the process known as “translation,” the binding of tRNA bearing specific amino acids to the mRNA permits the building of amino acid sequences that constitute the protein.

A series of three nucleotides is known as a “codon,” since each specifies incorporation of a specific amino acid into a protein with the use of what is known as the “genetic code.” Each amino acid is encoded by one or more unique triplet sequences, and the order of the DNA nucleotides will determine the exact amino acid sequence of the resulting protein. The DNA and mRNA transcribed from it are punctuated with a “stop codon,” which is a sequence that is associated with disruption of transcription and termination of the resulting protein. A piece of DNA that is large enough to encode an adequate number of amino acids to form a protein is known as an “open reading frame.”

The sum total of DNA of an organism is known as a “genome” (excluding pieces of DNA known as plasmids that may reproduce themselves separately from chromosomal DNA). The genome of HPV is quite small, consisting of only approximately 7,900 nucleotides compared to about 175,000 nucleotides in other viruses such as herpes viruses. To generate the requisite amount of information from such a small genome, HPV uses three different strategies used commonly by all higher organisms including humans: (a) each piece of DNA can be translated theoretically into three different proteins, depending on the site where transcription begins and may, therefore, contain up to three open reading frames. For example, the sequence “TAC GAA TCT CAG GTG” also could be read by the RNA polymerase as “TAC GAA TCT CAG TG,” or “TA CGA ATC TCA GGT G,” and each reading would result in different proteins; (b) human cells and many viruses such as HPV use another system to generate different amino acid sequences from the same DNA sequence, known as “RNA splicing.” When this occurs, mRNA is transcribed from the DNA strand (described earlier) but is processed prior to protein translation such that pieces of the RNA sequence are deleted, and the remaining pieces of RNA are spliced together to form a new strand of mRNA (Fig. 3B). The result is an mRNA sequence that is different from the original DNA sequence, and consequently a different protein will be encoded; and (c) a method called “posttranslational modification” is used by HPV to generate diverse proteins from the same DNA sequence. In this case, proteins are modified after they have been translated from the mRNA sequence; parts of the protein may be cleaved, some of the amino acids may have carbohydrate groups added.
to them, and some such as tyrosine or serine may have phosphate groups added to them (phosphorylation), all of which may modify the function of the protein. These three strategies are used efficiently by the small HPV genome to maximize its ability to generate the largest possible variation in information carried by its DNA.

The control of HPV gene expression reflects a complex interplay between host and viral factors that is critical to determine the outcome of viral infection. Certain RNA and DNA sequences serve specific functions in the processes of controlling transcription and translation of HPV genes. Because the same or very similar DNA sequences are used for the same purpose in many different species, these sequences are termed "consensus sequences." An example of such a consensus sequence is found where RNA polymerases bind DNA to begin transcription of mRNA from the HPV genome. Such sequences are known as "promoters." Promoters typically occur in papillomaviruses with the sequence TATAAA and are usually quite close to the start point for RNA transcription. To recognize the promoter site and to initiate mRNA transcription off the DNA strand, RNA polymerases require generally the presence of other specific proteins, known as "transcription factors," i.e., factors that influence the ability of the RNA polymerases to bind to the promoter site. These factors may determine the efficiency of expression of a particular protein, and several examples of these transcription factors have been demonstrated in papillomaviruses, as described below.

Other kinds of consensus sequences that may affect the efficiency of protein expression are known as "enhancers." Unlike promoter sequences, which are usually close to the gene being expressed, enhancer sequences may be thousands of nucleotides away from the gene being transcribed and may have the effect of greatly stimulating the promoter of that gene. These sequences have no promoter activity of their own and may be at one end or the other, or even in the middle of a transcribed gene. Enhancers tend to be effective only in certain kinds of cells, and the restricted host range of papillomaviruses may be, in part, a consequence of the existence of species-specific and host cell-specific enhancers, e.g., keratinocyte-specific enhancers. Papillomavirus enhancer sequences will be described in greater detail below. It is hypothesized that these promoters serve as the docking site for the assembly of molecular complexes that contain RNA polymerase. Presumably, the RNA polymerase complex, which is thousands of nucleotides away from the promoter site for the gene that will be expressed, is brought into close physical proximity to the promoter through induction of DNA bending. Thus, transcription factors interact cooperatively with a variety of regulatory sequences in giving RNA polymerase access to specific genes, ultimately determining the genes that are expressed, as well as the level at which they are expressed.

The influence of one gene over the expression of another may occur in one of two ways. In "cis-activation," a gene influencing the expression of another gene is usually in close proximity. Presumably the gene product of the influencing gene is acting directly on the gene that is being influenced, and the relative orientation of these genes is critical to their function. Another means of gene activation is called "trans-activation." It gets its name because the gene product of the activating gene is a soluble factor that can migrate to its site of action. When trans-activation occurs, the position of the activating gene relative to the gene sequence that is being influenced is not important. An example of a gene product with trans-activating functions might be a transcription factor that interacts with promoter and enhancer sequences for a given gene to determine its level of expression. Thus, the activating gene and its target do not even have to be on the same molecule (an example of this will be provided later in potential interactions between HIV and HPV). As described below, much of the regulatory activity of papillomaviruses occurs through trans-activation.

**Structure of the HPV Genome and the Function of HPV Genes**

HPV is a small, double-stranded DNA virus that is a member of the papovavirus group. As described previously, the viral genome consists of approximately 7900 nucleotides, and all viral gene transcription occurs off one strand (Fig. 4, A and B).

To date, more than 70 different HPV types have been isolated. These are not serotype viruses, such as HSV 1 or HSV 2, which, by definition, are distinguished from each other according to the ability of type-specific antibodies to bind them. Instead, the different HPV types are "genotypes," in which the typing scheme is based on the similarity of one HPV type to the other known HPV types at the DNA level. To determine if a new HPV genotype has been isolated, that is, a new genotype has been discovered, the DNA from this type is cloned and sent to a central HPV DNA repository in Heidelberg, Germany. The DNA from this putative new type is then used as a probe to "hybridize" or bind to the DNA of all the previously existing HPV types with the use of a technique known as "Southern blot hybridization." Hybridization will occur only if there is significant "homology" or gene sequence similarity between the purportedly new type and the other known target HPV types. If hybridization does not occur then the DNA sequence of the purportedly new HPV type must be sufficiently different from other HPV types to legitimately consider it a new type. If different, then it would be assigned the next highest number in the sequence of numbers assigned previously to HPV types.

This typing scheme has clinical significance for two reasons: (a) specific HPV types exhibit a remarkable degree of tissue tropism, or tissue selection. That is, some HPV types such as HPV 1 and HPV 2 are most often found in keratinized skin of the palms and soles in the form of plantar and palmar...
warts. Others, such as types 6, 11, 16, and 18 are most often found in the keratinized skin and mucosal surfaces of the anogenital region, including the cervix; and (b) as described below, specific HPV types such as HPV 16 or 18 exhibit a strong association with invasive cancer. These types are considered to have a "malignant" phenotype, while others such as HPV 6 or 11 are more commonly associated with benign disease such as condyloma.

HPV 16 is the type found most commonly in squamous cell cancers of the cervix in most studies to date, whereas type 18 is found most commonly in adenocarcinoma of the cervix (8, 9). The molecular basis for this genotype-associated tissue tropism is not well understood. Better understood, but still not completely, is the basis for the association between a specific HPV type and cervical cancer. As described below, the two most important HPV proteins in the pathogenesis of malignancy are the E6 and E7 proteins. Amino acid sequence differences in these proteins between low and high risk HPV types may in part explain their different behavior by virtue of their differing ability to inactivate cellular proteins involved in the negative regulation of cell growth.

Much of our understanding of the development of HPV-associated invasive cancer is derived from in vitro study of cell behavior and the expression of different HPV proteins. HPV cannot be grown easily in tissue culture, and large quantities of virus have been unavailable to conduct classic virological studies. To understand the role of HPV proteins in the pathogenesis of cancer, pieces of the HPV genome encoding different HPV proteins have been cloned and expressed in keratinocytes and in other cells to determine their effect. Many of these experiments were designed to measure the ability of these proteins to induce cell "transformation." Transformation includes several different phenotypic changes in cell behavior in vitro that correspond in varying degrees to the behavior of cancerous cells in vivo. These include cell "immortalization," where the cells continue to divide even after repeated passage in tissue culture; the ability of cells to grow in the absence of a solid surface to adhere to (anchorage-independent growth); loss of contact inhibition that ordinarily causes untransformed cells to cease dividing when they begin to touch each other in a Petri dish; and tumorigenicity, or the ability to cause tumors in animals such as mice.

The HPV genome may be divided into two parts, based on the function of the encoded genes: the early (E) region and the late (L) region. Early region proteins include E6, E7, E1, E2, E4 and E5, and late region proteins include L1 and L2. The viral genomic DNA in fully formed viral particles is surrounded by a protein coat known as the viral "capsid" that consists of the L-region proteins L1 and L2. In contrast to the L-region proteins, the E-region proteins are associated with cell transformation and viral gene regulation and are the most critical in the pathogenesis of invasive cancer. Between the E and L regions lies the LCR. This region does not include gene-encoding sequences but contains promoter and enhancer DNA sequences critical to regulation of viral gene transcription by both viral and cellular genes.

Patterns of HPV gene expression vary according to the grade of the clinical lesion. Early region HPV proteins may be detected throughout the entire spectrum of HPV-associated disease. In lower grade CIN biopsy material, the HPV E4 and E5 genes are often the most highly expressed (10, 11), with weaker signals from the E6 and E7 open reading frames, as well as the E2 open reading frame. In higher grade lesions, E6 and E7 gene expression becomes more prominent (4), and viral gene expression is higher in the basal cell layers than it is in the lower grade lesions, resulting in a more even distribution of viral gene expression throughout the epithelium.

**HPV E6 and E7 Proteins**

The most important HPV proteins in the development of malignancy are the E6 and E7 proteins. In several studies of HPV gene expression in cancerous tissues, expression of the E6 and E7 genes is preserved, suggesting that their continued expression is required to maintain the malignant phenotype (12). Recently, generation of transgenic mice with the use of either the E6 or E7 gene alone (13) or the entire early region (which also includes the E1, E2, E4, and E5 genes; Ref. 14) resulted in formation of condyloma, intraepithelial neoplasia, and invasive squamous cell cancer, although the cancer was not in the genital region. These data provide some of the strongest in vivo evidence for a link between expression of the E6 and E7 genes and development of invasive cancer.

Given this important role in the pathogenesis of cancer, differences between E6 and E7 proteins of low risk HPV types and those of high risk HPV types have been illuminating. These differences may be found in two forms: (a) differences in the regulation and levels of expression of these genes, in which E7 protein expression in tissues infected with HPV types associated with higher risk of malignancy is higher than that of tissues infected with low risk types (15, 16); and (b) as alluded to earlier, differences in the biological potency of the E6 and E7 proteins of high and low risk HPV types.

The E6 protein is an approximately 150-amino acid protein that is localized to the nuclear matrix, as well as to non-nuclear membranes (17, 18). E6 is not normally capable of inducing transformation by itself, but has been shown to induce immortalization of primary human keratinocytes in conjunction with E7 (19), as well as to promote anchorage-independent growth of rat cells (16). In contrast, the E6 proteins of HPV types not associated with invasive cancer such as HPV 6 are incapable normally of either of these functions, suggesting that the HPV 6 E6 protein may have lower intrinsic biological activity (20).

In the last five years, data have been obtained that may at least partially explain these differences in biological activity of the HPV E6 and E7 proteins. Portions of these proteins have strong homology with transforming proteins of other oncopgenic viruses, such as Polyomaviruses (SV40) and adenovirus (Fig. 5). These findings suggest that HPV belongs to a family of DNA tumor viruses that may have evolved from a common ancestor and that may exert their effects through common pathways, e.g., inactivation of one or more negative regulators of cell growth such as the p53 protein or the retinoblastoma gene product. For example, the E6 protein is partly homologous with the SV40 large T antigen and adenovirus E1B proteins. On the basis of the ability of the SV40 large T antigen and the E1B protein to bind the p53 cellular protein, it was hypothesized that E6 may function in a similar manner. This was confirmed in experiments on the binding of the E6 proteins of oncogenic HPV types such as HPV 16 to the p53 protein (21). Further, it was shown that one of the consequences of the binding of E6 to p53 was the degradation of p53 (22). The p53 protein has been shown to be an important negative regulator of cell growth and a tumor suppressor protein (23-25). Thus, by binding and degrading the p53 protein, the E6 protein may contribute to the pathogenesis of malignancy by removing this important negative regulator of cell growth from cellular circulation. Of note, the E6 proteins of HPV 16 and 18 bind p53 more efficiently than the E6 proteins of the low risk HPV types.
Similar to the differential binding of low and high risk HPV E6 proteins to p53, the E7 proteins of HPV 6 and 11 demonstrate lower binding affinity for RB than the E7 proteins found in HPV 16 and 18 (35–37).

One of the functions of the RB protein in the cell is to inhibit the effect of positive growth regulators (38). Binding of the HPV E7 protein to RB inactivates RB and may prevent RB from inhibiting growth. However, the exact role of E7-RB binding in cell transformation is unclear, since abrogation of RB binding through specific mutations of segments of the HPV E7 protein does not diminish its ability to induce cell transformation (39). The E7 protein also may play a more direct role in cell growth by interacting with proteins important in cell cycling (40).

**HPV E1 and E2 Proteins**

Most of the information about the HPV E1 protein was derived from studies in bovine papillomavirus-transformed mouse cell lines. In these studies, E1 has been shown to play an important role in the regulation of DNA replication, as well as in retaining the episomal form of the viral molecule (i.e., it remains separate from the host chromosome; Refs. 41, 42). Consistent with its role in the maintenance of the episomal state, mutations at the 3' end of the HPV E1 gene result in integration of the viral genome into the host chromosome, and as described in this section, this may be a key step in the progression to malignancy. Studies have shown that the E1 gene also may play a role in regulation of the HPV E2 protein activity, distinct from the functions described above (43). Since the E2 protein may influence the levels of HPV E6 and E7 gene expression, as described below, mutations at different sites of the E1 gene might affect both maintenance of the viral genome in episomal form as well as transformation potential. Consistent with this, almost all studies to date that have demonstrated the ability of HPV 16 DNA to immortalize keratinocytes were performed with an isolate of HPV 16 that was derived from a cervical carcinoma later shown to have a mutation within the E1 open reading frame. Correction of this mutation resulted in diminished efficiency of immortalization of primary human keratinocytes (44).

The HPV E2 protein also appears to be important in the process of cell transformation, apparently through its ability to influence levels of E6 and E7 gene expression. Several different versions of the E2 protein produced by different splice patterns have been shown to be expressed, and these may have either stimulatory or inhibitory effects on HPV E6 and E7 gene expression. In many situations, however, the predominant E2 species is one that has an inhibitory effect on E6 and E7 gene expression and would therefore limit the transformation potential of the HPV-infected cells.

Since the predominant effect of E2 protein is to downregulate expression of HPV E6 and E7 genes, the loss of E2 protein expression might be expected to enhance the transforming capability of the virus. Recent studies have confirmed this, with mutations of the HPV E2 gene resulting in increased immortalization capacity (44). Under normal conditions, the HPV viral DNA is a circular molecule and remains "episomal," i.e., it remains separate from the host chromosome. In vivo, one of the most common mechanisms by which HPV E2 expression might be disrupted is the loss of the episomal form and subsequent viral integration into the host chromosome. This is because HPV usually linearizes within the E1/E2 gene region when integrating into the host chromosome (Fig. 6). It is therefore of some interest that the viral genome is usually...
episomal in histologically normal tissues and cancer precursors but is often integrated into the host chromosome in invasive cancer. It is tempting to speculate that one of the events contributing to the development of cancer is viral integration, with subsequent loss of the HPV E2 expression, and enhanced HPV E6 and E7 expression. The integration status of the viral DNA also may be important because the viral genome may integrate into a site in the human genome that results in alteration of human gene expression or its regulation.

HPV E4 and E5 Proteins

In planar warts containing HPV 1a DNA, the HPV E4 proteins appear to constitute a family of proteins that is of considerable interest because they are expressed in great abundance, comprising up to one-third of the total cellular mass (45). Similarly, mRNA species encoded by the HPV E4 gene are among the most abundant viral transcripts in biopsies of CIN and condyloma (10, 46). The protein encoded by the E4 gene appears to accumulate in the cytoplasm (47–50), is expressed only in the most differentiated cells (51), and is not needed for viral transformation (52, 53). When expressed in human keratinocytes, the HPV E4 protein localizes to cytoplasmic inclusion granules and results in the collapse of the epithelial cell intermediate filament network (54). The significance of these findings for the pathogenesis of HPV-associated disease remains unclear, but suggests that the E4 protein may play a role in development of the cytopathic effects seen as koilicytosis.

Other transcripts encoding HPV E4 proteins with different splicing may exist as well, and appear to result in different cellular localization. With HPV 1, some species of E4 proteins were found in the cytoplasm, whereas others with different amino-terminal ends were found inside and around the nucleus (55). An intranuclear form of the HPV E4 protein also has been demonstrated in HPV 16-infected biopsy tissues (56), but the function and significance of these nuclear forms remain unknown.

Like the HPV E1 and E2 proteins, the E5 protein has been best characterized in the BPV system. The BPV E5 protein is the major transforming protein in established rodent cells (57, 58) and is located in cytoplasmic and plasma membranes (59). The mechanism by which the BPV E5 protein exerts its transforming activity is primarily by inhibiting the normal degradation of the cell surface receptors for cellular growth factors (60–62). The role of the HPV 16 E5 protein in the pathogenesis of malignancy remains uncertain since it does not appear to transform primary human keratinocytes by itself but it may play a facilitating role. Recently, the HPV 16 E5 protein has been shown to increase anchorage-independent growth in cells expressing high levels of epidermal growth factor receptors (63), and the effect of the HPV E5 protein may be through amplification of growth stimulatory signals from growth factor receptors (64), similar to the effects seen with BPV E5 protein.

The L1 and L2 Proteins

The L1 open reading frame encodes the “major capsid protein,” i.e., the protein that constitutes the majority of the viral protein coat (65), while the L2 open reading frame encodes the “minor capsid protein” (66). Formation of complete, infectious HPV particles requires assembly of the L1 and L2 proteins into a highly structured protein coat surrounding the viral DNA genome. It is likely that the most infectious form of HPV in vivo is the complete viral particle. As with E4 protein expression, L1 and L2 protein expression is linked strongly to keratinocyte differentiation, and high levels of L1 and L2 protein expression are most often confined to the more mature cell layers of relatively well differentiated low grade lesions. Consequently, the latter low grade lesions probably contain the highest number of infectious particles.

LCR

The LCR is a region of the genome adjacent to the E6 open reading frame that does not encode any proteins but rather contains promoters and enhancers that influence viral gene transcription. In the genital epithelium, HPV encounters a variety of cells as the cells change from a basal immature phenotype to a fully differentiated phenotype. Differences in the sequence of the LCR between HPV types may have profound effects on the malignant phenotype of that HPV type. For example, the higher immortalization efficiency of HPV 18 compared with HPV 16 has been mapped to LCR sequences adjacent to the E6 and E7 genes (67). The sequence of the LCR region also may influence the kind of keratinocyte in which the virus may be transcriptionally active. Thus, HPV 16 enhancer elements appear to function preferentially in genital skin and mucosal keratinocytes (68, 69) and may explain, in part, the tissue tropism of these viruses.

Several different transcription factors have been shown to bind to HPV enhancer elements (70–73). Since none of these factors is restricted to epithelial cells, binding of these factors alone does not account for the epithelial cell specificity of HPV. Instead, promoter activation appears to require cooperative activation of these factors, and cell specificity may in part reside in the concentration of these factors within a given cell type (70, 74). However, several other studies point to a possible role for keratinocyte-specific transcription factors (71, 75–78).
Enhancer elements that bind progesterone and glucocorticoid receptors (70) also have been identified, and although the exact role of these hormones in the pathogenesis of HPV-related disease remains unclear, they may explain hormonal influences on HPV gene transcription.

The LCR also may contain elements that are responsive to growth factors and cytokines. Thus, in addition to their effects on cell growth, these elements also may affect HPV gene expression. For example, retinoids and transforming growth factor $\beta_1$ and $\beta_2$ inhibit proliferation of normal epithelial cells (79, 80) and have been shown to inhibit expression of HPV genes as well (81, 82). Together these studies highlight the extremely complex nature of the interaction between HPV and its host cell.

Recent Epidemiological Findings Linking HPV, CIN, and Cervical Cancer

The association between a sexually transmissible etiological agent and cervical cancer has been recognized for some time (83, 84) and was supported by several lines of epidemiological evidence long before current molecular laboratory methods were in place to identify the agent. In brief, historical data include: (a) elevated rates of cervical cancer observed among wives of men diagnosed with penile cancer (85, 86); (b) clustering of cervical and penile cancer in various geographic areas (87-89); (c) higher number of sexual partners and earlier age at first sexual intercourse as primary risk factors for cervical cancer (83, 84, 90-93) providing somewhat more direct evidence of a sexually transmitted agent; and (d) a greater number of sexual partners, more extramarital affairs, and more extensive histories of sexually transmitted diseases among husbands of women with cervical cancer who had one sexual partner when compared with husbands of control subjects (91). Numerous excellent reviews and commentaries exist that discuss the work on the relationship between a sexually transmissible etiological agent and cervical disease (CIN and invasive cervical cancer) and on other risk factors for this disease and for HPV (84, 94-98). That work will not be discussed here except in the context of the molecular epidemiological association between HPV and CIN and invasive cervical cancer.

Supplementing classic epidemiological methods that used only interview data, with sensitive molecular detection techniques such as PCR, have changed dramatically the precision with which scientists arrive at conclusions regarding the relationship between CIN and invasive cervical cancer and sexually transmissible etiological agents like HPV. Serious difficulties relating to cross-reactivity among HPV types and problems in the use of laboratory methods have been encountered and overcome with the use of highly specific and well controlled techniques (99, 100).

Evidence that the sexually transmitted etiological agent that was associated with cervical cancer was HPV was reported 2 decades ago (101), and the association between HPV infection and cervical cancer has been argued as being causal in a summary presented following the Second International Workshop on the Epidemiology of Cervical Cancer and HPV (102). Principal arguments for causality were presented: (a) consistent reports of 85-100% prevalence of HPV DNA in cervical cancer specimens and high grade CIN with the use of PCR or high quality Southern hybridization methods; (b) strong and consistent association between HPV DNA detection and cervical cancer in case-control studies; (c) specificity of the association with a limited number of HPV types found commonly in the genital tract; and (d) dose-response relationship between estimates of viral load and the risk of cervical cancer (102).

The distinction between low grade squamous intraepithelial lesions and HGSIL as described earlier in “Histopathology of HPV-associated Disease” should be emphasized at the outset of the epidemiological portion of this paper as different HPV types are related to different lesion grades. Evidence is strongest for a link between the more “malignant” high risk HPV types 16 or 18 and high grade CIN and cervical cancer, which are less likely to regress spontaneously (90, 100, 103-106). In contrast, while there is some variation among studies, HPV types 6, 11, 42, 43, and 44 are associated primarily with more benign cervical diseases such as condyloma and low grade CIN with these lesions being more likely to regress than high grade lesions (90, 100, 103-105). HPV types 31, 33, 35, 39, 45, 51, 52, and 58 are associated with an intermediate risk of progression to malignancy (100, 103, 107, 108). In general, this work has borne similar conclusions in many geographic regions of the world (90, 100, 109-113), although there is some variation in the geographical occurrence of HPV virus types.

The underlying reasons as to why some HPV types are more strongly associated with invasive cancer are not yet fully understood. However, details of the role of HPV E6 and E7 proteins in carcinogenesis were discussed earlier, in which the ability of these two proteins to abrogate the normal physiological negative regulation of cell growth was reported as greater in the HPV types more strongly associated with cancer. Furthermore, the level of E7 expression has been shown to be greater in tissues infected with HPV types associated with higher risks of malignancy (15, 16).

In general, cervical HPV infection and its early cytological manifestations, koilocytic atypia and CIN 1, are relatively common among young women who are sexually active, while CIN 3 and invasive cervical cancer are relatively rare in the United States and are more likely to occur among older women (114). CIN is important clinically because in its more serious forms of CIN 2 or 3, it is thought to be a precursor to invasive cervical cancer with the risk of invasive cancer increasing with an increase in the degree of CIN (6, 7).

Many HPV infections are detectable transiently. They may become latent or cleared by the individual, possibly through immunological control mechanisms. Preliminary data have suggested that HPV DNA may become undetectable within 1-2 years of infection, although these estimates have been reported as not yet trustworthy (114). It is currently unknown whether an initial infection with a specific HPV type confers immunity against reinfection with the same or other HPV types (114), and there is uncertainty regarding those HPV types that persist in their expression over time and those that are more transient.

Some of the most compelling recent epidemiological evidence linking specific HPV types as etiological agents for CIN comes from work conducted in Oregon that used PCR to detect HPV DNA in cervicovaginal lavage specimens obtained from each study subject (90). Classic common risk factors for CIN (and for invasive cervical cancer) such as greater number of sexual partners, earlier age at first sexual intercourse, less education, lower family income, and cigarette smoking were reported in conjunction with HPV infection in this study that considered HPV in relation to CIN. When HPV types that were associated with low, medium, and high risk for CIN were adjusted for the common risk factors for this disease, the elevated odds ratios for HPV were unchanged. However, after statistical adjustment for HPV infection, particularly with types of HPV most commonly associated with high grade CIN and cervical cancer (types 16 and 18), the size of the risk estimates...
for the classic risk factors diminished substantially and were no longer significant, indicating the importance of the role HPV plays in the etiology of CIN. In addition, women with multiple HPV types detected were more likely to have been in the case group than women with single types in the Oregon study (90). The authors estimated that 76% of the entire CIN case group could be attributed to HPV infection. After classification into separate abnormalities, the authors estimated that 70% of condylomatous atypia, 90% of CIN 1, and 88% of CIN 2–3 could be attributed to HPV infection and concluded that “the great majority of CIN can be attributed to HPV infection, particularly with the cancer-associated types of HPV” (90).

Recent consequential results somewhat similar to the above study were found in a cohort study of 241 women that was conducted in Washington state with the use of dot-filter and Southern transfer hybridizations to detect HPV DNA. Prior to this study, the temporal relationship between infection with HPV and the incidence of HGSIL (CIN 2 or 3) and the temporal influence of other risk factors was unknown. The cumulative incidence of CIN 2 or 3 after 2 years of follow-up among women who began the study with negative Papanicolaou smears was 28% among the women who had tested positive for HPV and only 3% among those who had tested negative (111). The authors reported that CIN 2 or 3 was a “surprisingly early and common manifestation” of HPV infection, with all of the HPV-associated cases detected within 2 years of the initial detection of HPV DNA. The risk was highest for the more malignant types of HPV (for types 16 and 18; adjusted relative risk = 11) when compared to women without detectable HPV. It also is noteworthy that only 36% (10 of 28 women) had a smear that showed CIN 1 prior to the first smear that showed CIN 2 or 3. Elevated relative risks for HPV among women who began the study with cyologically negative Papanicolaou smears were not changed when adjusted for traditional risk factors for CIN 2 and 3 and for gonococcal infection and antibodies to chlamydia (111). There was no association with the development of CIN 2 or 3 with the detection of antibodies to HSV-2 or with the isolation of HSV from the cervix (111). However, when numerous common risk factors (e.g., cigarette smoking, number of sexual partners, and age at first intercourse) were adjusted for detection of HPV infection, they were no longer associated with CIN 2 or 3. Only gonococcal infection and antibodies to chlamydia remained as associated risk factors. The authors estimated that 78% of biopsy-confirmed CIN among the women in this cohort study could be attributed to one or more types of HPV DNA, with 52% attributable to types 16 or 18 (111). In a separate cross-sectional study conducted by the same group, testing for HPV types 42–45 added another 15% to the number of HPV infections detected by the same methods used in their cohort study (115). Further, newer more powerful tests such as PCR are more sensitive, but the authors point out that the clinical relevance of detecting HPV with the use of PCR as it relates to the ultimate risk of CIN grades 2 or 3 or cervical cancer is unknown currently.

Despite the evidence that HPV is related strongly to CIN and cervical cancer, there is a need to mention the role of other factors as they relate to HPV and cervical disease. Some factors have been discussed in the context of earlier studies and others will be mentioned here; those less well studied will not be discussed in any detail. Early age at first sexual intercourse and number of sexual partners have long been known to be risk factors for cervical disease and are mentioned in many of the studies already cited. The reason that early age at first sexual intercourse may be one of the more significant of the common risk factors for the development of CIN and cervical cancer is that the period of sexual maturation is a time of high regenerative activity that leads to lateral expansion of cells that harbor the viral genome and rapid proliferation of cells infected with HPV (116). The role that sexually transmitted diseases (other than HPV) play in CIN and cervical cancer has been reviewed recently (117). Considerably more work is needed to understand the possible role of sexually transmitted infections other than HPV, as well as the role that parity and nutritional factors may play in the incidence of CIN and cervical cancer; these and other cofactors need to be studied in relation to the presence or absence of specific HPV types.

A hypothesis linking smoking and cervical cancer was published in the late 1970s (118). Numerous subsequent studies have confirmed this association, and the topic has been reviewed recently (119). Work since this review has shown an inconsistent association with cigarette smoking and HPV among patients who had cervical cytological abnormalities. In the Oregon study, the association between smoking and risk of CIN was explained by adjusting for lifetime number of sexual partners (90). Further adjustment for all HPY types together provided no evidence that smoking remained associated with risk of all CIN levels together. However, in the same study, HPV-positive current smokers had estimated risk ratios of 2.7 for CIN 2 or 3 when compared to HPV-negative women who did not smoke, indicating that current smoking may be linked to the more advanced forms of CIN in ways that are not currently understood (90). Smoking was not found to be a risk factor for CIN grades 2 and 3 among current smokers in the cohort study conducted in Washington state after adjustment for HPV infection (111). In another study conducted in California, smoking was correlated with HPV infection in crude analyses, but the association was diminished after adjustment for number of male sexual partners (110).

Other results, with the use of PCR to characterize HPV types, showed an increase in the proportion of CIN patients with oncogenic HPV viruses (types 16, 18, or 33) present in cervical smears with an increase in the number of cigarettes smoked/day (120). To clarify the relationship between HPV and smoking among women with various levels of disease, the authors conducted further analyses. After adjustment for age at first intercourse and lifetime number of sexual partners, the authors reported an increase in the proportion of women who had evidence of oncogenic HPV types present in their cervical specimens with an increase in the number of cigarettes smoked daily. When patients were grouped into two groups, low grade lesions (up through CIN 2) and high grade lesions (CIN 3 and microinvasive carcinoma), the dose-response relationship between the number of cigarettes smoked/day and the occurrence of oncogenic HPV in the cervix was statistically significant in each of the groups. There was a higher proportion of patients with oncogenic viruses present among those with the most severe lesions who smoked the most, and conversely, the group who did not smoke and had low grade or no lesions had the smallest proportion of oncogenic viruses present (120). In recent work conducted in New Mexico, a statistically significant association was reported between smoking and high grade CIN (OR = 1.7) after adjustment for the effects of HPV (121). A dose-response relationship also was reported with number of cigarettes smoked/day and for pack-years of use with no association noted for former cigarette use.

Aside from studies that examined smoking in relation to HPV, other types of evidence provided support for the association between smoking and CIN and cervical cancer. In one clinical study, women who were seen for various levels of CIN or cervical cancer and who smoked were more likely than...
nonsmokers with these conditions to have mutagenic cervical fluids [Ref. 122 (although this effect was attenuated with the use of different laboratory techniques; Ref. 123)]. Further, nicotine and cotinine were found in the mucous secretions of the uterine cervix of women who smoked (124, 125). Tobacco by-products may produce a decrease in the concentration of antigen-presenting Langerhans cells in the normal cervical epithelium causing a smoking-mediated immunological defect (126) that allows HPV to infect and persist in the cervical epithelium (120). Tobacco by-products also may enhance chromosomal instability with or without the effects of HPV. More work is needed to: (a) ascertain the effects of smoking on the immune system and induction of chromosomal mutations; (b) thoroughly untangle the complicated relationship that smoking plays in the incidence of HPV infection and CIN and cervical cancer; and (c) determine whether the effects of smoking are potentiated by HPV.

The possibility of an association between OCs and CIN and cervical cancer that is not related to confounding is somewhat controversial, and comprehensive reviews have been published on the possible association (127, 128). However, most of the earlier studies on this topic have had little information on presence of HPV, leaving unresolved the relevance of the OC-CIN association after controlling for HPV. In four Latin American countries, ever use of OCs after control for HPV was shown to be related to adenocarcinomas of the cervix (adjusted odds ratio = 2.4) but not to squamous cell tumors (adjusted odds ratio = 1.1; Ref. 129), which provides support for earlier descriptive studies that have shown increasing incidence rates for adenocarcinomas among young women (130). However, the overall results for OC use in this study were not related to ever use, duration of use, or to years since first or last use (129). The authors concluded that the effect of OC use on CIN could not be excluded entirely, although it is likely to be limited only to a small proportion of all cases (129). Other authors in Colombia and Spain reported a somewhat elevated risk for CIN 3 with use of OCs among women who were HPV positive, although the association could have been due to chance and there was an inconsistent dose-response relationship (131). Later work in the same population that separated HPV-positive from HPV-negative women showed an elevated risk for CIN among the HPV-positive women for ever use of OCs. However, there were only two women in the HPV-positive control group among the ever users of OCs and, as the authors noted, risk estimates based on such small numbers are extremely unstable with any misclassification of the variables causing a substantial impact on the risk estimates and possibly on interpretation of the data (92). In Oregon, when HPV-positive and HPV-negative women were analyzed separately, odds ratios for risk of CIN among women who were HPV negative were 0.5 for current use and 0.7 for past use of OCs, and 2.1 for current use and 2.0 for past use among those who were HPV negative, although confidence limits for all values overlapped unity (90). Use of OCs was not found to be a risk factor for CIN 2 and 3 in the cohort study conducted in Washington state after adjustment for HPV infection (111), nor were they a risk factor for CIN in a cohort study conducted in Washington OC after a similar adjustment for HPV (132).

Other work that explored the reputed relationship between OC use and CIN at the molecular level found no support for OCs affecting gene expression of HPV. It is thought that the HPV-16 transforming ability in CIN is mediated by the E6 and E7 proteins, which were described earlier in more detail in the section on these proteins. Detailed studies of E6 and E7 gene expressions were conducted in relation to HPV 16 and OCs and showed that their expression was not influenced by OC use. These results suggest that if OCs do play a role in HPV-mediated carcinogenesis, it is not through quantitative changes in gene expression in vivo (133).

Another, relatively recently recognized cofactor for development of CIN and cervical cancer is infection with HIV. HIV as the causative agent for the AIDS has emerged as a leading cause of death in the United States over the decade of the 1980s (134). Since 1985, the largest increase in mortality from HIV-associated disease has been in women (134), with relatively little known about the course of AIDS in this group (135). Because cervical disease has emerged as an important cause of morbidity in women infected with HIV, the Centers for Disease Control has included cervical cancer as an AIDS-defining illness in women (136). The full extent of the relationship between HIV and HPV in conjunction with an increase in cervical pathology is unclear currently. Several studies have considered this association with varying results and a clearly presented commentary has been published recently (135).

In recent years, there have been numerous reports describing increased prevalence of both CIN and cervical HPV infection among HIV-positive women compared to HIV-negative women (137–143). In a meta-analysis of five studies, an odds ratio of 4.9 was reported for the association between HIV infection and cervical neoplasia (144). Once cervical cancer develops in HIV-positive women, the disease may be more aggressive and less responsive to treatment (141, 145). There also is evidence to suggest that HIV-positive women respond inadequately to standard therapy for CIN when compared with HIV-negative women and that recurrence rates after standard therapy are higher (146).

A similar picture has begun to emerge for anal HPV infection, anal intraepithelial neoplasia, and HIV-related immunosuppression in homosexual and bisexual men. However, unlike cervical cancer there is some evidence to suggest that the incidence of anal cancer has increased since the onset of the HIV epidemic, although the precise contribution of HIV-related immune suppression remains unknown (147). In a study of HIV-negative and HIV-positive men in San Francisco, the prevalence of both anal HPV infection and disease were highest among HIV-positive men, especially those with CD4 cell counts <250/mm³ (148), consistent with earlier studies of men with symptomatic HIV disease (149). These findings have been confirmed in other populations (150–153), and preliminary studies of the natural history of anal disease in men with group IV disease suggest that anal disease in this population may progress rapidly (154).

Two primary means by which HIV infection may influence the pathogenesis of HPV-associated cervical pathology are through effects on the function of the immune system in HIV-positive women or through molecular interactions between HIV and HPV genes (135). Although these have not yet been characterized fully, one theoretical mechanism of interaction, described earlier, may be through the transactivation of HPV gene expression by HIV proteins, such as the effect of the HIV-1 tat protein on up-regulation of HPV E6 and E7 expressions (155). Use of sensitive techniques, such as PCR, combined with better methods to evaluate immune status will be required in population-based epidemiological studies to explore further the relationship between HPV infection in HIV-positive women who have CIN and cervical cancer (135).

Taken together, recent studies using techniques such as PCR suggest: (a) only a small proportion of HPV-infected individuals develop clinically detectable disease (94); (b) HPV-associated diseases usually take several years to progress from
benign to malignant (6); and (c) HPV infection alone may be insufficient for development of disease and cofactors may be necessary. These results are consistent with the notion of requirement for accumulation of chromosomal mutations. Because these mutations occur over time, there is a rather long period to progression. The mechanism currently is unclear for the role of risk factors such as smoking and how they might contribute to chromosomal mutations. Interestingly, several HPV-negative cervical cancer cell lines and cervical cancer biopsy tissues have been examined and found to have mutations in the p53 genes (156). These results suggest that cervical cancer may be divided into two groups: (a) the most common form that contains HPV; and (b) the rarer form that has mutations in genes that regulate cell growth such as p53, thereby resulting in the same functional phenotype as inactivation of the p53 protein by the E6 protein. Women with HPV-negative tumors usually are older than the women with HPV-positive tumors, possibly reflecting time to have had this mutation occur versus early age of exposure to HPV in the women with HPV-positive tumors.

Conclusion

The evidence supporting a role for HPV in the pathogenesis of cervical cancer is exceedingly strong. This evidence includes (a) detection of HPV DNA in most cases of cancer and CIN; (b) evidence of transcriptional activity in the tissues; and (c) evidence that viral genes have transforming ability and will generate tumors in animal models, including transgenic mice. However, only a small proportion of HPV-infected individuals will develop HPV-associated cervical disease and, therefore, the role of cofactors also must be considered. The functions of some of these factors and their effect on HPV gene expression have been described here and provide a theoretical framework for the biological interaction of these cofactors with HPV in the pathogenesis of cervical disease. In general, these interactions fall into three categories: (a) cofactors associated with attenuation of immune response to HPV; (b) cofactors that up-regulate E6/E7 gene expression; and (c) cofactors that contribute to genetic damage independently of their effect on HPV gene expression. In many cases, these cofactors operate in more than one of these modes. For example, HIV infection may result in generalized attenuation of immune response. As secondary effects on HPV through its action on CD4 cells and other immune effector cells. Additionally, a more direct interaction between HIV and HPV was described earlier (155). Similarly, the effects of cigarette smoking on CIN and cervical cancer may operate on several levels simultaneously, both by affecting local Langerhans-cell immune response and by contributing to genetic damage that may be additive to that induced by E6-mediated abrogation of p53 function. Clearly, acquisition and persistence of HPV infection, development of cervical disease, and progression of cervical disease represent the net effect of many interwoven processes. Knowledge of both the epidemiological risk factors and the effect of these factors on the molecular biology of the cell and the HPV virus have led to great progress in comprehending the pathogenesis of cervical disease. This new knowledge of mechanisms promises to provide further insight that will enhance understanding and will be likely to provide methods for prevention and new approaches to therapy.

References


Molecular virology and epidemiology of human papillomavirus and cervical cancer.

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