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Human Cancer Viruses

Principles of Transformation and Pathogenesis

Editors
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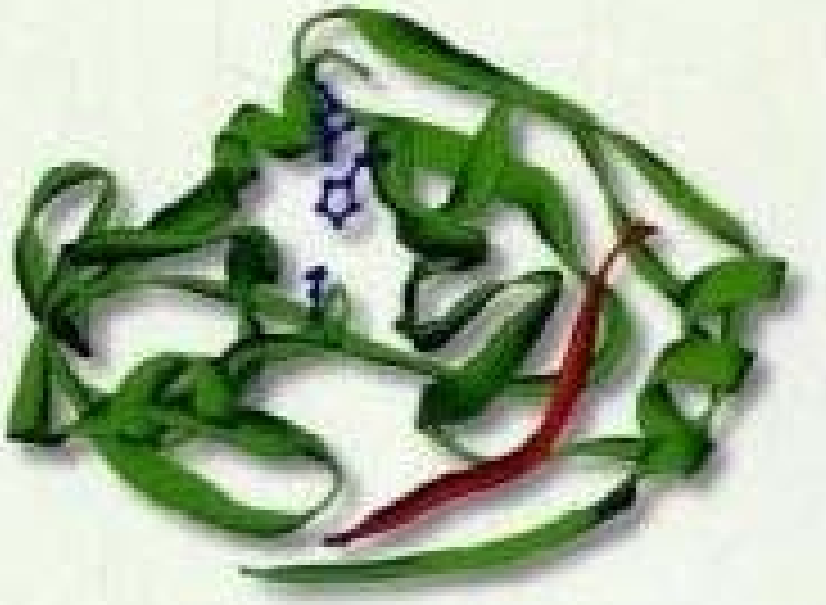
K.-T. Jeang
T.-C. Wu



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Translational Research in Biomedicine

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Contents

Foreword

Welcome to *Translational Research in Biomedicine*, a new book series dedicated to the dissemination of seminal information in contemporary biomedicine with a translational orientation.

Translational research (TR) is now a household word in the arena of contemporary biomedical research, although a universal definition for this term is currently wanting. In a more restricted sense, TR is often associated with research and development based on the classical bench to bedside approach. Thus, it has been said that 'the goal of TR is to implement in vivo measurements and leverage preclinical models that more accurately predict drug effects in humans' [1]; or 'TR describes a uni-directional effort to test in humans novel therapeutic strategies developed through experimentation' [2]. The current enthusiasm for the application of genomic or stem cell research to therapeutic strategies is also grounded on a similar premise. In a broader sense, TR is taken as a bench to bedside and back approach to foster communication between the scientific community and clinical practitioners [1]. It is a concept that needs the attention from everyone and should be the foundation of a modern understanding of health provision [3].

If we subscribe to the philosophical connotation that medical research is for the betterment of humankind, then we should realize that there is no real demarcation between clinical (bedside) and preclinical (bench) research. This is because the only difference is that human subjects instead of animals, tissues or cells are employed in the studies. Nonetheless, governed by the same ethical principles and guidelines, all of them will reveal information in some aspects of biomedicine. Thus, this monograph series shall take a holistic view on TR that transcends the boundaries between bench and bedside research. Each volume shall be a synthesis of ideas, technologies and research outcomes that are associated with a particular theme in contemporary biomedicine, to be edited by experts in that field. Some chapters may be up-to-date reviews on fundamental

principles that underlie this theme, to be followed by their clinical applications. Other chapters may begin with clinical observations on this theme, to be followed by their underlying cellular and molecular mechanisms. The word 'translation' is most commonly defined as expression of words in another language. Its definitions can be extended to encompass expression in simpler language and uncomplicated interpretation. In this spirit, all volumes in this series will be presented in a fashion that is amenable to nonexperts, be they scientists or clinical practitioners.

My sincere thanks go to Drs. John Nicholas, Kuan-Teh Jeang and T.-C. Wu, Editors for this inaugural volume, for their unfailing efforts to make this series a reality. I am particularly in debt to Dr. Jeang for his timely advice during the planning stage of this project. I also wish to acknowledge the capable hands of Rolf Steinebrunner, Stefan Goldbach and Esther Bernhard at S. Karger AG during the development and production of this series. Last but not least, the publication of *Translational Research in Biomedicine* would not have been possible without the foresight, enthusiasm and whole-hearted support of my dear friend, Dr. Thomas Karger.

Samuel H.H. Chan
Series Editor

1 Hörig H, Pullman W: From bench to clinic and back: perspective on the 1st IQPC Translational Research Conference. J Transl Med 2004;2:44–51.
2 Mankoff SP, Brander B, Ferrone S, Marincola FM: Lost in translation: obstacles to translational medicine. J Transl Med 2004;2:14–18.
3 Sonntag KC: Implementations of translational medicine. J Transl Med 2005;3:33–35.

Preface

The genesis of this book stems from a long-standing collaboration between many of its authors in the teaching of viral oncology to postgraduate students at the Johns Hopkins School of Medicine. Recognizing that a number of these students had little or no exposure to the history, landmark contributions or current research in this important and exciting field was the prime motivation for teaching the course and, subsequently, for compiling this book. Studies of human and nonhuman oncogenic viruses have made enormous contributions to our understanding of crucially important aspects of cell biology and transformation. For many of us who have lived through pivotal advances in viral oncology over the past 4 decades, there is a strong desire, perhaps a feeling of obligation, to share the past and present excitement of the field with a new generation of scientists. This, along with the wish to present a review of human viral oncology as a cohesive topic, represents the mission of this book.

The first identification of a tumor-causing virus, Rous sarcoma virus, occurred almost 100 years ago, but it was not until the 1970s that the genetic basis for oncogenesis by this and other acutely transforming retroviruses of avian and rodent species was appreciated. Numerous viral oncogenes and their corresponding cellular proto-oncogene counterparts were identified in rapid succession from these altered and defective viruses. These studies launched a new era of research forging insight into the basic mechanisms of cell cycle control and the functions of key genes involved in its regulation. Later studies of DNA tumor viruses simian virus 40 and human adenovirus, found to be transforming in culture and animal models, provided the next advances in our understanding of viral oncogenesis and virus manipulation of the cell cycle and cell survival. These viruses served as crucially important models for the study of oncogenesis, linking processes normally utilized by viruses for replication to mechanisms of oncogenesis occurring under conditions nonpermissive to productive replication. The importance of these discoveries to the elucidation of normal cellular

control pathways and the aberrancies occurring in nonvirally associated as well as virus-induced cancers is difficult to overstate. They exemplify what all virologists know to be true, that viruses, by virtue of evolutionary selection processes, target and manipulate centrally important cellular control pathways by elegantly efficient means that can be utilized by the experimental biologist to elucidate their architecture. It is entirely understandable, then, that p53 and E2F and Rb/E2F associations, for example, were first identified via the study of viruses and viral proteins, SV40 T-Ag and adenovirus E1A, respectively.

Viruses are not only tools for discoveries in cell and molecular biology. However, they are worthy research objects in their own right, not least because they are significant pathogens, including oncogenic agents in humans. Leaving aside the unresolved (and often contentious) issue of the potential role of polio-vaccine-introduced SV40 in human malignancies, there are several human viruses that are strongly associated epidemiologically with human cancers and that represent significant health concerns. However, from the high infection rates of these viruses coupled with the relatively low incidence of virus-associated cancers, it is evident that oncogenic transformation is not part of the normal life cycle of these viruses. In addition to virus exposure, predisposing cofactors can include immunosuppression, host genetics and particular environmental criteria. This highlights the multifactorial, multistep nature of viral oncogenesis in the natural host, the viral agent being a necessary or contributory factor but clearly not the only one. The relevant viruses with regard to human cancers are human T lymphotropic virus 1, human papillomavirus, hepatitis viruses B and C, and the human γ-herpesviruses Epstein-Barr virus and human herpesvirus 8 (also called Kaposi's sarcoma-associated virus); these comprise the focus of this book. The particular mechanisms relevant to transformation by these viruses are varied, but all appear to involve the influence of viral proteins whose roles are to provide the conditions for efficient virus productive replication or, in the case of Epstein-Barr virus and human herpesvirus 8 at least, for the maintenance of the latently infected cell pools. Understanding the mechanisms leading to cellular transformation and oncogenesis by these viruses, in addition to the roles of particular viral proteins in these processes and the normal virus life cycle, is essential for the development of highly directed and specific therapeutic and antiviral treatments. This said, it is also evident that vaccination to prevent primary infection by oncogenic viruses can provide a means of eliminating the possibility of cancers in which viruses play a necessary role. Cancer vaccination is already here with regard to the 70% of cervical carcinomas caused by human papillomavirus types 16 and 18, and further research is ongoing to utilize protein- and DNA-based vaccines to provide broadly based protection against all oncogenic human papillomaviruses. Similar strategies could conceivably be used for tackling cancers caused by other viruses, although this is easier said than done.

This book provides a comprehensive overview of the human oncogenic viruses, with respect to their molecular biology and epidemiology and to clinical aspects of disease, therapy and prevention. As outlined in the chapters collated here, work on

these viruses has greatly aided our appreciation of the diversity and details of mechanisms likely to be involved in oncogenesis as well as virus biology and has, along the way, revealed new paradigms in cell and molecular biology. These human oncogenic viruses, then, join their historical predecessors in serving as tools for understanding normal cell regulatory processes. It seems evident that further seminal discoveries of general import will be made through the study of these significant human pathogens, and that such study will also provide the means to prevent and treat associated human disease.

John Nicholas, Kuan-Teh Jeang and *T.-C. Wu*
Volume Editors

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Molecular Epidemiology of Human Papillomavirus Infection

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Abstract

At least 5% of the global burden of human cancers each year have been attributed to human papillomavirus (HPV) infection. All cervical cancers as well as distinct subsets of vulvar, vaginal, anal and oral cancers among women, and penile, anal and oral cancers among men are causally associated with HPV infection. Multiple lines of evidence indicate that HPV-16 is uniquely carcinogenic in human subjects. Compared to other high-risk HPV infections, cervical infections by HPV-16 are more common, persist longer and are more likely to result in the development of high-grade cervical dysplasia. Additionally, HPV-16 is the most common HPV type isolated from cervical cancers worldwide and from all other human cancer types associated with HPV infection. Taken together, these data indicate that HPV-16 is unique in its ability to evade the host immune response and transform the infected cell. The prevention of infection by HPV-16 via prophylactic vaccines already demonstrated to reduce the incidence of cervical, vaginal and vulvar dysplasias therefore holds tremendous promise for reducing the overwhelming majority of the worldwide burden of cancers caused by HPV.

Human Papillomaviruses

Papillomaviruses are nonenveloped, double-stranded DNA viruses with a circular genome of approximately 8,000 base pairs. Human papillomaviruses (HPV) are the subset of *Papillomaviridae* that specifically infect humans. Phylogenetic assemblages based on sequence have defined 2 distinct higher-order genera for the HPV: the α-papillomaviruses, which infect primarily, although not exclusively, the mucosal epithelia, and the β-papillomaviruses, which infect primarily cutaneous epithelia (fig. 1) [1]. Within these genera, lower-order phylogenetically related types constitute species, which have a sequence identity of approximately 60–70%. HPV within the same species group share 71–89% sequence identity, and an HPV 'type' is defined by 10% or greater sequence variability in the conserved L1 major capsid protein sequence. Sequence variability of less than 10% within an HPV type defines a variant or subtype. Approximately 120 different HPV types have been cloned and sequenced to date from human subjects [1]. The genomic diversity of HPV types in different

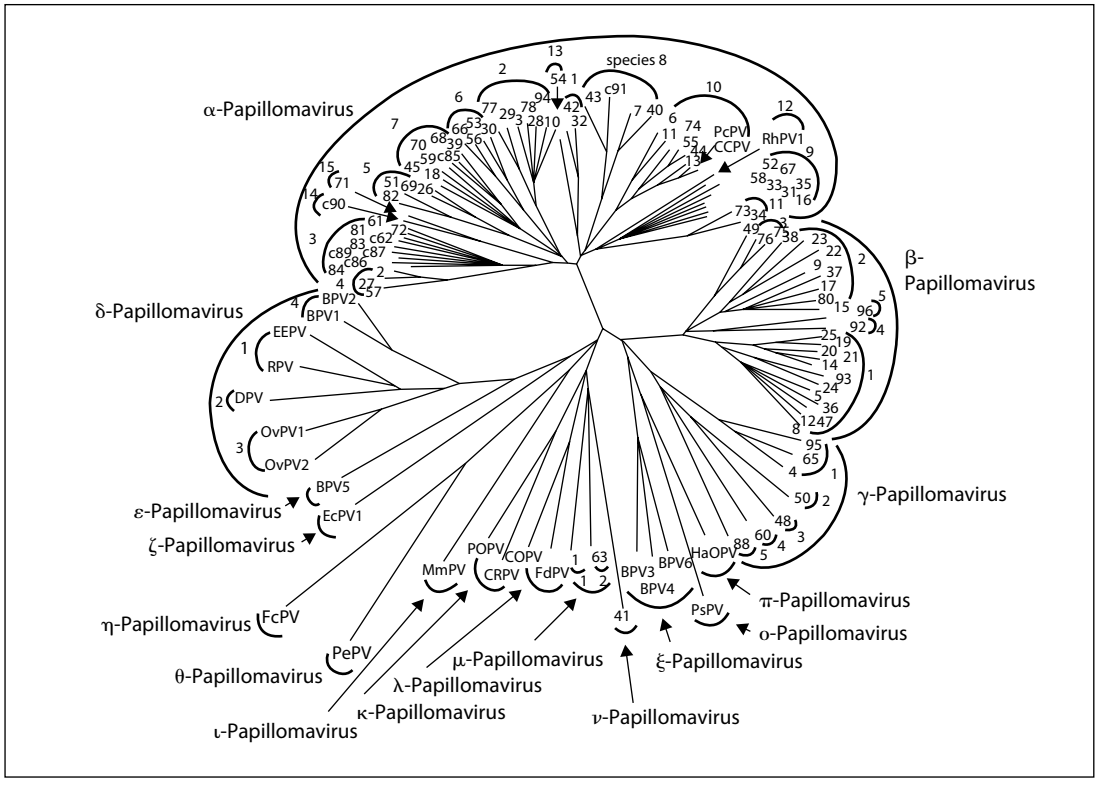

Fig. 1. The current phylogenetic tree for papillomaviruses based on analysis of the sequence of the L1 open reading frame, inclusive of 118 different types. The outermost and innermost semicircles delineate genera and species, respectively. The numbers at the ends of each of the branches identify an HPV type; c-numbers refer to candidate HPV types. The HPV are within the α- and β-papillomavirus genera. From de Villiers et al. [1].

world populations indicates that HPV have comigrated with humans from a common African ancestor [2–4].

This manuscript will focus on the α-papillomaviruses because these viruses are clearly established as carcinogenic in human subjects. α-Papillomaviruses are classified by type as oncogenic or 'high-risk' and nononcogenic or 'low-risk' based upon epidemiological associations with cervical cancer. Initially, the high-risk designation was dependent on whether the particular type had been detected in a cervical cancer specimen. More recently, a designation of high-risk has been based on epidemiological associations with cervical cancer in case-control studies [5, 6]. HPV types strongly associated with cervical cancer in case-control studies and therefore designated as high-risk include HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82. Those classified as probably high-risk include HPV-26, -53 and -66, and the low-risk types include HPV-6, -11, -40, -42, -43, -44, -54, -61, -70, -72, -81 and CP6108 [5, 6].

In a recent monograph published by the International Agency for Research on Cancer [7], HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66 were classified as established carcinogenic HPV types in humans. The high-risk classification derived from epidemiological associations tends to correlate well with clustering based on viral sequence [8]. The majority of high-risk types fall within a limited number of species, in particular the A9 species, those most closely related to HPV-16 (e.g. 31, 33, 35, 52 and 58), and the A7 species, those most strongly linked to HPV-18 (e.g. 39, 45, 59). In contrast to the high-risk HPV types, the low-risk types are associated with low-grade cervical dysplasias and benign hyperproliferations of the infected epithelium (e.g. warts). For example, 90% of the genital warts are attributable to low-risk HPV types 6 and 11 [9].

Studies of the genomic organization of various types of papillomavirus reveal a well-conserved general organization. The genome of the majority of high-risk α-papillomaviruses encodes 8 proteins. The early (E) genes encode proteins involved in viral DNA replication, maintenance of the viral episome, regulation of viral and host cell gene expression and host cell proliferation. The 2 late (L) genes L1 and L2 encode the major and minor structural proteins of the viral protein capsid, respectively. The viral genome also contains the long control region, which includes the viral origin of replication and transcriptional regulatory regions. The E1 and E2 genes are DNA binding proteins that form a complex at the viral origin of replication to recruit cellular polymerases necessary for viral replication and are also important for the maintenance of the viral episome during cell division [10]. The E1 gene is also an ATP-dependent helicase that unwinds DNA during viral replication [11], whereas the E2 protein also functions as a transcription factor that negatively regulates viral E6 and E7 expression [12]. The E2 gene is frequently disrupted during viral integration, one of several mechanisms that result in deregulated expression of the viral E6 and E7 oncoproteins [13], thereby promoting tumorigenesis. The E4 protein is expressed primarily in differentiated epithelial cells and arrests cells in the G_2 phase of the cell cycle, modulates late viral genome expression and viral replication and may interact with the cellular cytokeratin network [14, 15]. The E5 protein is an integral membrane protein that promotes cellular transformation via the EGFR pathway [16], including via phosphorylation and inhibition of degradation of the receptor [17]. It also functions to downregulate MHC class I expression on the infected cell, perhaps aiding in the evasion of the host immune response [18].

The transforming potential of oncogenic HPV types is largely attributable to the viral E6 and E7 proteins that are capable of inactivating 2 human tumor suppressor proteins, p53 and pRb, respectively [19]. The E6 protein combines with a cellular protein, E6-AP to form an ubiquitin ligase which targets p53 for degradation via the proteosome [20]. Another major function of E6 important for immortalization is the activation of the catalytic subunit of telomerase, hTERT [21]. The E7 protein binds the tumor suppressor pRb and blocks its binding to the E2F transcription factor, leading to the constitutive activation of S phase genes [22]. E6 and E7 are necessary for

viral transformation and stimulate cellular proliferation, delay cellular differentiation, increase the frequency of spontaneous and mutagen-induced mutations and induce chromosomal instability (i.e. gene amplification, polyploidy and aneuploidy) in transfected cell lines [19].

Human Papillomavirus Presence and Type Distribution in Cervical Cancer

A paradigm for HPV-mediated carcinogenesis has evolved from studies of the relationship between HPV and cervical cancers. Large, international studies of cervical cancer worldwide have detected HPV genomic DNA in 99.9% of the cases [23, 24], indicating that HPV infection is necessary for the development of cervical cancer. Case-control studies have consistently observed a strong association between HPV infection and cervical cancer. HPV infection confers an estimated 90- and 81-fold increase in the risk for squamous cell and adenocarcinomas of the cervix, respectively [6]. Prospective cohort studies of cervical HPV infection have also described the histopathological progression of cervical cancer, from cervical HPV infection without cytologic abnormality to mild, moderate and severe cervical dysplasia/carcinoma in situ [25, 26].

HPV-16 is most frequently isolated from invasive cervical cancers, regardless of geographic region (fig. 2) [27]. HPV-18 consistently ranks second in all regions, and then the rank order varies by geographic region, with HPV types 31, 33, 52, 58 and 45 predominating [28]. HPV-16 and -18 account for approximately 70% of the cervical cancers worldwide. A recent analysis of cervical cancer specimens over calendar time from 1930 to 2005 also revealed that the predominance of HPV-16 and -18 has not changed over time [29]. HPV-16 is most strongly associated with cervical squamous cell carcinoma, while HPV-18 is most closely related to adenocarcinoma [30]. The majority of cervical cancers worldwide are squamous cell carcinomas, but adenocarcinomas account for a higher proportion among developed than undeveloped countries, possibly because squamous cell dysplasias are more readily detectable with cervical cytology screening [31].

Prevalence and Type Distribution of Genital Human Papillomavirus Infection in Women

HPV infection is the most common sexually transmitted disease in the USA [32]. In US population-based surveys, approximately 26.9% of the 18- to 25-year-old women in 2001–2002 [33] and approximately 26.8% of the 14- to 59-year-old women in 2003–2004 had a prevalent genital HPV infection [34]. The Centers for Disease Control have estimated that approximately 80% of the women will acquire an HPV infection by the age of 50 years [35].

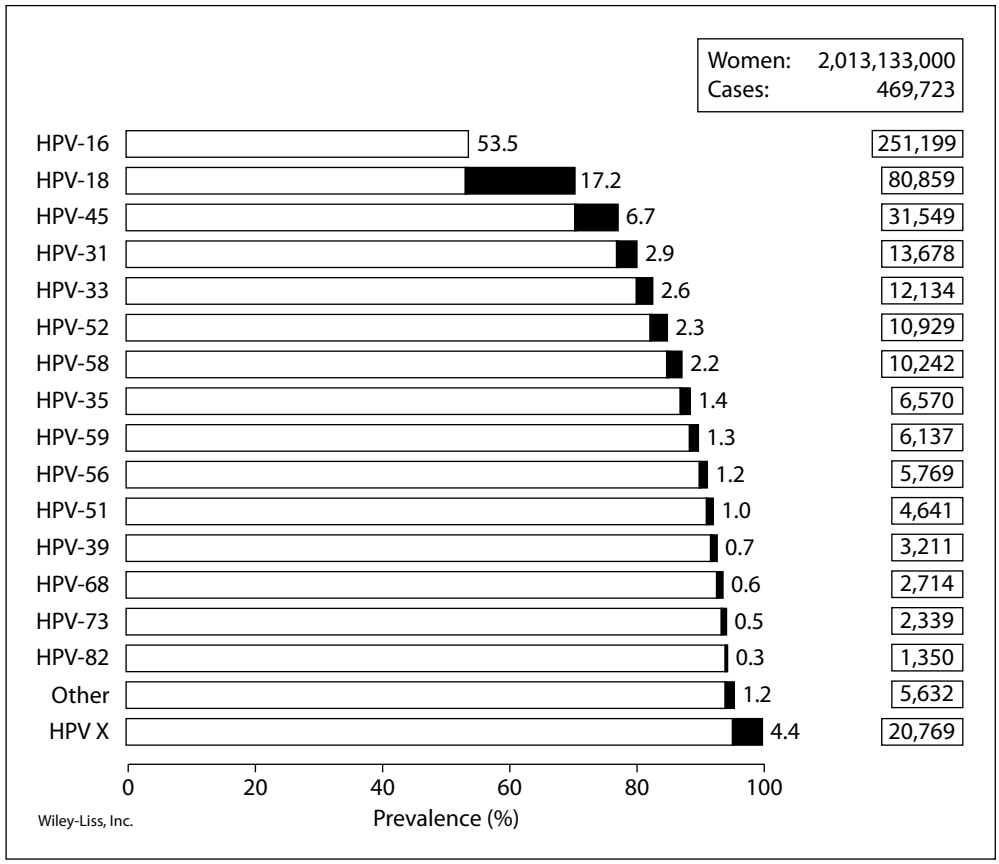

Fig. 2. The cumulative type-specific prevalence of HPV type in approximately 470,000 cervical cancer cases worldwide. From Munoz et al. [28].

Recent meta-analyses estimate that approximately 10% of the women worldwide with normal cervical cytology are HPV infected. Thus, 291 million women worldwide have a genital HPV infection [36, 37]. It is important to note, however, that there is substantial regional variation in prevalence (fig. 3) [36, 37]. For example, in an analysis of 15,613 women aged 15–74 years from 11 countries, the age-standardized prevalence among women with normal cervical cytology varied by 20-fold, from 1.4% in Spain to 25.6% in Nigeria [37].

The prevalence of cervical HPV infection declines with age in most populations [36]. In a US population-based study, the National Health and Nutrition Examination Survey, the HPV prevalence increased with each year of age to 24 years, then declined through 59 years [34]. However, age-specific prevalence curves significantly differ by geographic region [36, 38]. In some populations, the prevalence of cervical HPV infection initially declines with age and then a second peak is observed among women 55 years and older. In a few populations the prevalence remains largely unchanged with increasing age [38].

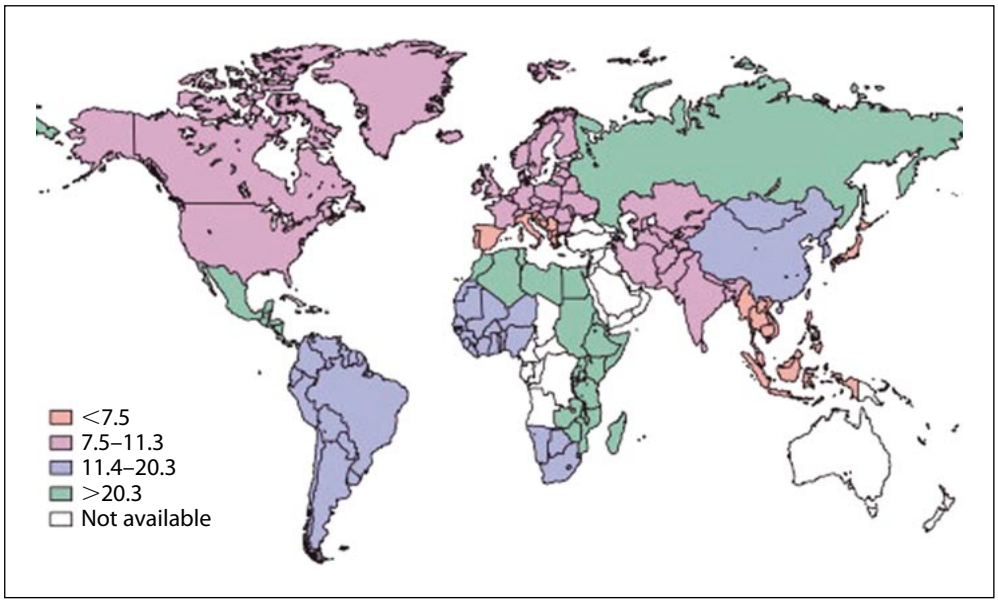

Fig. 3. Estimated prevalence of cervical HPV infection worldwide among women with normal cervical cytology based upon a meta-analysis of 157,879 women from 78 studies. The colors represent the age-adjusted prevalence in the region. From de Sanjose et al. [36].

Analogous to the age distribution, the HPV type distribution among women with normal cytology varies by geographic region [36, 37, 39]. HPV-16 is the most prevalent HPV infection worldwide, and with few exceptions it is the most prevalent type identified in all geographic regions [36, 37]. In general, the prevalence of cervical infection by high-risk HPV types exceeds low-risk types and approximately 30% of the infected women are concurrently infected by more than 1 HPV type [37]. The HPV type distribution among HIV-infected women with normal cervical cytology is more broad than among HIV-uninfected women [40]. The HPV type distribution is also dependent upon cervical cytology. The prevalence of HPV-16 and -18, and perhaps -45, increases with lesion severity from normal through to high-grade dysplasia and cancer, whereas the prevalence for all other HPV types declines [39].

Risk Factors for Cervical Human Papillomavirus Infection

Current data indicate that HPV infection is transmitted overwhelmingly via sexual contact. The most important and consistent risk factors for HPV infection are the lifetime number of sexual partners and the sexual behavior of one's partners [25, 41]. Cervical HPV infection among young women is strongly associated with the number of recent sexual partners and among older women (aged 40–50 years) with the lifetime

number of partners [41]. Cohort studies in which sexually inexperienced women are prospectively followed are particularly informative. Among US college-aged women, the cumulative prevalence of cervical HPV infection among women with a single, first partner was 28.5% at 1 year and increased to 50% by 3 years [42]. A similar cumulative prevalence of 45% was observed within 3 years of first sexual intercourse with a single partner in another cohort of women aged 15–19 [43]. The incidence rates for new infection are similar among virgins and nonvirgins with a new partner [44]. A 2-year cumulative prevalence of 82% was observed in a small prospective cohort of adolescent women in which cervical sampling was performed weekly, indicating that the standard sampling interval of 4–6 months may underestimate the incidence [45]. Smoking appears to increase the risk [44], whereas consistent use of condoms appears to reduce it [43, 46]. Young age at first intercourse has been associated with an increased risk of infection but may serve as a surrogate for a higher number of partners and high-risk partners [43, 47]. Incident cervical HPV infection among virginal women is rare [48] and likely attributable to nonpenetrative sexual contact [44].

The dynamics of HPV transmission among sexual partners is largely unknown, as prospective cohort studies of HPV transmission among sexual partners have not been performed. The type-specific concordance among sexual partners in cross-sectional prevalence studies is highly variable depending on the population studied but appears greater than would be expected by chance [49–51]. The concordance of infection is particularly high when restricted to partners of women or men with known HPV infection [52, 53]. Incidence data from a cohort study of female college students in Montreal were recently used to model the risk of HPV transmission during a single act of sexual intercourse. A median probability of infection of 0.4 (or 40% per episode of sexual intercourse) was most consistent with the observed data [54]. However, there was considerable uncertainty around this point estimate.

Natural History of Cervical Human Papillomavirus Infection

The majority of cervical HPV infections clear without histopathological consequence within 1–2 years [55]. The average duration of incident infection among immunocompetent, college age women is approximately 8 months [25]. The median time to clearance tends to be longer for high-risk than low-risk infections [45, 56] and in particular for HPV-16 [57]. HIV-related immunosuppression significantly alters the natural history of cervical HPV infection. The increased prevalence of HPV infection among HIV-infected individuals relative to HIV-uninfected individuals is due to both an increase in incidence and a reduced rate of clearance in the setting of declining immune function (e.g. increasing HIV viral load and decreasing CD4 cell count) [58]. However, the natural history of HPV-16 infection is less affected than other HPV types by HIV-related immunosuppression, indicating that HPV-16 may be unique in its ability to evade the immune response among immunocompetent women [59].

Factors Associated with Risk of Progression to Cervical Dysplasia

Persistence of type-specific HPV infection is a strong and consistent risk factor for the development of abnormal cervical cytology [25, 60, 61]. For example, the incidence of abnormal cytology among women with 2 consecutive HPV-negative specimens is 0.7 per 1,000 woman months versus 8.7 per 1,000 among twice HPV-DNA-positive women [60]. The incidence of abnormal cervical cytology among HPV-positive women at 1 year is about 25–50% and then declines [62]. While the development of abnormal cervical cytology among HPV-infected women is common, the growth of high-grade cervical dysplasia/carcinoma in situ is much less frequent. Approximately 4.5% of the women with any cervical HPV infection or abnormal cervical cytology will develop high-grade dysplasia within 10 years [26]. The risk is greater among women infected by high-risk types: among cytologically normal women with a high-risk HPV infection, the cumulative prevalence of high grade cervical dysplasia was 28% at 14 years in a large British cohort study [63].

The single most important factor associated with risk of cervical dysplasia among HPV-infected women is HPV type. HPV-16 appears to be particularly carcinogenic, as it is both more likely to persist and to progress to cervical dysplasia [8]. Women infected with HPV-16 are significantly more prone to progress to a cervical dysplasia than those infected with the other high-risk HPV types 18, 35, 39, 45, 51, 52, 56, 58, 59 and 66 [64]. In a prospective cohort study in Portland, Oreg., USA, the 10-year cumulative incidence rate for high-grade dysplasia or cervical cancer among HPV-16-infected women was 17.2%, among HPV-18-infected women 13.6%, among 11 other high-risk types 3.0% and among high-risk HPV-negative women (as detected by use of Hybrid Capture II) 0.8% (fig. 4) [65]. Similarly, approximately 20% of the women infected by HPV-16 developed high-grade dysplasia or cancer within 5 years in a large cohort study in Costa Rica [8]. In addition to the HPV type, the HPV variant sequence within types may affect the risk of progression. Women infected with non-European variants of HPV-16, in particular those with Asian-American variants, were at increased risk for progression to high-grade cervical dysplasia [66, 67]. Interestingly, African-American variants of HPV-16 are less likely to clear among African-American women than European variants, and visa versa, indicating the presence of race- and variant-specific viral-host interactions [68]. Sequence variability within an HPV type may result in differential control over viral oncogene expression, oncoprotein function or viral immune evasion [69].

Case-control studies have identified increased parity, tobacco smoking and oral contraceptive use as potential cofactors for the development of cervical cancer [70]. In a recent analysis of 16,573 women with cervical cancer and 35,509 controls, the risk of cervical cancer significantly increased among current users of oral contraceptive pills and increased with years of use (test for trend, $p < 0.0001$). The risk declined with years since cessation of use [71]. A similar analysis revealed current smoking and number of cigarettes smoked per day were associated with risk of

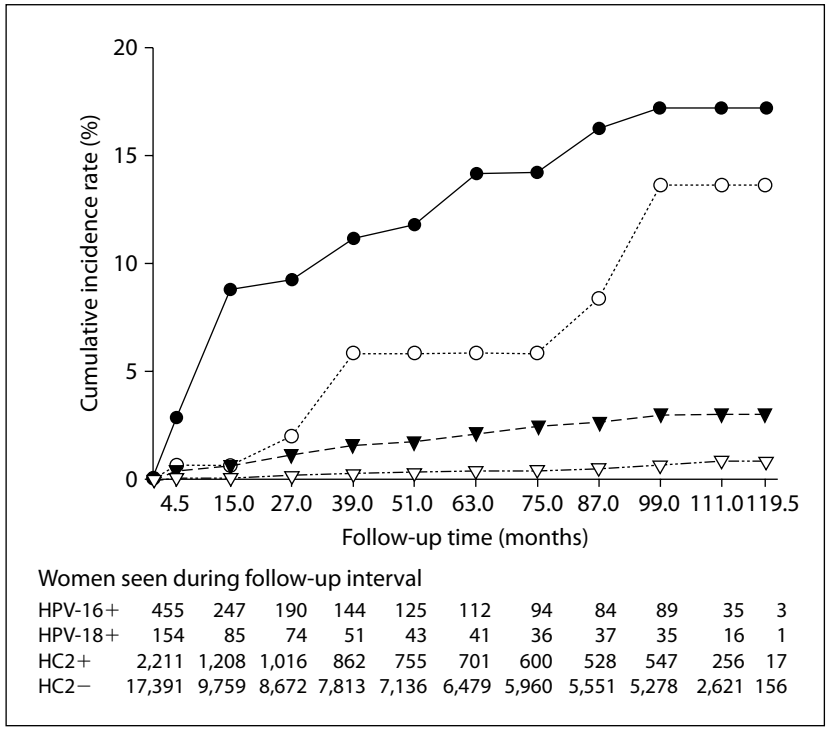

Fig. 4. The cumulative incidence of high-grade cervical dysplasia or cancer among 12,514 women in a prospective cohort study in Costa Rica. Data are stratified by the presence of cervical HPV infection at enrollment and by the type of HPV infection detected among HPV-infected women. The cumulative incidence among women without infection by 13 different high-risk HPV types as measured by Digene Hybrid Capture II assay is represented by open triangles. The cumulative incidence among women infected by HPV-16 is indicated by closed circles, for those infected by HPV-18 by open circles and for women infected with the non-HPV-16/18 oncogenic types in Hybrid Capture 2 by closed triangles.

cervical cancer [72]. Because of potential confounding by cervical HPV infection, prospective cohort studies which examine the risk of cervical dysplasia among HPV-infected women are particularly informative [73]. The risk of cervical cancer among HPV-infected women is associated with the use of oral contraceptives, with dose-response relationships observed for the use of 5 years or more [71]. Tobacco smoking also appears to increase the risk of high-grade cervical dysplasia or cancer among HPV-infected women [74] and the risk increases as a function of intensity (cigarettes per day) and duration (in years) of tobacco smoking [75]. Parity is inconsistently associated with increased risk of high-grade dysplasia among HPV-positive women [76]. Concurrent cervical infection by *Chlamydia trachomatis* has been reported to increase the duration of cervical HPV infection [77–79] but not the risk of cervical dysplasia in HPV-infected women. Additional viral factors that have been found to

increase the risk of progression to cervical dysplasia include multiple concurrent infections [80] and HPV-16 viral load [81]. Condom use may facilitate regression among women with cervical dysplasia [82] and clearance among infected women, perhaps by preventing reinfection from an infected partner [83, 84]. Among HIV-infected women, highly active antiretroviral therapy significantly increased the rate of regression of an incident cervical dysplasia. However, the overwhelming majority of lesions persist [85, 86].

Genital Human Papillomavirus Infection Prevalence and Risk Factors for Infection in Men

The prevalence of genital infection in men has not been as extensively studied as it has been in women. However, recent studies indicate that the prevalence of infection is as high or higher among men than women [87]. As an example, a cross-sectional prevalence of 65.4% was recently reported among 463 US men aged 18–40 years [88]. Infection by high-risk HPV types and concurrent multiple infections are common [87]. Circumcision has been associated with a lower prevalence of genital HPV infection in men [89]. The effect of age on genital HPV infection prevalence in men has not been reported.

As is the case for women, genital HPV infection in men is strongly associated with sexual behavior. The number of recent sexual partners [89–91], anal sex with a man [92] and current smoking [90, 91] all increase the risk of genital HPV infection in men. As is the case for women, the incidence of infection among sexually active young men is quite high. For example, among 18- to 20-year-old heterosexual male college students in the USA, the cumulative incidence of new genital HPV infection was 62.4% at 24 months and that of HPV-16 infection was 19.5% [91]. Concurrent, multiple HPV infections and current smoking appear to increase the infection persistence in men [89] and circumcision may reduce it [92]. As for women, consistent condom use may decrease the risk of infection among men [90, 93].

Anal Human Papillomavirus Infection Prevalence and Risk Factors for Infection

Data on the prevalence of anal HPV infection among men and women are sparse in comparison to those for cervical HPV infection in women. Limited data among immunocompetent women indicate that the prevalence of anal HPV infection may approximate that for cervical infection [94, 95]. In a cohort of women in the USA, the 27% prevalence of anal HPV infection closely approximated the 29% prevalence of cervical HPV infection among women [95]. Anal HPV infection was 3-fold more common among women with a cervical HPV infection [95]. A recent study reported

a 20% prevalence of anal HPV infection among immunocompetent men aged 18–40 [88]. The prevalence of anal HPV infection is high (approximately 60%) among HIV-negative homosexual men and is higher among HIV-infected when compared to HIV-uninfected men and women [94, 96, 97]. A few studies have evaluated the age-specific prevalence of anal HPV infection: the prevalence among HIV-negative homosexual men did not appreciably change with age [98] and declined with age in immunocompetent women, depending on the presence of a concurrent cervical HPV infection [95].

The cross-sectional prevalence of anal HPV infection is associated with a history of anal sex and the number of lifetime and recent sexual partners [95, 98, 99]. However, a history of receptive anal intercourse is not necessary for anal HPV infection. The incidence of anal dysplasia among homosexual men with anal HPV infection is equally high or higher than the incidence of cervical dysplasia among women with a high-risk cervical infection. The 4-year cumulative incidence of high-grade anal dysplasia was 17% among HIV-negative and 49% among HIV-positive homosexual men [100]. As for cervical infection, the risk of anal dysplasia is associated with smoking and severity of HIV-related immunosuppression as measured by CD4 cell count [99, 101].

Oral Human Papillomavirus Infection Prevalence in Men and Women

Very little is known about the prevalence of oral HPV infection or risk factors for infection. The majority of prevalence estimates are derived from control populations in case-control studies that have investigated associations between HPV and oral cancers. In these studies, the prevalence of high-risk HPV infection in adult populations ranges from 1.5 to 14% [102–109]. As is the case for anal HPV infection, the oral HPV infection prevalence is 3-fold greater among women with a cervical HPV infection [110]. In a cross-sectional study in which oral and cervical samples were collected from HIV-infected and -uninfected women, the HPV type distribution was significantly different at the 2 sites [110].

In contrast to cervical and anal HPV infection, nonsexual transmission of HPV to the oral cavity is well documented and associated with clinical disease. Nonsexual vertical transmission of HPV infection to the oral cavity of newborns has been documented [111] and is strongly associated with the risk of juvenile-onset respiratory papillomatosis [112]. However, the majority of studies indicate that peripartum transmission of oral HPV infection is infrequent (0–2%) [113–116]. In a recently reported study of approximately 1,000 children aged 2 weeks to 20 years, a bimodal age distribution for oral HPV infection was observed, consistent with peripartum transmission followed by gradual acquisition later in childhood. Among 16- to 20-year-olds, the oral HPV infection prevalence was approximately 3% and was associated with female gender, a history of genital warts and current smoking [117]. Factors associated with

elevated odds of prevalent oral HPV infection in adults to date have included increasing age, male gender, HIV infection, immunosuppressive medical therapy, the presence of a cervical HPV infection (in women), a history of sexually transmitted disease and the number of oral sex partners [102, 107, 110, 118, 119].

There are no published natural history studies of oral HPV infection, and therefore the risk factors for incident and persistent infection are unknown.

Cancer Burden Attributable to Human Papillomavirus Infection

In a recently published monograph summarizing the evidence for the carcinogenicity of HPV in human subjects, the International Agency for Research on Cancer concluded that there is sufficient evidence for an oncogenic role for HPV in cervical, vulvar, vaginal, penile, anal and oral cancers in humans [7]. In contrast to cervical cancers, where HPV is necessary for cancer development, for all other cancers now accepted as causally related to HPV, HPV is associated with a distinct subset of cancers at each site [120]. Data in support of a causal association at each site include consistent detection of high-risk HPV in tumors, elevated odds of cancer in association with detectable HPV infection and sexual behavior in case-control studies, and seroepidemiological associations with risk of cancer in nested case-control studies (reviewed in [7]). Among noncervical sites, prospective cohort studies in which HPV infection has been observed to precede the development of high-grade dysplasia have been reported only for anal dysplasia.

Approximately 5.2% of the incident cancers worldwide each year are attributable to HPV infection, accounting for approximately 561,100 cases in 2002 [121]. The burden is substantially higher in undeveloped than developed countries and higher among women than men [121]. Among men, there were approximately 33,900 cases of penile, anal and oral cancers attributable to HPV worldwide in 2002, as compared to 527,200 cases of cervical, vulvar vaginal, anal and oral cancers among women. HPV-16 is the HPV type most frequently detected in human cancers, regardless of the anatomic site of development of the cancer.

Among the estimated 561,000 cancers worldwide each year that are caused by HPV infection, 72% or a total of 402,900 cases are attributable to HPV-16 and -18 infection. Among these, 64% or a total of 257,700 cases occur among women at anatomic sites where a recently developed prophylactic vaccine has demonstrated high efficacy in the prevention of high-grade dysplasias. The discovery that the L1 major viral capsid protein of HPV can self-assemble into empty virus-like particles in in vitro systems led to a subsequent phase I trial of HPV-16 virus-like particles in human subjects [122]. The vaccine induced high titer serum neutralizing antibodies and was subsequently demonstrated to have 100% efficacy for the prevention of an incident and persistent HPV-16 infection [123]. Randomized, placebo controlled trials of bivalent (for HPV types 16 and 18) and quadrivalent (for HPV types 6, 11, 16

and 18) HPV virus-like particle vaccines have now demonstrated 90–100% efficacy in the prevention of high-grade cervical, vulvar and vaginal dysplasias caused by HPV-16 and -18 [124, 125].

Whether the vaccines will be similarly effective in preventing anal, oral, and penile infections and cancers, is currently unknown. However, it is clear that the vaccines will significantly alter the molecular epidemiology of cervical HPV infection in vaccinated populations. The vaccines have been demonstrated to prevent incident cervical infections and dysplasias by HPV-16 and -18 and to some extend those caused by the HPV types most closely related to HPV-16 and -18 [126]. There is no evidence for clustering by type among prevalent HPV infections [127]. Additionally, although concurrent acquisition of multiple HPV type infections occurs more frequently than expected by chance [128], there is little evidence for clustering by HPV type among concurrently or sequentially acquired HPV infections [128], with the exception that reported incident infections by HPV-58 may be more frequent among women with a preceding incident infection by HPV-16 or -18 [129]. There is also little evidence for interaction among HPV types with respect to clearance of infections in multiply infected women [130, 131]. Given infections by HPV-16, and to some extent also HPV-18, appear biologically unique with regard to the risk of development of cervical dysplasia, other high-risk HPV types do not seem to be biologically capable of filling the niche currently occupied by HPV-16 and -18.

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Molecular Pathogenesis of the Human Papillomavirus: Cervical Cancer as a Model

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Abstract

Infection of the cervical epithelium by human papillomavirus (HPV) is an essential event in the development of cervical cancer, but it is not sufficient. The immune status of the host, type of cell infected and expression of viral-encoded oncogenes are all important factors that determine whether HPV causes relatively mild productive viral infection or invasive carcinoma. In this chapter, the molecular interactions between HPV and the infected host cell upon viral entry are discussed, both in the development of productive viral infection and cervical cancer. The hallmarks of cervical cancer cells – as observed in cell culture and in the clinic – are then described to develop a model of cervical cancer progression. Finally, we explore how the information garnered from studies of HPV pathogenesis may be translated into the clinical arena in the form of new diagnostic, preventive and therapeutic tools.

Human Papillomavirus as an Essential Causative Agent of Cervical Cancer

The family of human papillomaviruses (HPVs) is sizable and diverse. It contains over 100 distinct types of the virus, distinguished by their DNA sequence [1]. HPVs are commonly classified into genera based on their evolutionary origin, with the α- and β-papillomaviruses comprising the vast majority (about 90%) of currently identified HPV types. These viruses cause lesions at different epithelial sites.

β-Papillomaviruses are both less common and not as likely to cause human disease compared to the α-genera. β-Papillomaviruses cause cutaneous infections which are typically clinically inapparent except in immunocompromised patients [2, 3]. Certain α-papillomaviruses (e.g. HPV-2) are also cutaneous viruses and as such do not represent a great health threat to the patient [4]. However, many α-papillomaviruses infect the genital epithelium and represent the most common sexually transmitted infectious agents. While most α-papillomavirus infections are benign, some (e.g. HPV-16) are known to inflict lesions in the cervical epithelium that can progress to cancer. These genital tropic cancer-associated HPVs are classified as high-risk. In fact, it is well established that

high-risk HPV is a necessary, but not sufficient, causative agent of cervical cancer. Viral DNA from these high-risk types is almost universally detectable in cervical cancers, including in over 99.7% of the cervical squamous cell carcinomas (SCCs) [5, 6] as well as 95% of the cervical adenocarcinomas and adenosquamous carcinomas [7, 8].

HPV-16 and HPV-18 are clearly the most prevalent high-risk HPV types found in cervical cancer [6]. The genomes of these viruses encode the oncoproteins E6 and E7, which critically mediate the induction and maintenance of transformation of cervical cells. The loss of these oncoproteins triggers apoptosis or senescence of cervical cancer cells. Importantly, E6 associates with and promotes the degradation of the tumor suppressor protein p53 [9], while E7 inhibits the activity of the retinoblastoma protein (Rb) [10]. The decrease in levels of p53 renders cells incapable of undergoing growth arrest or apoptosis in response to DNA damage, and the loss of Rb abolishes the cell cycle checkpoint at G_1. Thus, in combination these events create a cellular environment that promotes the development of a cancerous phenotype.

Of the various histological classifications of cervical cancer, SCC is the most widespread and develops from precursor squamous intraepithelial lesions (SILs), also referred to interchangeably as cervical intraepithelial neoplasias (CINs). As shown in figure 1, the severity of the lesion is determined histologically according to the degree of abnormality of the epithelial cells that extend from the parabasal region of the squamous epithelium towards the surface. Low-grade SIL (also CIN-1) indicates a status of mild dysplasia. Of the high-grade SILs (i.e. CIN-2, CIN-3), CIN-2 correlates with moderate dysplasia and CIN-3 with severe dysplasia or, in more extreme cases, carcinoma in situ [11].

Development of Squamous Intraepithelial Lesions from Human Papillomavirus Infection

Infection with HPV is a crucial event in the onset of cervical cancer but it is not sufficient. In fact, while many individuals contract HPV sometime during their lives [12], the vast majority of these infections are transient and never progress to a CIN stage [13]. The precise reasons for this still remain elusive; however, a variety of plausible explanations have recently emerged. First, the ability of the immune system to eliminate HPV from the host is likely a key determinant in the prevention of CIN formation. It has been found that certain alleles of the polymorphic human histocompatibility leukocyte antigen (HLA) class I and class II as well as the immunoglobulin-like receptor genes may favor protection against or susceptibility towards HPV infection [14, 15]. Indeed, HPV-positive individuals who possess the protective forms of HLA class II alleles consistently exhibit short-term viral persistence, decreased viral load and reduced incidence of development of SIL or carcinoma in situ [15, 16]. Likewise, HPV-associated malignancy is more commonly detected in immunocompromised patients.

A second, distinct possibility that may account for the failure of HPV to cause CIN lies in the type of host cell it infects. It is believed that viral persistence results when the

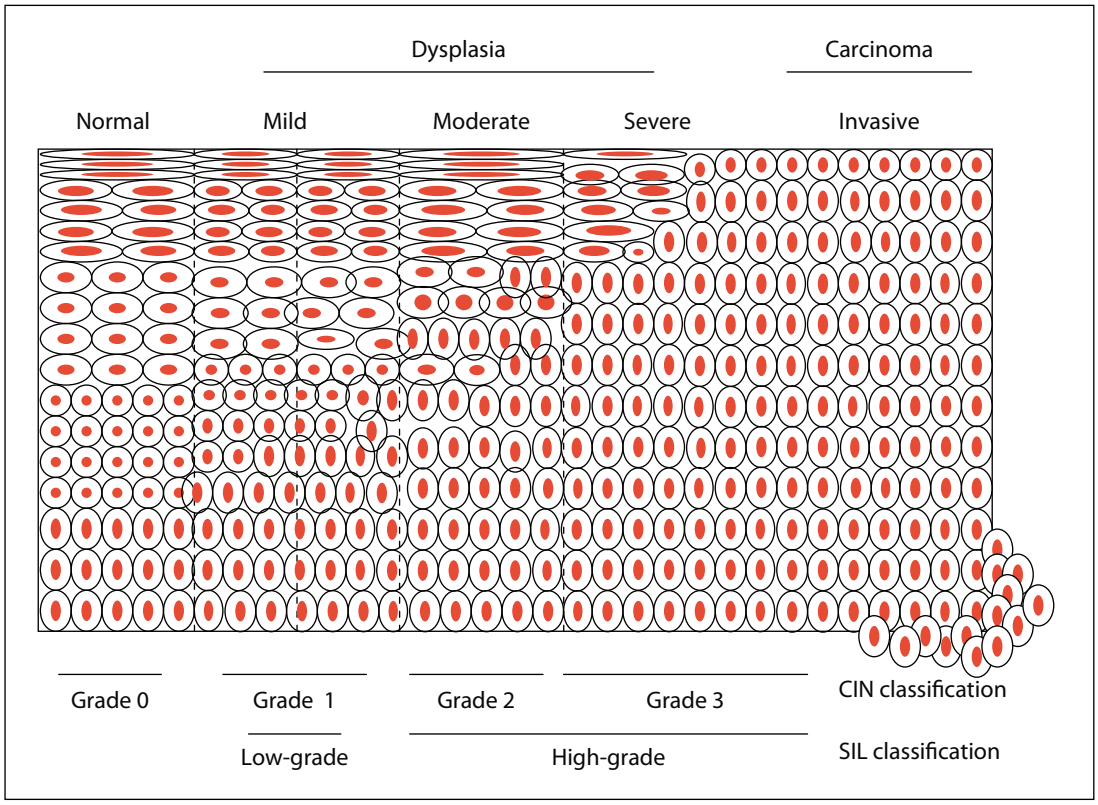

Fig. 1. The progression of CIN lesions following infection with HPV. Normal cells of the epidermis have a differentiated phenotype as they progress towards the surface, while those with a transformed morphology retain the morphology of basal cells. The correlation between the CIN and SIL classifications is also shown.

primary HPV infection originates from the basal proliferating cells of the squamous epithelium, possibly transit-amplifying or stem cells. By contrast, primary invasion of the more differentiated, surface cells of the epithelium is less favorable for viral maintenance and CIN progression because as these cells further differentiate, they are eventually shed from the host, leading to loss of viral DNA [11]. Therefore, in summary the transition from initial HPV entrance into the epithelium to the development of CIN is an improbable event that is potentially influenced by the host immune response to the virus as well as the differentiation status of the primary infected cells.

Do Squamous Intraepithelial Lesions Lead to Productive Viral Infection or Cancer?

When CIN does occur, the onset of cervical SCC is still exceedingly uncommon and depends predominately on the deregulation of viral gene expression. Indeed, 10–15

years are usually required for carcinoma to develop from a successful HPV infection [17, 18]; additionally, most low-grade as well as some high-grade SILs have often been reported to spontaneously regress [19]. Taken together, these observations suggest that several sequential steps are involved in the gradual progression from CIN to SCC. However, it is controversial as to whether high-grade CIN develops from low-grade CIN or high-grade develops de novo from a high-risk infection.

The currently accepted model for HPV-associated pathogenesis is that CIN-2 and CIN-3 lesions may appear within 2–3 years following infection [20] but need over 10 years to develop into overt carcinoma [17, 18]. This theory has led to the classification of HPV-induced diseases as either productive viral infections (which include low-grade SILs and do not progress to neoplasia) or cancerous precursors (which include high-grade SILs). Figure 2 illustrates the program of HPV gene expression or its deregulation during a productive viral infection or a cancerous precursor lesion. Support for this classification is provided by reports showing that low-grade lesions (i.e. CIN-1 and in some cases CIN-2) often contain low-risk HPVs which have negligible likelihood of causing cervical cancer [21]. Even when these lesions are found to harbor the high-risk HPV types, viral gene expression analysis frequently indicates a status of productive viral infection as opposed to cancerous precursors [22, 23].

Thus, the standard pathway of HPV pathogenesis (i.e. productive viral infection) may be seen as one in which the virus productively amplifies itself within the host but does not induce transformation of the cells it infects. Development of cancer represents a nonproductive aberration that kills the host from the viruses' perspective. The HPV first enters the basal cells of the epithelium through endocytosis [24], by recognizing and binding to heparan sulfate proteoglycans [25]. Viral particles dissociate in the late endosomal or lysosomal compartments, and the HPV DNA is transported to the nucleus in a process assisted by the minor capsid protein L2 [24]. After infecting the basal cells in this way, the HPV proceeds to establish stable episomes by expressing the viral replication proteins E1 and E2, which are required for genomic amplification. E2 is a DNA-binding protein specific for a palindromic region occurring at multiple distinct sites in the noncoding region of the HPV genome; the binding of E2 to this genome is necessary for the recruitment of the E1 helicase at the viral origin of replication. The E1 protein then associates with other molecules involved in DNA replication, such as replication protein A and DNA polymerase α-primase [26–29]. Through this process, amplification of the viral genome is tightly coupled to DNA replication during the S phase of the cell cycle. E2 causes the genome to associate with mitotic chromosomes such that copies of the HPV genome are equally divided among the 2 daughter cells when mitosis occurs.

In most productive HPV infections, cells above the basal layer are coerced into a state of increased proliferation in order to support further production of HPV virions. Typically, these suprabasal cells exit the cell cycle and begin the process of terminal differentiation to form the protective barrier of the skin. However, in

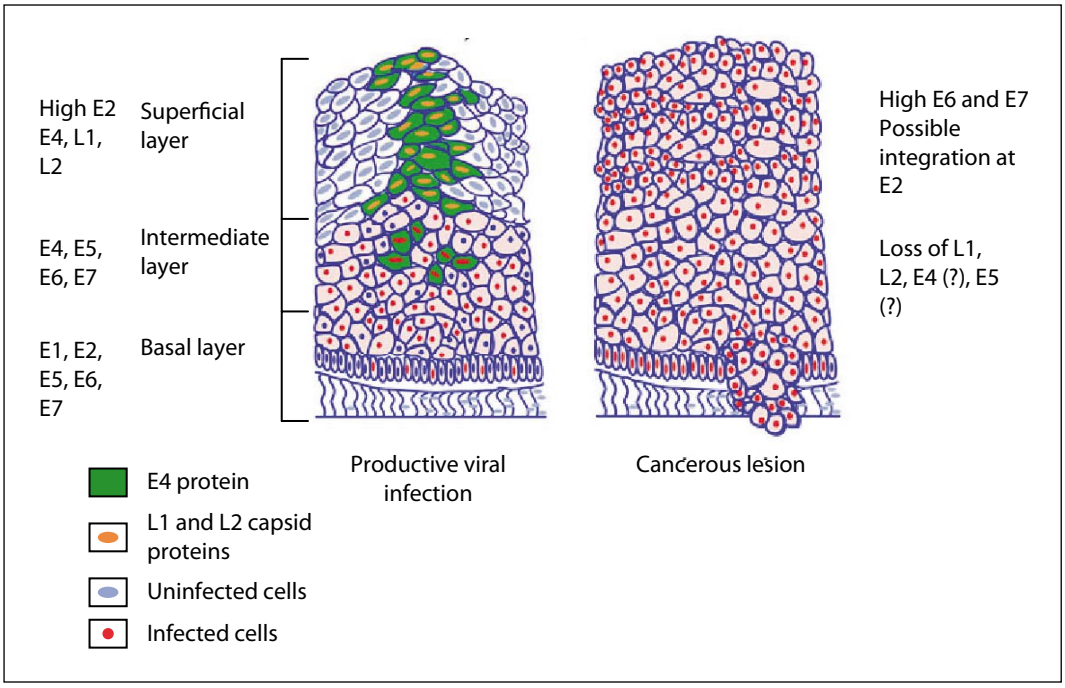

Fig. 2. HPV gene expression in HPV-mediated productive viral infection or cervical carcinogenesis. The HPV gene expression in the different epithelial cell layers is depicted in the case of productive viral infection or in cancerous lesions. In most cases of cervical cancer, the HPV genome integrates into the host chromosomal DNA and leads to the disruption of the viral E2 gene. Since E2 is a transcriptional repressor of E6 and E7, loss of E2 leads to upregulation of E6 and E7 genes. The uncontrolled expression of E6 and E7 proteins disrupts normal cell cycle regulation by interacting with p53 and Rb, thereby prolonging the cell cycle and suppressing apoptosis, contributing to the progression of HPV-associated cervical cancer.

HPV-infected cells, such a differentiation program is suppressed in part due to expression of the viral oncoproteins E5, E6 and E7.

The E5 protein is primarily present on the endoplasmic reticulum of the infected cell but can also associate with and disrupt the function of ATPase-driven proton pumps on the endosome membrane, delaying the process of endosomal acidification [30, 31]. This enhances the recycling of internalized receptors, such as the epidermal growth factor receptor to the cell surface, which enables prolonged signaling through the epidermal growth factor receptor and helps maintain an environment favorable to cellular proliferation [32].

The HPV oncoproteins E6 and E7 play important roles in the course of productive viral infection by promoting cell division. They are also necessary and sufficient for the induction and maintenance of a transformed phenotype in cervical cells. Here we consider the molecular mechanisms by which these oncoproteins promote cell division, both in the case of productive viral infection and cervical carcinogenesis.

Through interaction with and subsequent degradation of Rb activity, E7 allows differentiated cells to progress to the S phase of the cell cycle [33]. The removal of Rb liberates the transcription factor E2F, which stimulates progression through the S phase of the cell cycle. E7 also enhances the expression of the S phase cyclins A and E [34], and inactivates the cyclin-dependent kinase (CDK) inhibitors p21 and p27 [35–37]. In addition, recent studies have shown that through inhibition of Rb, E7 also causes upregulation of the serine/threonine kinase AKT, which has an important antiapoptotic function [38]. Despite these growth- and survival-promoting activities of E7, the accumulation of E2F may trigger apoptotic pathways which ultimately result in cell death. Therefore, E7 may be considered to exert both prosurvival and apoptotic effects on the infected cell, and it is presently unclear which is the dominant effect. E7 alone is capable of inducing a transformed phenotype in cells but does so with diminished efficacy.

The E6 oncoprotein binds to the ubiquitin ligase E6-associated protein (E6-AP), and the E6/E6-AP complex then specifically ubiquitinates the tumor suppressor protein p53, targeting it for proteosomal degradation. E6 also interacts with the proapoptotic protein Bak and stimulates its degradation. Together, the downregulation of p53 and Bak by E6 mediates progression through the cell cycle, resistance to apoptosis and chromosomal instability [39, 40]. In addition, E6 activates telomerase and may inhibit proteolysis of the SRC family of kinases, enabling them to further stimulate cellular division [41]. However, the transforming properties of E6 are counteracted by p16, a protein inhibitor of cyclin D1- CDK complexes, which blocks the expression of cyclin E and prevents the progression into the S phase of the cell cycle. Thus, like E7, E6 also has a low transformation capacity when expressed by itself in cells.

The functions of the HPV oncoproteins E6 and E7 complement one another in an interesting fashion to induce efficient transformation of normal cells. While the growth-promoting activities of E6 are blocked by p16, the presence of E7 bypasses this obstacle by directly stimulating the expression of cyclins E and A. The cell is therefore driven through the cell cycle without much resistance. Furthermore, although E7-mediated activation of E2F may contribute to the onset of apoptotic signals in the cell, E6 causes degradation of the apoptosis-promoting proteins p53 and Bak, thereby disrupting the transmission of these signals to downstream effector molecules [34, 42]. Thus, the proliferative activities of E6 and E7 synergize to cause cervical carcinogenesis. These molecules behave identically in productive viral infection; however, in these cases, E6 and E7 are invariably downregulated as the disease progresses.

Indeed, as infected cells finally terminally differentiate in the course of productive viral infection, the expression of E6/E7 is effectively repressed by the E2 molecule as that of the capsid proteins L1/L2 is induced. It is believed that E2 behaves as a transcriptional activator when present at low levels in the cell [43]; but as the amount of E2 increases, binding of this protein to HPV DNA causes displacement of transcription factors (i.e. Sp1, TATA-binding protein) which are essential for promoter activation, thereby inhibiting the expression of the oncoproteins E6 and E7 [44]. By this

carefully regulated, differentiation-dependent mechanism, HPV is able to replicate continuously within the host until it reaches the terminally differentiated cells of the squamous epithelium, at which point the viral genome is encapsidated and newly assembled virions are released as these cells are shed from the skin.

The process of HPV departure from the infected cells is dependent on the protein E4 and subsequently mediated by the capsid molecules L1 and L2. Although the precise role of E4 is presently unclear, this protein appears to induce cell cycle arrest at G_2 – thereby counteracting the effects of E6/E7 – as noted by an increase in the cytoplasmic concentrations of cyclin B/Cdk2, potentially facilitating overreplication of the viral genome [45, 46]. The L2 protein contains nuclear localization signals and associates with HPV DNA at the promyelocytic leukemia bodies in the nucleus, where it efficiently packs the viral genome into preassembled pentavalent L1 capsomeres with the assistance of the chaperone protein Hsp70 [47]. In the final stage of the productive viral infection, the HPV is released from the host through a process likely mediated by E4, which can disrupt the cytokeratin of the cell, thereby facilitating viral escape [48].

The above described pathway of HPV pathogenesis (i.e. productive viral infection), is characteristic of CIN-1 diseases which do not progress to cancer. By contrast, cases of HPV infection which result in CIN-2 and CIN-3 lesions (i.e. cancerous precursors) are most distinguished by, and likely a result of, a dramatic, uncontrolled increase in E6/E7 expression [22, 23]. The exact molecular mechanisms accounting for deregulated E6/E7 production are not completely understood; nonetheless, a few interesting concepts have been proposed. Experiments with epithelial raft cultures have pointed towards aberrant histone deacetylation as a potential factor underlying this change [49]. Furthermore, CIN-3 disease is frequently accompanied by integration of the HPV genome into that of the infected host cell [50], an event which could disrupt the regulation of E6/E7 expression. For instance, the viral protein E2 is an important transcriptional repressor of E6/E7, and loss of E2 following integration could lead to unchecked synthesis of E6/E7 [51]. An additional consequence of HPV integration, and E6/E7 activity, may be genomic instability of the infected cells or centrosome duplication (for a review, see [52]). Indeed, HPV-mediated cellular transformation is usually associated with significant changes in the host cell gene expression profile, with the molecules involved in carcinogenesis being upregulated, while those that suppress tumor formation are silenced. We devote the next few sections to examining some of these molecules and their major functions as they have been revealed to molecular pathologists through in vitro models and subsequently validated in the clinical arena.

In vitro Models as Valuable Tools for Studying the Pathogenesis of Human Papillomavirus

The development of in vitro systems for studying HPV infection has shed some light on the properties of HPV-transformed cells and their molecular abnormalities. For

example, the ability of high-risk HPV types to transform epithelial cells was discovered by findings which demonstrated that human primary keratinocytes could be immortalized by the expression of E6 and E7 [53–56]. Furthermore, culturing these cells for extended periods of time resulted in the emergence of tumorigenic clones [57, 58]. These and similar studies using in vitro models have identified 4 principal characteristics of HPV-transformed epithelial cells: prolonged lifespan, immortalization, anchorage-independent proliferation and tumorigenicity. The introduction of antisense RNA targeting E6/E7, or the indirect repression of these oncoproteins through ectopic expression of E2, in HPV-infected cell lines caused the cells to undergo growth arrest and show signs of senescence, indicating that E6 and E7 are necessary for a prolonged lifespan [55, 59]. The causes of immortalization, anchorage-independent proliferation and tumorigenicity have been investigated with somatic cell fusion experiments, which showed that all of these characteristics are recessive and arise from deregulation of tumor suppressor pathways [57, 58]. Also, for all of these properties, in vitro complementation assays have been performed, and the chromosomal changes likely responsible for each one have been proposed.

Remarkably, these changes correlate closely with those observed in cervical cancer samples derived from human patients, hinting that a conserved set of genetic alterations underlie both in vitro HPV-induced cellular transformation and in vivo cervical carcinogenesis [57, 58]. These interesting findings allow for the creation of a molecular model of cervical cancer progression based on in vitro data. Below we focus on 3 of the main properties of HPV-transformed cell lines – immortalization, anchorage-independent proliferation and tumorigencity – and explain how each represents a clinically important feature of cervical carcinogenesis. This discussion is summarized in table 1.

Immortalization

Human keratinocytes typically divide 50–100 times before transitioning into a senescent phase. Although HPV-infected cells with deregulated E6/E7 expression – and hence reduced p53 and Rb activity – are able to resist entry into senescence [39, 60], these cells are often observed to undergo crisis, during which the majority of cells die and immortal clones emerge at low frequency. Passage through crisis and the attainment of an immortal status are likely attributable to the activation of the telomerase reverse transcriptase enzyme [61, 62]. Telomerase appends 6 base pair repeats to the telomeric ends of chromosomes, which progressively shorten during DNA replication and thus provide an intrinsic checkpoint to the proliferative potential of cells [63]. Telomerase activity, which is absent in most normal cells but strongly induced in cancer cells, depends on the expression level of human telomerase reverse transcriptase (hTERT), the catalytic subunit of this enzyme [64]. The importance of hTERT in the immortalization of high-risk HPV-infected cells is illustrated by experiments in which this protein was ectopically expressed in HPV-16- and HPV-18-containing epithelial cells before they underwent crisis [65]. These cells were largely immune to telomere erosion as well as the apoptotic effects of crisis.

Table 1. Evidence for the 3 most commonly observed characteristics in cervical cancer both in the laboratory and in the clinic

Source of evidence	Immortalization	Anchorage-independent proliferation	Tumorigenicity
In vitro and preclinical data	ectopic expression of hTERT immortalizes HPV-trasformed cells; transfer of portions of chromosomes 2, 3, 4, 6 and 10 into HPV-infected cells causes growth arrest and apoptosis	absence of TSLC1 in epithelial cells eliminates their adhesive potential	treatment of cervical cancer cells with chromosome 11 or TSLC1 abrogates their tumorigenicity in nude mice; ectopic expression of c-Fos confers tumorigenic properties on cervical cells
Clinical specimens	elevated hTERT expression and telomerase activity are detected; deletions are observed at chromosomes 3, 4 and 10 in cervical carcinomas and high-grade SILs		deletions on chromosome 11 are observed; TSLC1 is repressed in ~50% of the human cervical cancer cases; c-Fos upregulation is reported in cervical carcinomas

hTERT = Human telomerase reverse transcriptase; TSLC1 = tumor suppressor in lung cancer 1.

While studies have shown that hTERT production may be induced by E6 [66], the susceptibility to crisis of high-risk HPV-infected cells (without ectopic introduction of hTERT) suggests that other cellular changes are necessary for telomerase activation [61, 65, 67]. Several groups have conducted experiments in which regions of human chromosomes 2, 3, 4 and 6 were transferred into HPV-immortalized cells, leading to growth arrest and characteristics resembling crisis [65, 68–71]. These findings suggest that some host cell genes have the potential to suppress telomerase activation and that the loss of these genes may facilitate, in conjunction with E6, the immortalization of high-risk HPV-infected cells. Later reports identified portions of chromosomes 3, 4 and 6 to directly exert a suppressive effect on telomerase activity [65, 68]. For instance, ectopic expression of hTERT counteracted the growth arrest mediated by chromosome 6 in HPV-16+ cells, showing that parts of this chromosome interfere with telomerase function.

These in vitro data correlate strongly with information derived from clinical specimens. Interestingly, elevated hTERT expression and correspondingly enhanced telom-

erase activity have been documented in nearly all cervical SCCs and in about 40% of the CIN-3 lesions but not in CIN-1 and CIN-2 disease [72]. Furthermore, in the considerable population of cervical carcinomas and CIN-3 which displayed increased telomerase activity, allelic imbalances were widely observed on chromosome 6 [73], providing convincing evidence that a telomerase suppressor lies on this chromosome. Also in line with the in vitro experiments, deletions at chromosomes 3, 4 and 10 are commonly found in cervical carcinomas and in high-grade SILs [69, 70, 73]. Although additional studies are necessary to pinpoint on these chromosomes the specific genes responsible for their presumed antiproliferative function, it is clear that they play important roles in repressing cellular immortalization, both in vitro and in vivo.

Tumorigenicity and Anchorage-Independent Proliferation

Studies conducted by Koi et al. [74] have suggested that the tumorigenicity of cervical cancer cells is dependent on loss or suppression of certain genes at chromosome 11, as treatment of the cells with this chromosome rendered them incapable of establishing tumors in nude mice. These results are verified by clinical observations of chromosome 11 deletions in a variety of cervical carcinomas [75]. Further exploration of this phenomenon has recently revealed that the tumor suppressor in lung cancer 1 (TSLC1) gene in particular may be lost in cervical cancer cells [76], which confers the properties of both tumorigenicity and anchorage-independent proliferation on these cells. The TSLC1 gene encodes an immunoglobulin-like cell surface protein which helps mediate cell-cell adhesion by homotypic or heterotypic interactions [77]. As one would expect, the lack of TSLC1 in epithelial cells eliminates their adhesive potential and contributes to their anchorage-independent growth. In addition, TSLC1 associates with class-I-restricted T-cell-associated molecule – a receptor protein expressed on activated CD8+ T cells and natural killer cells – and can thereby promote an anticancer immune response [78]. Thus, the loss of this molecule in cervical cancer cells is likely to increase their tumorigenicity by facilitating a state of immunological privilege.

The importance of TSLC1 in HPV-mediated cervical cancer progression is supported by several clinical studies. For example, it has been shown that this gene is repressed in approximately 90% of the cervical cancer cell lines due to allelic loss or promoter hypermethylation [76]. Similar results were noted in about 60% of the cervical carcinomas and 40% of the high-grade SILs but not in low-grade SILs. Additionally, ectopic TSLC1 expression protected against tumor formation in nude mice and abolished anchorage-independent proliferation of cervical cancer cell lines [76]. Altogether, these findings suggest that loss of TSLC1 occurs during cervical carcinogenesis, disrupting cell-cell contacts and potentially facilitating tumor immune escape. However, it is unlikely that changes in the expression of this one gene alone are sufficient for inducing tumorigenicity in cervical cancer cells.

In fact, it has been found that alterations in the composition of the AP-1 complex are also important for tumorigenicity [79–81]. The AP-1 transcription factor, which

consists of the subunits c-Jun, c-Fos or Fra-1 associated as homo- or heterodimers, regulates multiple cellular pathways, including differentiation and proliferation. In normal cells, AP-1 exists predominately as Jun/Fra-1 complexes, but in cervical cancer cells, c-Fos is constitutively expressed (with concomitant reduction in Fra-1 levels), resulting in a significant shift towards Jun/c-Fos [79, 80]. It is probable that this change affects the ability of transformed cells to form tumors in vivo, since ectopic expression of c-Fos in nontumorigenic cell lines drove the cells towards a tumorigenic phenotype [79]. Reports of upregulation of c-Fos in human cervical carcinomas [82] also support a role for abnormal AP-1 composition in HPV-mediated cervical carcinogenesis.

Furthermore, gain of chromosomal segment 3q is frequently observed in cases of cervical carcinoma and oftentimes marks the shift from dysplasia to invasive cancer [83]. Although the specific genes present on 3q which contribute to tumorigenesis are currently unknown, it is likely that this region of the chromosome contains 1 or more oncogenes.

An Emergent Model of Cervical Cancer Progression

There is a striking consensus between the information about HPV pathogenesis derived from in vitro data and from clinical samples. Thus, a model of cervical carcinogenesis can be generated to account for the genetic changes that occur in the host which favor cellular transformation from the time of HPV entry to the onset of overt carcinoma. First, the productivity of infection is critically determined by the ability of the host immune system to clear the virus. Once a CIN lesion is established, deregulated expression of the oncoproteins E6/E7 represents the primary mechanism which pressures cells towards a transformed state. At this point, developing cervical carcinomas tend to downregulate MHC class I molecules [84, 85], an event which is closely tied to loss of portions on chromosome 6 [86–88]. This phenomenon provides a likely strategy for the HPV-infected cell to evade immunological surveillance and further selects for the most aggressive, tumorigenic cells. Finally, after a CIN-3 lesion has developed, subsequent genetic alterations give rise to carcinoma in situ, as the cancerous cells become immortalized, lose their anchorage dependence and begin to invade through the basement membrane.

Human Papillomavirus in the Clinical Arena and Concluding Remarks

The great wealth of knowledge regarding HPV pathogenesis garnered over the past years from in vitro experiments as well as clinical specimens has unleashed numerous opportunities for the effective screening, diagnosis and treatment of cervical cancer.

One especially promising approach, which may help reduce the incidence of false-negative results derived from presently employed screening techniques (i.e. cytological analysis of cervical smears), is to assay for the presence of viral markers (e.g. HPV genomic material, viral oncoproteins) in human tissue samples. High-risk HPV DNA can be efficiently detected by hybridization or by polymerase chain reaction. Furthermore, the persistence of this DNA could indicate with reasonable conclusiveness whether certain histological abnormalities pose a significant risk for developing into cervical carcinoma, or whether they will remain at the low-grade SIL stage and then spontaneously regress [89, 90]. These examinations may be confirmed by analysis of E6/E7 mRNA amounts [91–93] or by amplification of host-viral chimeric transcripts to detect viral integration [50]; positive readings for either of these factors would imply that the individual is at high risk of developing cervical cancer and should receive appropriate therapeutic interventions.

An attractive approach to the diagnosis of HPV-mediated carcinogenesis is the identification of host cell markers which are deregulated in cervical cancer. For instance, the cell cycle regulatory protein $p16^{INK4A}$ is induced by inactivation of Rb. Therefore, the presence of E7, a suppressor of Rb, would lead to accumulation of $p16^{INK4A}$, which could be detected by immunohistochemical staining of tissue samples [94, 95]. Furthermore, similar assays could be used to determine hTERT expression and telomerase activity [96–98]; and the repression of TSLC1 could be studied by promoter hypermethylation assays [76]. Taken together, these tests should reveal whether cervical cells from the HPV-infected patient show the characteristic molecular patterns of carcinogenesis (i.e. immortalization, tumorigenicity, anchorage-independent proliferation); and if so, the patient would be treated accordingly. It is finally worthy of note that the immense body of data collected about the molecular biology of HPV pathogenesis could also be translated into novel therapeutic remedies for cervical cancer, such as drugs targeting HPV proteins.

The requirement of the oncoproteins E6 and E7 for the maintenance of a transformed phenotype in tumor cells provides an excellent opportunity to develop therapeutic interventions which suppress these proteins or their functions. For example, the use of a molecular inhibitor to block E6/E6-AP interactions prevented p53 degradation mediated by E6-AP and restored the levels of this tumor suppressor in cervical cancer cells [99]. Furthermore, recent in vitro experiments have shown that posttranscriptional silencing of E6 and E7 by small interfering RNA inhibits the growth of and induces death in HPV-18+ cervical cancer cells [100]. Additionally, due to the universal expression of virally encoded E6 and E7 in tumor cells, but not at all in normal cells, these oncoproteins represent ideal tumor-specific antigens that can be targeted by therapeutic cancer vaccines. Numerous types of vaccines have been developed using this principle, and several have achieved considerable success in preclinical models (for a review, see [101]). Similarly, the viral capsid proteins L1 and L2 serve as excellent targets for prophylactic HPV vaccines, some of which have achieved great success in human patients (for a review, see [102]). Taken together, these research

programs, combined with concurrent efforts to generate improved screening, diagnostic and therapeutic methods create strong optimism that soon the number of people worldwide suffering from cervical cancer and other HPV-induced diseases will be on the decline.

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Human Papillomavirus Vaccines

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Abstract

Persistent high-risk type human papillomavirus (HPV) infection has been identified as a necessary, albeit not sufficient, cause of cervical cancer and it is responsible for ~5.2% of all cancer deaths worldwide. Since there are currently no HPV-specific therapies, effective vaccination against HPV and ablation of for precursor lesions (high grade cervical intraepithelial neoplasia) are used for the control of cervical cancer. The newly licensed preventive HPV vaccines have a remarkable safety profile and clinical efficacy against the 2 most common high-risk HPV genotypes, HPV-16 and HPV-18. However, these vaccines will only protect against up to 70–80% of cervical cancer cases and also lack therapeutic effect against established HPV infection and HPV-associated lesions. Thus, the future focus should be on the development of a new generation of preventive and therapeutic vaccines that are capable of protecting against all oncogenic HPV types and eliminating pre-existing disease.

Human papillomavirus (HPV) infection is responsible for 1 in 20 of all cancers worldwide [1]. It is now known that persistent infection with HPV is the primary factor in the development of cervical cancer [2]. HPV is one of the most common sexually transmitted diseases in the world and HPV DNA has been detected in 99.7% of the cervical cancers [2]. More than 200 HPV genotypes have been identified and are classified as cutaneous or genital types based upon their tropism. Further, the genital types are divided into low- or high-risk types (also called benign and oncogenic respectively), depending on their propensity to cause cervical cancer [3]. Of the ~15 known high-risk types, HPV-16 and HPV-18 are the 2 most commonly detected in cervical cancer. Worldwide estimates suggest that HPV-16 and HPV-18 cause ~50 and ~20% of cervical cancers respectively. High-risk HPV types cause squamous intraepithelial lesions, also known as cervical intraepithelial neoplasia (CIN), the precursor lesions of cervical cancer (for review see [4]).

HPV has a circular, double-stranded DNA genome made up of ~8,000 base pairs that is maintained as an episome (i.e. not integrated) at ~100 copies/nucleus. HPV has a 2-stage transcriptional program producing the early and late proteins. The early proteins

are involved in the regulation of viral DNA replication (E1, E2), viral transcription (E2), cytoskeleton reorganization and G_2 arrest (E4) and driving the host cell into S phase to provide the cellular factors necessary for viral replication (E5, E6, E7). The late proteins, including L1 and L2, form the structural components of the viral capsid. The expression of viral proteins is tightly regulated and associated with the differentiation of infected epithelial cells. The early proteins are expressed throughout the life cycle, whereas late proteins and E4 are expressed only during terminal epithelial differentiation in the upper layers of the skin. E2 is the master regulator that modulates the expression of all the other viral genes via the upstream regulatory region of the genome that contains multiple binding sites. The viral oncogenes E6 and E7 are responsible for transformation. In almost all cases of cervical cancer, the HPV genome integrates into the host chromosomal DNA, leading to the disruption of the viral E2 gene. Since E2 is a transcriptional repressor of E6 and E7, loss of E2 leads to upregulation of E6 and E7 gene expression. The elevated expression of E6 and E7 proteins further disrupts normal cell cycle regulation by interacting with p53 and Rb respectively (amongst numerous other important interactions), thereby driving the cell cycle and suppressing apoptosis, and contributing to the progression of HPV-associated cervical cancer (for a review, see [5]). Indeed persistent expression of E6 and E7 is necessary to the viability of cervical cancer cells. E5 is a third viral oncogene that acts via the epidermal growth factor and platelet-derived growth factor receptors, but it is inconsistently expressed in cervical cancer and is not considered to be a major factor in transformation by HPV.

A clear understanding of the biology of HPV is integral to the development of vaccines against HPV. Vaccination represents a cost-effective approach to reduce the mortality of HPV. The clear association between HPV infection and cervical cancer indicates that HPV antigens are clear targets for the development of preventive and therapeutic vaccines. Vaccination could be implemented to prevent infection by generating neutralizing antibodies to block HPV viral infection (preventive vaccines) or to eliminate infection by inducing a virus-specific T-cell-mediated response (therapeutic vaccines), although the latter might also be used in a preventative context to eliminate new infections or precursor lesions prior to the onset of disease.

Human Papillomavirus Vaccines That Prevent Infection

Cervical cancer is potentially completely preventable through frequent cytologic screening and intervention. Complementary to this, vaccines that prevent HPV infection offer great promise in reducing the incidence of cervical cancer especially in resource-limited regions of the world where screening programs and Pap smears are not currently implemented [4]. A preventive vaccine typically acts by generating neutralizing antibodies to HPV infection. Both papillomavirus capsid proteins, L1 and L2, have great potential as prophylactic vaccine antigens. Indeed, vaccines based on L1 have recently been licensed for the prevention of HPV infection and the anogenital diseases it causes.

Mechanisms of Viral Neutralization by Preventive Vaccines

Vaccines have had a huge impact in preventing human diseases caused by many microorganisms, even eliminating some diseases. However, despite their efficacy, we do not have a precise understanding of how vaccines work. The possibilities range from the notion that antibodies are required primarily to control bacterial, rather than viral, infection and are dispensable for the control of some viral infections to the view that antibodies are the only identified agent of successful vaccine protection; the truth probably lies somewhere between these extremes [6, 7]. How do antibodies protect the host? The papillomavirus system provides an important model to study this deceptively simple question because the capsid proteins are not detectably expressed by the basal epithelial cells that harbor the viral infection but only by terminally differentiated epithelial cells about to slough off. Thus, cell-mediated immunity targeting the capsid antigens does not impact the outcome of infection, and antibodies are the primary mediators of protection.

Possible mechanisms by which antibodies neutralize infection include that viruses are neutralized extracellularly by the binding of one or a few antibody molecules [7], that conformational changes in envelope or capsid molecules are inactivating, or that viral inactivation by antibody can occur after entry to infected cells by, for example, blocking virus uncoating or altering receptor interactions and trafficking [8]. Clearly, their mechanisms may differ between infectious agents given their diverse receptors and mechanisms of cell entry, and multiple mechanisms may be used given the differing specificities and isotypes in a polyclonal antibody response. Burnet proposed the 'occupancy model' in 1937, in which neutralization occurs as a significant proportion of available epitopes on the virion are covered by antibody, thereby blocking the attachment of virus to host cells or interfering with the process of entry. A marked linear relationship between the surface area of a virus and the number of antibody molecules that are required to bind to the virus for neutralization supports this proposal. Burnet's model predicts that the neutralizing efficacy of an antibody should relate to its affinity for the virion. However, evidence from the vesicular stomatitis virus system indicates that only low threshold avidity is required for protection [9] although this has not yet been addressed for HPV. Furthermore, the relatively large size of the antibody molecule, approximately similar to that of a typical capsid unit, is proposed to be crucial [7]. Indeed, in a 3-dimensional reconstruction of the L1-specific neutralizing monoclonal antibody, mAb9 bound to bovine papillomavirus type 1 (BPV-1) shows the antibody completely coating the surface of the virion [10]. Consistent with this hypothesis, it has been demonstrated that mAb9 prevents binding of BPV-1 to the cell surface [11]. Nevertheless, it was shown that L1-specific antibody 5B6 neutralizes but does not block surface binding. Structural analysis reveals that this antibody does not cover the pentavalent capsomers on the 5-fold axes. Importantly, no evidence of structural changes in L1 was found in virions coated with antibody. Structural analysis demonstrates that the 5B6 antibody cross-links the capsomers and it has been shown that 14–72 antibodies per virion were necessary for

neutralization, suggesting that it blocks a conformational change needed for infection, or prevents virus uncoating or redirects the virus along a noninfectious entry pathway [11, 12]. Like 5B6, neutralizing antibodies to L2 do not prevent the virus from binding to the cell surface. L2 plays a critical role in virion trafficking in infection and numerous nonneutralizing L2 mAbs of the same isotype that bind to the virus surface have been identified, suggesting that Abs must bind to the appropriate locations on the capsid surface to effect neutralization.

Role of HPV Capsid Proteins in Infection and Neutralization

L1 is sufficient for initial particle binding to cells, since L1 virus-like particles (VLPs) bind to somatic cell monolayers in culture and also compete for infection by native virus [12]. Furthermore, neutralizing antiserum to L1 but not L2 inhibits binding of virions to cell monolayers [12] and L1 VLPs are sufficient to hemagglutinate mouse erythrocytes [13]. L1 VLPs of all papillomavirus types bound most somatic cells tested [12], except lymphoid cells [14], suggesting that the primary receptor is broadly expressed. The addition of HPV VLPs competed for both cell surface binding and infection by BPV-1 and vice versa, indicating that the papillomaviruses share a common primary cell surface receptor [11, 15, 16]. Furthermore, the cell surface receptor is evolutionarily conserved, since papillomavirus binds to and infects cells derived from many different species and origins [12]. Prior treatment of cells with trypsin reduced binding of virus to cells [15, 16] and erythrocytes [13], suggesting that the receptor is proteinaceous. Binding is mediated by polar interactions with a K_d of 84 pM to ~26,000 molecules/HeLa cell [15]. CV-1 cells express ~10,300 receptor molecules/cell with a K_d of 140 pM. Linear L1 bound CV-1 cells with a K_d of only 40 mM [14]. Monoclonal antibody GoH3, which binds to α_6 integrin, blocks binding of HPV-6 L1 VLPs to HaCaT and CV-1 cells [17]. VLP binding is also inhibited by laminin, the natural ligand of α_6 integrin, leading Evander et al. [17] to propose α_6 integrin as the primary receptor for HPV [18]. However, α_6 integrin is not an obligate receptor [19, 20]. Importantly, Culp et al. [21, 22] suggest that a molecule overlapping laminin-5 in the extracellular matrix may act as a transreceptor that represents a sink for the virus until contact with basal keratinocytes during wound healing. Interaction with the cell may occur via a C terminus of L1 containing a heparin-binding domain that mediates binding of VLPs to cell surface heperan sulfate glycosaminoglycans [23]. High-molecular-weight dextran sulfate competes strongly for VLP binding (IC_{50} of 12 nM for 500,000-Da dextran sulfate) and heparinase or heparitinase pretreatment of cell surfaces abolishes binding [23] and also infection [24]. Indeed, syndecan-1 was recently suggested as the primary receptor [25]. This story has been complicated by the observation that heparan sulfate is not required for HPV-31 infection [26] but is for HPV-16 and -33 infection, despite the close evolutionary relationship of these viruses [27]. Interestingly, Day et al. [28] recently observed that L1-specific neutralizing monoclonal antibody H16.U4 or soluble heparin cause HPV-16 virions to be

trapped on the extracellular matrix. In contrast, H16.V5 and H16.E70 permit transfer of the virions to the cell surface but block their uptake and entry into cells.

Although L1 VLPs bind to cell surfaces as for virion, L2 is also able to bind to cell surfaces via HPV-16 L2 residues 108–120 [29] or 13–31 [30]. Indeed, these regions of L2 bound to a wide variety of cells and competed with infection. Furthermore, residues 108–120 of L2 promoted the uptake of L2 and form a conserved neutralizing epitope [31] and residues 17–36 also represent a conserved protective epitope. L2 specific neutralizing antibodies do not inhibit virion binding to cell surfaces [12], suggesting that this N-terminal domain of L2 performs some significant post-surface binding function that constrains antigenic variation. This data is consistent with binding to a secondary viral receptor by L2 to facilitate the uptake of virion [32].

Polyclonal antisera to L2 completely neutralized BPV-1 infection, yet did not prevent virus from binding to cell surfaces [11, 13]. This is indicative of a role for L2 in infection only after the virus has bound the cell surface [11]. However, studies using pseudotype virions assembled in vitro suggest that L2 is dispensable for infection [33]. These data are very surprising, since they show that L2 has no role in the life cycle for papillomavirus assembly or infection. In contrast, we observe that L2 is absolutely required for infection using an in vivo system that produces virions morphologically and immunologically consistent with to those obtained from warts. We generated virions comprising L1 and mutant L2 that are rendered noninfectious by 9 residue deletions from either terminus of L2 [34]. We obtained a similar result when scrambling the sequence of the N-terminal 9 amino acids or deleting residues 91–129 [35]. Furthermore, knockout of L2 in the raft culture system also demonstrates the importance of L2 [36]. This emphasizes the significance of using high-quality virion preparations and the appropriate model to study papillomavirus biology.

The events after binding are also controversial. Virus may be neutralized as many as 4–8 h after addition to cells [10, 20, 37] and uncoating begins at ~6 h after infection [38]. This suggests that the virions may remain on the cell surface during this time. However, VLPs or virions added to cultured cells and followed by electron microscopy and immunofluorescent staining are taken up very rapidly [39] in an L2-independent manner, although it is not clear if this represents a true infectious pathway or a dead end [30]. Transport occurs via phagosomes (not clathrin-coated vesicles [40]), yet another study indicates the importance of clathrin-dependent pathways [41] and a third shows the importance of caveosomes for some HPVs [42]. The uptake is inhibited by cytochalasin B and taxol, suggesting involvement by microfilaments and microtubules in the rapid uptake pathway [39]. Indeed, random diffusion of virions through the cytosol represents a very inefficient method of reaching the nucleus and so many viruses exploit the cytoskeleton for transport [43]. L1 has been shown to associate with tubulin [44]. Cytochalasin D, and inhibitor of actin polymerization, was reported to inhibit papillomavirus infection [41]. We recently demonstrated that L2 binds to β-actin, indicating that papillomavirus may use microfilaments

to reach microtubules and traverse the cytoplasm towards the nuclear membrane [45]. Other findings propose that interaction between L2 and syntaxin-18 may be critical to correct routing through the cell within vesicles [46] (see fig. 1). Escape from intracellular vesicles is dependent upon furin cleavage at the N terminus of L2 [47] as well as sequences at the C terminus of L2 [48]. Furin cleavage can occur extracellularly [47]. The uptake into the nucleus is extremely inefficient after microinjection of the SV40 viral genome-histone complex into the cytoplasm, suggesting that a viral capsid protein facilitates minichromosome entry to the nucleus [49]. L2 also contains a DNA-binding domain at its N terminus [50], and deletion or scrambling the sequence of this domain prevents infection [34, 51]. Furthermore, peptides comprising these regions disrupt infection [42] and the C terminus of L2 has also been implicated in exiting of the late endosomes during infection. In vitro and in vivo data suggest that whole papillomavirus particles cannot enter the nucleus [39] but L1 import, probably as capsomers, occurs via the karyopherin-α_2/β_1 pathway [52]. Interestingly, L2 enters the nucleus in association with the viral DNA and the C-terminal nuclear localization sequence is critical to infection. L2 dumps the viral DNA at ND-10/POD and thereby enhances early viral transcription [38]. Indeed, yeast 2-hybrid screening has been used to identify several L2-interacting, ND-10-associated proteins, although their significance in infection is unclear [53]. L2 residues 390–420 have been identified as important for ND-10 localization with Daxx and exit of Sp100 [54]. However, promyelocytic leukemia protein deficiency reduces the efficiency of infection by only an order of magnitude despite disrupting the ND-10 structure, suggesting that this targeting is not obligatory for infection [38]. Clearly, the uptake process of papillomavirus is poorly defined and controversial despite its significance. A concerted, quantitative analysis in multiple model systems, with biochemical and structural studies, is essential to better address the molecular mechanisms of papillomavirus infection.

L1-Based Vaccines

It has been shown that the expression of the recombinant major capsid protein L1 in various cell types generates VLPs that are very similar to native virions with respect to their morphology, epitope display and immunogenicity [55–57]. Vaccination with L1 VLPs induces both high titers of neutralizing antibodies and protection in a number of animal papillomavirus challenge models [58], including cottontail rabbit papillomavirus and rabbit oral papillomavirus infection of domestic rabbits [59, 60], BPV-4 in cattle [61] and canine oral papillomavirus in dogs [62]. Furthermore, early-phase clinical trials involving intramuscular vaccination with HPV L1 VLPs suggested that the approach is safe and provided immunogenicity data for selection of the appropriate L1 VLP dose. A number of efficacy trials have been conducted using L1 VLP vaccines. Among these, the first and landmark clinical trial conducted by Koutsky et al. [63] showed that HPV-16 L1 VLPs were 100% effective in protecting uninfected women from HPV-16 infection and HPV-16-associated CIN over a

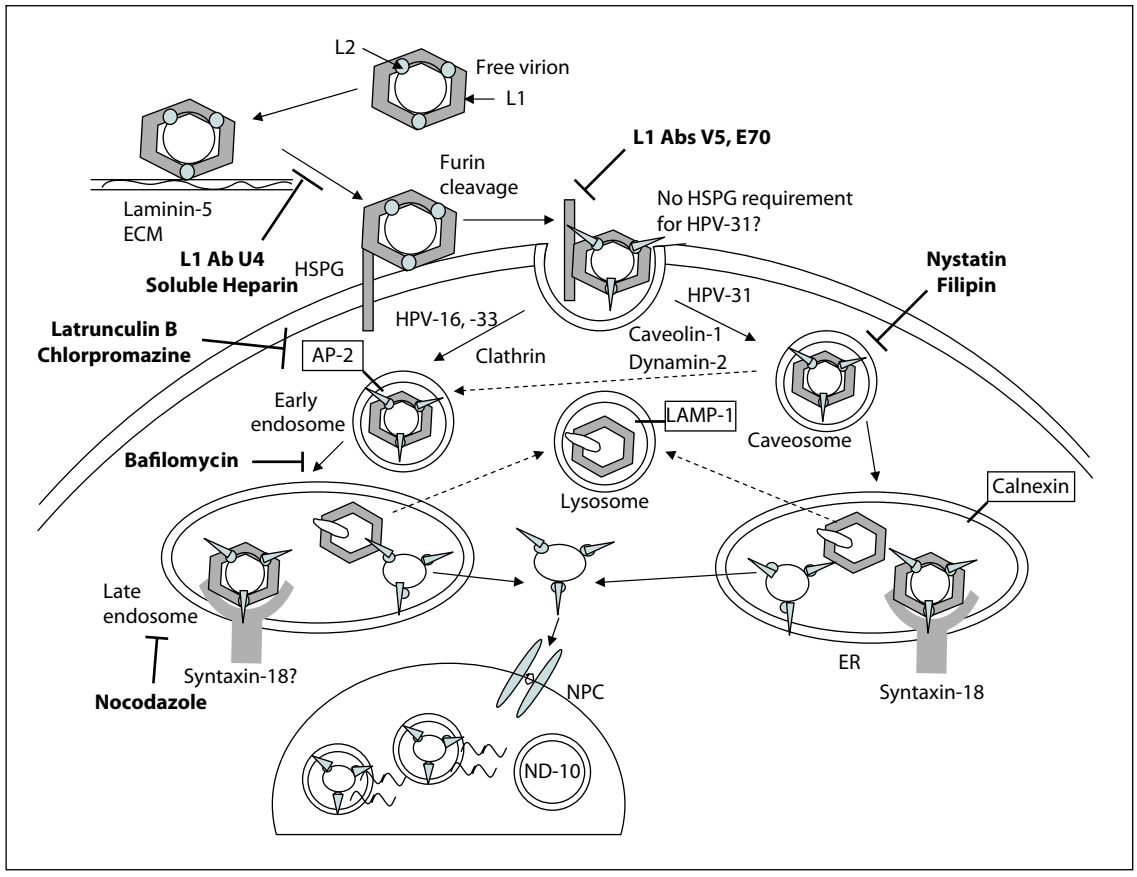

Fig. 1. Model for papillomavirus infection. Free virions containing L1 and L2 around the supercoiled and histone-bound viral genome reach the basement membrane via microabrasions in the epithelium. The virions interact with laminin-5 in the basement membrane via L1. A slow transfer from laminin-5 in the extracellular matrix (ECM) to cell surface heparin sulfated proteoglycans (HSPG) occurs via binding of L1 to heparin sulfated proteoglycans. The process of HPV-16 infection is blocked by soluble heparin (although not for HPV-31) and the HPV-16-specific neutralizing antibody H16.U4. Binding to a potential secondary receptor that signals virus uptake occurs, possibly after extracellular furin cleavage of L2 and a change in L2 and/or L1 conformation/surface availability. Uptake is blocked by the HPV-16-specific neutralizing antibodies H16.V5 and H16.E70. Although L2 binds to the cell surface, L1-only VLPs are taken up. The existence of a noninfectious/ default pathway in which particles lacking L2 or otherwise defective traffic to the lysosomes (marked by LAMP-1) is proposed. Divergent pathways for uptake have been suggested; clathrin-mediated uptake into early endosomes for HPV-16, HPV-33 and BPV-1, but caveolin-1 and dynamin-2-depednent uptake for HPV-31 presumably into caveosomes. Divergent later events have also been described, including uptake into late endosomes from early endosomes, and transfer from caveosomes to an endoplasmic reticulum (ER)-like location containing syntaxin-18 and calnexin. Interaction between L2 and syntaxin-18 via its luminal tail mediates appropriate trafficking. Syntaxin-18 is a component of the fusion pore complexes. It is presumed that the particle undergoes proteolysis/reduction in these late compartments near the nucleus and that the L2/genome complex escapes from late endosomes or the ER and passes into the cytoplasm. L2 is bound to the viral genome via a nonspecific DNA-binding domain and ultilizes its nuclear localization signals (NLS) to pass through the nuclear pores without L1. L2/genome complexes traffic to subnuclear domain ND-10/POD, whereupon transcription of the early genes and viral DNA replication is initiated. This figure was modified from one provided by Patricia Day. NPC = Nuclear pore complex.

one and a half year period. However, protection against HPV infections other than HPV-16 was not examined in this initial study, although the same number of CIN related to types other than HPV-16 were present in both the placebo and vaccine arms.

An HPV L1 VLP vaccine, 'Gardasil', developed by Merck has recently been licensed. This vaccine targets 4 of the most common HPV genotypes, HPV-16 and HPV-18 for cervical cancer and HPV-6 and HPV-11 for benign genital warts. The HPV types 16 and 18 are detected in ~70–75% of all cervical cancers and HPV types 6 and 11 cause ~90% of the genital warts. Gardasil has been extremely successful in inducing nearly complete protection from persistent HPV infection and disease (i.e. cervical, vaginal and vulval intraepithelial neoplasia and genital warts) associated with these 4 HPV genotypes. Another HPV L1 VLP vaccine, Cervarix, developed by Glaxo Smith Kline that contains HPV types 16 and 18 has already been approved in Europe and Australia and is currently under review by the US Food and Drug Administration. The results of trials have indicated that the vaccines are well tolerated, highly immunogenic and capable of generating high titers of neutralizing antibody to the HPV types included in the vaccine, thus inducing protection from HPV-related CIN [64, 65]. There is also some amount of cross-protection with the closely related HPV types 31 and 45, thus suggesting the possibility of protection against ~80% of the cervical cancers [64, 66]. In addition, these vaccines have maintained their efficacy over the 5-year period analyzed thus far [66, 67]. Table 1 summarizes a comparison of the 2 L1 VLP vaccines. The ability of the vaccines to induce some level of cross-protection indicates that even relatively low titers of neutralizing antibodies are protective as cross-neutralization occurs ~10–100 times less efficiently in vitro than for homologous type neutralization. This implies that homologous type protection is likely to be longer lasting than heterologous type protection.

HPV L1 VLP vaccines provide protection primarily against infection by the homologous papillomavirus type(s) targeted (i.e. one VLP type protects against the homologous type infection), consistent with the type specificity of the neutralizing antibodies that are believed to mediate protection. Type-restricted immunity is not absolute [66] but renders comprehensive vaccination against cervical cancer with L1 VLPs of >15 HPV types more difficult and increases the cost and complexity of vaccine development. Indeed, the current vaccines contain VLPs of only 2 oncogenic HPV genotypes. An octovalent HPV vaccine is currently under development and contains VLPs of the 6 most common oncogenic HPV types. Currently, the manufacture of L1 VLP vaccines is expensive and there is a requirement of refrigeration for storage and needles for injection. Thus, these vaccines may not be feasible in low-resource and remote areas, where they are most needed. These obstacles may be overcome by cheaper production of the L1 VLP vaccines using *Escherichia coli* or production of the L1 capsomer vaccine, which is potentially more stable at room temperature. The expression of L1 in *E. coli* produces high levels of capsomers that can

Table 1. Comparisons between Cervarix and Gardasil

Parameters	Gardasil	Cervarix
Manufacturer	Merck & Co	Glaxo Smith Kline
HPV types included	HPV-16, -18, -6, -11	HPV-16 and -18
Production system	yeast	insect cells infected with recombinant baculovirus
Adjuvant	alum	ASO_4 [aluminium salt + MPL (3-O-desacyl-4′-monophosphoryl lipid A)]
Dose	0.5-ml dose containing 20 μg HPV-6 L1, 40 μg HPV-11 L1, 40 μg HPV-16 L1 and 20 μg HPV-18 L1	0.5-ml dose containing 20 μg HPV-16 L1 and 20 μg HPV-18 L1
Recommended regimen and route of administration	3 intramuscular injections at 0, 2 and 6 months	3 intramuscular injections at 0, 1 and 6 months
Recommended age for vaccination, years	9–26	10–25
Price, USD	~120 per dose	~100 per dose
Diseases covered	anogenital cancers, including cervical, vulval, vaginal and anal cancers and their associated precursor lesions and a subset of head and neck cancers, genital warts and laryngeal papillomas	anogenital cancers, including cervical, vulval, vaginal and anal cancers and their associated precursor lesions and a subset of head and neck cancers
Available data regarding length of protection	at least 5 years	at least 5 years

self-assemble in vitro [68–70] and vaccination with such capsomers induces neutralizing antibodies and demonstrates protection in animal models [69, 71, 72]. Clinical trials are currently being planned to evaluate the safety and immunogenicity of L1 capsomere vaccines formulated in alum [Drs. Robert Garcea and Warner Huh, pers. commun.]. A potentially even more cost-effective approach is to express L1 from a live vector, although this comes with additional safety concerns. For example, the expression of L1 in the typhoid vaccine, live attenuated *Salmonella typhi*, has great potential. It could be delivered orally and is currently being developed for clinical trials [Drs. Denise Nardelli-Haefliger and John Schiller, pers. commun.].

At present, there is ongoing development of an alternative low-cost vaccine candidate comprising a conserved and cross-protective antigen, L2, that induces broad protection and can be expressed in *E. coli*.

L2-Based Vaccines

L2 represents an attractive but unproven candidate antigen for broadly protective vaccination. Indeed, it has been shown that immunization of rabbits or cows with L2 or its peptides [73–75] protects from experimental papillomavirus infection at mucosal and cutaneous sites, and this protection is mediated by L2-specific neutralizing antibodies [76]. Importantly, several groups have demonstrated that vaccination with L2 induces cross-neutralizing antibodies that are likely to afford broad protection against oncogenic and low-risk HPV types [77, 78]. In addition, since L2-based antigens can be produced in *E. coli* as opposed to the current L1-VLP vaccines that are expressed in yeast (Gardasil) or insect cells (Cervarix), manufacture could be easier and less expensive. The production of clinical-grade HPV L2 vaccines in *E. coli* for phase I/II clinical trials is currently underway. Clinical trials are being planned to evaluate the safety of HPV L2 polypeptide vaccination in healthy women [Drs. Richard Roden and Warner Huh, pers. commun.]. These studies may eventually lead to a locally manufactured, low-cost but broadly effective preventive HPV vaccine that could have the greatest impact in developing countries.

Therapeutic Human Papillomavirus Vaccines

There are several factors that highlight the need for the development of therapeutic vaccines for the control of HPV-associated malignancies in addition to preventive HPV vaccination. First, since the capsid antigens (L1 and/or L2) are not expressed by infected basal epithelial cells, it is unlikely that preventive vaccines targeting L1 or L2 would be effective in eliminating pre-existing HPV infection, and clinical studies to date have born this out. Furthermore, there is currently a considerable burden of existing HPV infections worldwide and it is estimated that it would take decades for preventive vaccination to impact the cervical cancer rates. Thus, in order to accelerate the control of cervical cancer and treat currently infected patients, the continued development of therapeutic vaccines against HPV is essential. Finally, such therapeutic vaccines could also be used in a preventive context, i.e. eliminating new infections before disease becomes apparent.

The choice of target antigen is a key factor in the designing of therapeutic vaccines. The HPV early viral proteins, E6 and E7, are obvious target antigens since they are essential for transformation, expressed early in viral infection and only in HPV-infected cells, although other early viral proteins (E1, E2, E4, E5) may be useful targets for therapy of premalignant lesions [79]. Thus, early genes, and E6 and E7 is particular, have been extensively used in the development of various kinds of therapeutic HPV vaccine,

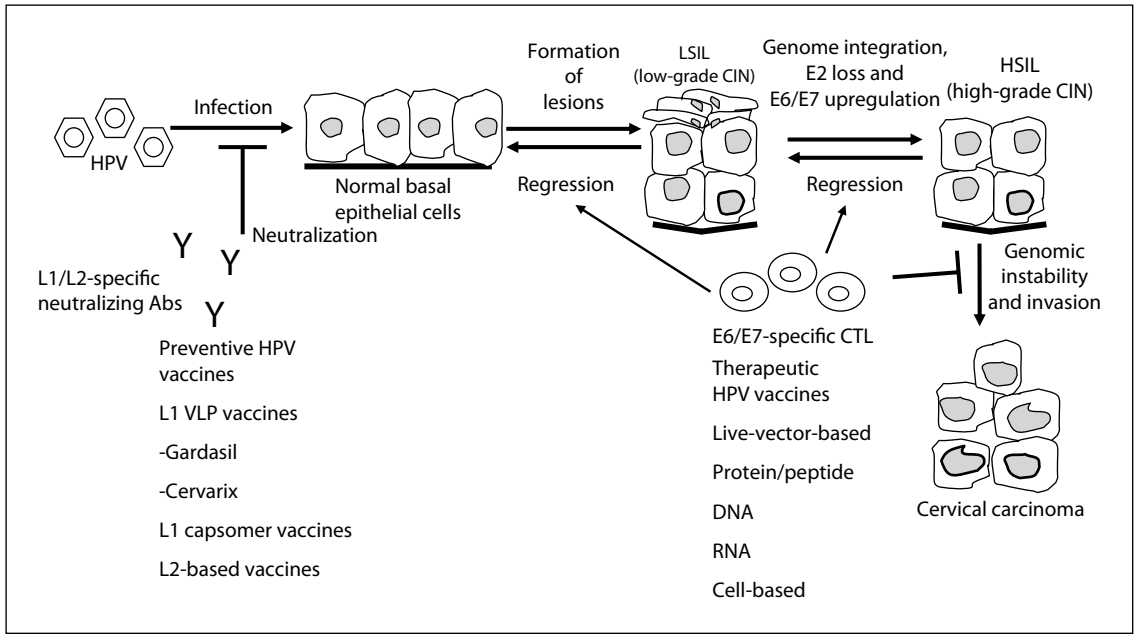

Fig. 2. HPV vaccines and disease progression. Microtrauma during intercourse is believed to allow HPV to access the basal epithelial cells. HPV infection promotes epithelial cell proliferation, leading to low-grade squamous intraepithelial lesions (SIL) or CIN, which generate progeny virions. The majority of these infections are self-limiting and cleared by the immune system and the epithelium returns to normal. In some cases, however, high-grade lesions progress to microinvasive then invasive cervical carcinoma, and this is associated with integration of the viral genome, loss of E2 and upregulation of E6/E7 expression and the emergence of chromosomal abnormalities such as 3q gain. This diagram provides an overview of the immunologic effects of preventive and therapeutic vaccination against HPV. Preventive vaccines, including L1 VLP, capsomer and L2-based vaccines, act by generating L1- or L2-specific neutralizing antibodies that prevent HPV infection of the basal epithelial cells. Therapeutic HPV vaccines, such as live-vector-based, protein/peptide-based, DNA-, RNA- and cell-based vaccines targeting early protein expression, e.g. E6 and E7, generate cell-mediated immune responses (CTLs) that block progression of CIN lesions to cervical cancer by inducing regression of CIN lesions.

such as live vector vaccines, peptide- or protein-based vaccines, cell-based vaccines, DNA vaccines as well as combination approaches (fig. 2). Table 2 discusses the advantages and disadvantages of the different therapeutic HPV vaccine approaches.

Live Vector Vaccines

Several live-vector-based vaccines have been employed for HPV vaccine development. Live vector vaccines, including recombinant viral and bacterial vectors, are highly immunogenic because they can replicate within host cells and facilitate the spread and replenishment of antigen within the host [80–85]. However, the production of

Table 2. Comparison of the various therapeutic HPV vaccine approaches

Approach	Pros	Cons
Vector-based vaccines	high immunogenicity, wide variety of vectors available, easy to deliver	risk of toxicity, potential of spreading, potential pre-existing immunity, inhibited repeated immunization
Peptide-based vaccines	safe, easy to produce, stable	HLA-restricted, poor immunogenicity, requires injection
Protein-based vaccines	no HLA restriction, easy to produce	poor immunogenicity, better induction of antibody response than cytotoxic T lymphocyte response, requires injection and refrigeration
DC-based vaccines	high immunogenicity, generation of large quantities of dendritic cells	expensive, labor-intensive production
Tumor-cell-based vaccines	likely to express relevant tumor antigens, useful when tumor antigen is unknown	safety concerns, difficulty in production, weak antigen presentation by tumor cells
RNA vaccines	noninfectious, multiple immunizations possible, RNA replicons replicate in the cell and enhance antigen expression	Unstable, difficulty in production
DNA vaccines	easy to produce, store and transport, multiple immunizations possible	poor immunogenicity

vector-specific neutralizing antibodies in the host during vaccination could reduce the potency of repeat immunizations. This is also a problem for some vectors with a high prevalence of pre-existing vector immunity within the target population. There is also a potential risk of toxicity associated with the use of live vectors in patients, particularly those with weakened immune systems. Nevertheless, this approach has been widely applied.

Phase I/II clinical trials have been conducted using recombinant vaccinia encoding an HPV-16/18 E6/E7 fusion protein, termed TA-HPV, and the vaccine was shown to be well tolerated and induced T-cell-mediated immune responses in CIN and vulval intraepithelial neoplasia patients [86–91]. More recently, a recombinant vaccinia vector encoding the E2 viral protein, termed MVA-E2, has been tested in patients with CIN [92, 93] and flat condyloma lesions [94]. Although it is not clear if this vaccine is capable of generating E2-specific immune responses, they have shown some evidence of efficacy.

Peptide/Protein-Based Vaccines

Peptide-based vaccines are considered to be safe, easy to produce by direct chemical synthesis and stable. One limitation, however, is that they tend to be poorly immunogenic. Another drawback is that peptide vaccines are major histocompatibility complex (MHC)-specific. Protein vaccines, on the other hand, have the ability to bypass MHC restriction [95–97]. Like peptide vaccines, they are also safe but are somewhat more complex to produce. However, they are weakly immunogenic and usually induce a better antibody response than cytotoxic T lymphocyte response [98, 99]. Consequently, most of the research in this area has focused on the use of adjuvants and fusion protein strategies to enhance vaccine potency. Preclinical studies suggest that the potency of these vaccines can be enhanced by using liposome encapsulated formulations [100], fusion proteins with heat shock protein 70 (Hsp70) [101, 102] or by employing the intranasal route of administration with a strong mucosal adjuvant [103]. Several peptide/protein-based HPV vaccines have also been shown to be safe and well tolerated in multiple clinical studies [104–106]. Recently, an approach using long overlapping peptides of HPV-16 E6 and E7 sequences in Montamide ISA 51 adjuvant generated promising results in vulval intraepithelial neoplasia patients [107]. Several such strategies employing synthetic peptide vaccines are currently being developed (for review see [108]).

Cell-Based Vaccines

Dendritic-CellBased Vaccines

Dendritic cell (DC)-based vaccines are potentially advantageous in that they can possibly circumvent some types of tumor-mediated immunosuppression by directly presenting antigenic peptides to the immune system [109, 110]. However, these individualized DC-based vaccines are costly and cumbersome to generate and this makes large-scale production challenging. Nevertheless, DC-based HPV vaccines have been tested in clinical trials. For example, subcutaneous injection of HPV-18 E7-pulsed DCs has been employed in patients with cervical cancer without significant side effects [111, 112].

Tumor-Cell-Based Vaccines

Tumor-cell-based vaccines possess the advantage that the tumor antigen(s) need not be identified. However, in the case of HPV, the relevant tumor antigens are already defined and thus tumor-cell-based vaccines do not hold any particular advantage for the treatment of HPV-associated malignancies. Furthermore, the employment of tumor-cell-based vaccines creates safety concerns, since it involves the risk of introducing new cancers. In addition, the production of individualized autologous vaccines in large scale is difficult. Thus, tumor-cell-based vaccines have not been further explored in clinical studies of cervical cancer and have a limited scope for HPV vaccine development in our opinion.

Nucleic-Acid-Based Vaccines

RNA Replicon Vaccines

The employment of RNA-replicon-based vaccines against HPV infections has been explored in preclinical models. RNA replicons are naked RNA molecules that can replicate in transfected cells and can potentially produce sustained levels of antigen. Since RNA replicon vectors do not contain viral structural genes, no infectious particles are produced and thus the host immune response to these vectors is likely to be limited. RNA replicons can also be administered as naked suicidal DNA that is transcribed into RNA replicons, which replicate as RNA in transfected cells. These DNA-launched RNA replicons combine the advantage of the inherent stability of DNA with the ability of the RNA replicons to enhance vaccine potency [113]. Another replicon system uses a flavivirus termed Kunjin as a stable noncytopathic RNA replicon vaccine vector. Vaccination of mice with Kunjin replicons expressing an HPV-16 E7 epitope induced specific T cell responses and protected mice from tumor challenge [114]. These vaccines are advantageous in that they do not cause cell death and thus demonstrate long-term presentation of antigen, unlike lytic RNA replicons [115]. However, due to the low general stability of naked RNA compared to DNA and difficulties in large-scale production, RNA replicons have not yet been explored in clinical trials.

DNA-Based Vaccines

Naked DNA vaccines are an alternative strategy for the development of therapeutic HPV vaccines. DNA vaccines have several advantages including safety, stability, ease of manufacture and administration. Furthermore, they can be used for the longer-term expression of antigen in cells (for review see [116, 117]). However, one drawback is that DNA vaccines are not particularly immunogenic because DNA lacks the intrinsic ability to amplify or spread from transfected cells to surrounding cells in vivo.

Several strategies are being developed to enhance the potency of DNA vaccines. Since DCs play an integral role in DNA-vaccine-mediated immune responses,

Table 3. Strategies to enhance HPV DNA vaccine potency by modification of properties of APCs (For review see [118, 119])

Strategies	Methods	Study
Strategies to increase the number of antigen-expressing DCs	• Epidermal administration of DNA vaccines via gene gun	[144, 145]
	• Intercellular antigen spreading	[146, 147]
	• Linkage of antigen to molecules capable of binding to DCs	[148]
	• Employment of chemotherapy-induced apoptotic cell death	[132]
Strategies to improve antigen expression, processing, and presentation in DCs	• Employment of intracellular targeting strategies to enhance MHC class I and class II antigen presentation in DCs	[149–153]
	• Codon optimization	[154, 155]
	• Bypassing antigen processing – MHC class I single-chain trimer	[156]
Strategies to enhance DC and T cell interaction	• Prolonging DC survival	[157, 158]
	• Promoting in vivo DC expansion	[159]
	• Employment of cytokines and costimulatory molecules	[160]
	• Induction of CD4+ T cell help	[153]

modification of DCs represents an excellent method to improve DNA vaccine potency. Some of the strategies to enhance DNA vaccine potency by modification of the properties of DCs include: (1) increasing the number of antigen-expressing DCs; (2) improving antigen expression, processing and presentation in DCs; (3) promoting DC activation and function, and (4) enhancing DC and T cell interaction, to improve T cell immune responses (for review see [118, 119]). Table 3 summarizes the various strategies that have been used to enhance HPV DNA vaccine potency by modification of the properties of DCs.

The successes in preclinical models have resulted in several therapeutic HPV DNA vaccine clinical trials. For example, an encapsulated plasmid DNA vaccine encoding HLA-A2-restricted epitopes derived from HPV-16 E7 protein has been tested in patients with high-grade anal intraepithelial lesions [120] and high-grade CIN [121]. The vaccine (ZYC101) is composed of plasmid DNA encapsulated in biodegradable polymer microparticles. The trials in patients with high-grade CIN lesions showed significant immune responses and no serious side effects. Subsequently, a study using ZYC101a, an encapsulated plasmid DNA vaccine encoding protein peptides derived from E6 and E7 proteins of HPV-16 and HPV-18, was conducted in women with high-grade CIN [122] and was well tolerated in all patients.

Another DNA vaccine that is currently undergoing clinical examination is the Sig/E7(detox)/Hsp70 DNA vaccine. It encodes a signal sequence linked to an attenuated form of HPV-16 E7 [with a mutation that abolishes the Rb-binding site E7(detox)] and fused to Hsp70 [Sig/E7(detox)/Hsp70]. This vaccine is being tested on HPV-16-positive patients with high-grade CIN lesions at Johns Hopkins. The phase I trial tests a homologous prime-boost vaccination regimen of 3 DNA vaccinations per patient, at 3 dose levels. No adverse or dose-limiting site effects were observed at any dose level of the DNA vaccine and the vaccination was considered to be feasible and tolerable in patients with CIN-2/3 lesions. The patients in the highest-dose cohort generated IFN-γ-secreting CD8+ T cell immune responses to E7 of greater magnitude in peripheral blood mononuclear cells than subjects in lower-dose cohorts. In the highest-dose cohort, disease regression was observed in 3 of 9 patients after vaccination, whereas no regressions were seen at lower doses. Another phase I trial using the same naked DNA vaccine [Sig/E7(detox)/Hsp70] is currently ongoing in HPV-16-positive patients with advanced head and neck squamous cell carcinoma at the Johns Hopkins University. Likewise, no significant adverse effects were observed in this study. Some of the DNA-treated patients developed appreciable E7-specific immune responses.

Another candidate DNA vaccine that is currently being prepared for clinical trials conducted at the University of Alabama at Birmingham in collaboration with Johns Hopkins is a DNA vaccine encoding calreticulin (CRT) fused to HPV-16 E7(detox). Intradermal administration of the CRT/E7 DNA vaccine has been shown to generate significant E7 antigen-specific immune responses in preclinical models (see above). This therapeutic HPV DNA vaccine trial will be performed in HPV-16-positive patients with stage 1B1 cervical cancer using a PowderMed/Pfizer proprietary individualized gene gun device (ND-10). Improving the delivery in patients is likely to be key to obtaining successful immunization with DNA vaccines. This study aims to investigate whether the repeated, cluster (short-interval) intradermal CRT/E7 DNA vaccination is safe and able to generate E7-specific CD8+ T cell immune responses in patients with stage 1B1 resectable cervical cancer. DNA vaccination using the cluster vaccination regimen has been shown to rapidly induce antigen-specific CD8+ T cell immune responses in preclinical models. The proposed cluster vaccination regimen will make it possible to complete the vaccination regimen before tumor resection allowing the assessment of the influence of the DNA vaccination on the tumor microenvironment without compromising the standard care of the patient.

Combination of Preventive and Therapeutic Vaccines

An ideal HPV vaccine should aim at preventing new HPV infections as well as treating established HPV infections and HPV-associated lesions. Several approaches that

include both early and late HPV antigens to induce both neutralizing antibodies and early protein-specific cellular immunity have been tested in clinical trials. For example, fusion proteins containing HPV capsid proteins and HPV early proteins such as TA-GW, a fusion of HPV-6 L2 and E7 [123–125], and TA-CIN, a fusion of HPV-16 L2, E6 and E7 [126], have been shown to be well tolerated and immunogenic in a number of clinical trials. Both generate E6- and E7-specific T cell responses, although TA-GW had no therapeutic efficacy against genital warts. Healthy volunteers vaccinated with TA-CIN induce L2-specific serum antibodies that neutralized across HPV species [127]. A recent clinical study was conducted using HPV-16 L1E7 chimeric VLPs in women suffering from high-grade cervical intraepithelial neoplasia (CIN 2/3). The vaccine was shown to be safe and well tolerated and induced high titers of antibodies against HPV-16 L1 and low titers against HPV-16 E7 as well as cellular immune responses against both proteins [128].

Combination Modality Vaccines

The effect of therapeutic HPV DNA vaccines may be enhanced through a combination approach using heterologous prime-boost strategies. Prime-boost regimens utilizing vaccines of different modalities are one of the most effective strategies for boosting specific immune responses. Because nucleic acid vaccines often generate relatively weak cytotoxic T lymphocyte responses, and boosting with live vectors is not always possible, combinatorial vaccination approaches are used to circumvent these limitations. Priming with a DNA or RNA vaccine and then boosting with a viral vector vaccine has been shown to result in enhanced immune responses relative to single-modality vaccinations. For example, we have demonstrated that vaccination with DNA prime followed by vaccinia boost regimen generates a significantly higher antigen-specific immune response compared to DNA vaccination alone [129]. Prime-boost combinations involving priming with fusion protein (TA-CIN) and then boosting with recombinant vaccinia virus encoding E6 and E7 (TA-HPV) have been tested in a number of trials. This approach was well tolerated and most immunogenic when the protein vaccine is given first [91, 130, 131]. A clinical trial using pNGVL4a/Sig/E7(detox)/Hsp70 DNA prime followed by E7-expressing vaccinia boost is currently being planned at Johns Hopkins University in patients with CIN-2/3 lesions. Unfortunately the downside of this approach is increased complexity of manufacture (i.e. 2 products) and potentially more side effects.

Combination approaches including chemotherapy, radiation or other biotherapeutic agents together with HPV therapeutic vaccination may also serve to enhance the therapeutic HPV vaccine potency. For example, it has recently been shown that the chemotherapeutic agent epigallocatechin-3-gallate, a chemical derived from green tea, could induce tumor cellular apoptosis and enhance the tumor antigen-specific T cell immune responses elicited by DNA vaccination [132].

The combination of HPV therapeutic vaccines with agents that influence the tumor microenvironment may also potentially be used to generate enhanced therapeutic effects against HPV-associated malignancies. Several factors in the tumor microenvironment suppress immune responses including the expression of STAT-3 [133], MIC-A and -B [134], B7-H1 [135] and galectin-1 [136] on tumor cells and release of indoleamine 2,3-dioxygenase enzyme, arginase or reactive oxygen species [137], immunosuppressive cytokines such as IL-10 [138] and TGF-β [139], T regulatory cells [140] and myeloid-derived suppressor cells [141]. Blockade of factors that inhibit T cell activation or action in tumor microenvironments, such as anti-CTLA-4 and PD-1, may also be used in combination with HPV vaccination in order to enhance the therapeutic effects against cervical cancer [142, 143].

Conclusions

The implementation of Pap screening and ablation of high-grade squamous intraepithelial lesions has reduced the incidence of cervical cancer by ~70–80% in the USA. Nevertheless, there are still ~5,000 deaths in the USA each year due to cervical cancer, predominantly in those without access to adequate preventive healthcare. Most significantly, developing countries currently lack the resources to implement such screening programs and therefore cervical cancer has remained the second leading cancer killer of women in these countries. The recent demonstrations of the efficacy of L1 VLP vaccines and their licensure will further impact the cervical cancer rates in developed countries over the next few decades. However, it is critical that we continue to develop HPV vaccines that are practical for use in developing countries and hard to reach populations. An alternate protective antigen, L2, and many new technologies for the delivery of L1 are being considered to overcome these practical difficulties and protect against the transmission of all oncogenic HPV types.

The development of therapeutic vaccines remains a priority for 2 important reasons. Firstly, HPV infection and disease is widespread and current vaccines are not effective against pre-existing disease. As a result, preventive vaccines will not impact cervical cancer rates for about 2 decades, and current patients urgently need effective virus-specific treatments. Secondly, because we know the etiologic agent and relevant tumor antigens, therapeutic vaccination against cervical cancer represents an important system in which to develop antigen-specific cancer immunotherapy. While many vaccine studies have demonstrated the ability to induce antigen-specific T cell responses in patients, most show little or no evidence of therapeutic efficacy against cancer (although there is some progress against premalignant disease). Clearly, more work is needed to counter or overcome the immune suppression and evasion by cancers.

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Hepatitis C Virus Genetics and the Discovery of Mechanism-Based Inhibitors of the NS3/4A Protease and NS5B Polymerase

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Abstract

Hepatitis C virus (HCV) is a major cause of chronic liver disease frequently resulting in cirrhosis or hepatocellular carcinoma. The many genetic variants of HCV circulating world-wide are organized into genotypes based on sequence analysis. There is approximately 25% variation between any 2 genotypes at the amino acid level, and this genetic variation impacts antiviral treatment. Current therapy consists of various forms of interferon usually co-dosed with ribavirin. Many patients, especially non-genotype 2 ones, do not respond to this therapy, accentuating the need for more treatment options. This review describes the organization of the HCV genome and biological systems useful to examine viral replication. Two viral enzymes, the NS3/4A serine protease and the NS5B RNA-dependent RNA polymerase, have been the subject of intensive efforts to find small-molecule, mechanism-based inhibitors of these functions. The structures of both enzymes have been solved by X-ray crystallography and this aids in understanding how small molecules interact with these enzymes. The discovery of inhibitors active against HCV replicons in cell culture allows for the selection of replicons resistant to inhibitors. In this review, emphasis is placed on the analysis of inhibitor resistance and the parallel genotypic variation in the amino acids which confer resistance.

It had been noted that many cases of infectious hepatitis could not be attributed to hepatitis A or B virus. The causative agent of these non-A, non-B hepatitis infections remained elusive until 1989. The first description of a new virus, termed by convention hepatitis C virus (HCV), was based on library expression cloning of chimpanzee infectious plasma samples followed by elimination of cloned sequences hybridizing to human and chimp DNA and screening of the expression library using a non-A, non-B hepatitis patient serum [1]. In a companion paper, one of the expression library clones was used as the source of viral antigen for the detection of HCV-specific antibodies in patient sera [2]. One major result of this work was the development of a test to screen blood prior to transfusion. The institution of blood screening to detect HCV led to a

dramatic decline in transfusion-mediated new infections from an estimated 150,000 new cases per year worldwide before screening to about 33,000 new cases after screening [3]. Worldwide estimates provide for approximately 170 million infections and a 2–3% seropositivity rate with the USA tending towards the lower seropositivity value. Given the long usually chronic nature of HCV infection and the decades long incubation period prior to the development of hepatocellular carcinoma, there remains the need for effective antivirals and vaccines for the control or prevention of HCV infections. This review will focus on the use of biological systems to quantitate HCV replication and current efforts to define mechanism-based inhibitors of HCV enzymes essential for viral replication. Special emphasis will be placed on an analysis of amino acid substitutions which confer resistance to these inhibitors.

Virology

The initial physical characterization of HCV suggested that it was closely related to the flaviviridae, that family of viruses which is now broken into the flaviviruses, the pestiviruses and the hepaciviruses. The flaviviruses include the yellow fever virus, West Nile virus, the dengue viruses and several other arthropod-borne viruses. The pestiviruses comprise several viruses such as bovine viral diarrhea virus and hog cholera virus, which are important agricultural pathogens. For many years, bovine viral diarrhea virus served as a cell culture surrogate model for HCV as it was the mostly closely related virus that can be replicated in cell culture. Subsequent to the discovery of HCV several related viruses, GBV-A, GBV-B and GBV-C, which appear to be more closely related to HCV, have been described, but these offered no advantages for cell culture studies.

The HCV-1 isolate consisted of 9,401 bases (Genbank accession number M62321) of (+) strand RNA polarity, meaning that the RNA acts as messenger RNA and can be directly translated to produce a polyprotein of 3,011 amino acids. The organization of the viral genome and the polyprotein will be described in detail below.

Following the discovery of HCV-1, many closely related viral isolates from diverse geographic locales were isolated and sequenced. After extensive analysis of viral sequences, the vast majority of HCV isolates have been divided into 6 major genotypes, 1–6, with further division into subtypes [4]. There may be other minor genotypes and subtypes, but these often consist of only a single sequence from a defined geographic location. Genotypes 1 and 2 are by far the most predominant and have the greatest geographic distribution. Genotypes 1 and 2 and to a lesser extent genotype 3 are the focus of most antiviral efforts. In the USA, genotype 1 is responsible for about 70% of the HCV infections, with genotype 2 just under 20% and genotype 3 about 10%. All other genotypes account for $<1\%$ of the HCV infections in the USA.

Analysis of multiple isolates from a single patient has shown that there is some genetic variation within HCV genomes found in an individual patient. Within a

patient, infection by a single strain of virus results in the replication and genetic selection of a highly related but nonidentical population of HCV genomes termed quasispecies. Variation within a single patient is not surprising given that the virus encodes an RNA polymerase which does not have proofreading capability. The error rates for the polymerase are estimated at 1 mismatch per 10^4 bases, a size consistent with the viral genome length. Thus one can argue that every genome in a patient differs from that patient's consensus sequence. Patient viral loads of 10^6 are not uncommon. In addition, infected individuals may produce approximately 10^{10} genomes per day [5]. Both factors may contribute to the wide diversity of circulating genomes. The error rate of the viral polymerase also impacts viral replicative fitness. A misincorporation into a region important for the maintenance of RNA secondary structure may be selected against due to decreased replication capability. A misincorporation into a codon for an amino acid important for protein function may result in a protein that is misfolded, prematurely terminated or functionally deficient. Such viruses may be defective and incapable of further replication. The high mutation rate coupled with the replication capability to produce high virus titers has a tremendous impact on the selection of virus resistant to mechanism-based antivirals. This topic will be discussed in detail below.

The vast majority of patients appear to be infected with a single isolate of a single genotype. For those infected with multiple isolates evidence for recombination is rare. As a generalization, at the amino acid level there may be approximately 1% divergence within a single patient, 5% divergence within a subtype (e.g. 2 different genotype 1b isolates), 10–12% divergence within a genotype (e.g. genotypes 1a and 1b) and 25% between genotypes (e.g. genotypes 1 and 2).

Genome Organization

The original isolate of HCV-1 has a 5′ nontranslated region of 341 bases. This sequence has been shown to act as a structurally ordered internal ribosome entry sequence (IRES). Two other important characteristics are that unlike most cellular mRNAs, the HCV IRES does not contain a 5′ cap and translation does not initiate at the first AUG initiation codon. Lidenbach and Rice [6] have written an excellent review of the organization and replication of the HCV genome and this reference can be used as the source of many of the primary citations.

The HCV-1a polyprotein consists of 3,011 amino acids. Following the termination codon, the 3′ nontranslated region can be broken into 3 elements; a variable sequence of approximately 40 bases, followed by a polypyrimidine region, and a 3′ conserved region. For some years, it was thought that the polypyrmidine region represented the authentic 3′ end of the genome. It was subsequently determined that the actual 3′ end of the HCV genome consists of a 98-base sequence termed 3′ X [6]. This sequence is also structurally ordered in that it folds into 3 stem loop structures as shown in figure 1.

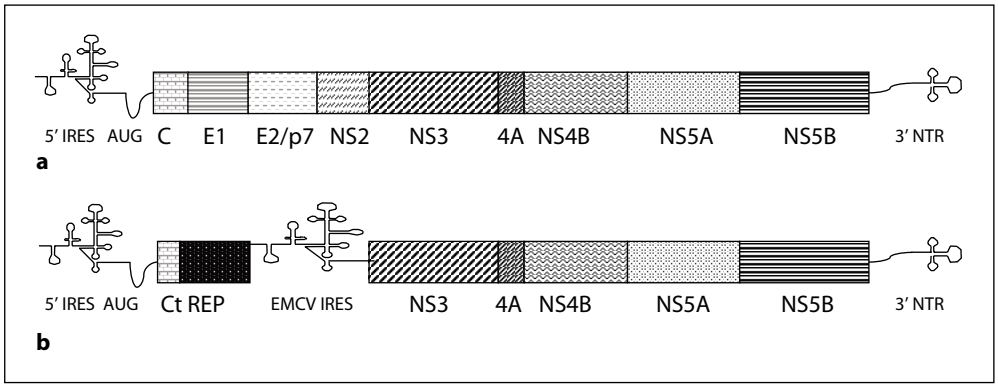


Fig. 1.a Organization of the HCV genome. The order from left to right is 5′ IRES, core (C), envelope glycoproteins E1 and E2, p7, and nonstructural proteins NS2, 3, 4A, 4B, 5A and 5B, followed by the 3′ nontranslated region (NTR). The location of the initiation codon, AUG, is shown as part of the IRES to denote that the IRES functionally extends into the C protein. Structural elements of the 5′ IRES and 3′ NTR are noted as stem loop structures. Internal vertical bars within the polyprotein represent cellular or viral protease cleavage sites. **b** Organization of the HCV NS3 replicon. REP indicates the reporter or the selectable neomycin phosphotransferase coding region which is part of a fusion protein with the Core N-terminus. The structure of the encephalomyocarditis virus IRES is just used to present secondary structure without corresponding to the authentic structure of the encephalomyocarditis virus (EMCV) IRES.

Given the necessity to preserve the folded structure of the 5′ IRES and the 3′ X sequence, there is generally good sequence conservation across genotypes [4].

The HCV-1 polyprotein can be divided into structural and nonstructural regions [6]. The N-terminal one third constitutes the structural region and this is cleaved by host cell peptidases into the C, E1, E2 and p7 polypeptides. C represents the core protein, the major structural protein of the virion. E1 and E2 are envelope glycoproteins. The E2 glycoprotein contains a small domain of 18 amino acids termed the hypervariable region, which evolves over time to escape host cell immune responses [7]. E2 appears to interact with cell surface receptors, predominantly CD81 [6]. A coreceptor may be an essential component necessary for cell entry and recently the role of claudin-1 as a coreceptor may help explain the hepatotrophism of HCV [8]. The interaction of HCV with host cell receptors may provide a useful antiviral target for the discovery of inhibitors of HCV infectivity. The small p7 protein is important late in the virus replication cycle and may contribute to efficient viral assembly and release of infectious virions [9].

The nonstructural region is represented by the C-terminal two thirds of the polyprotein. NS2–3 is an autocatalytic protease which cleaves internally to split off the NS2 moiety. The structure of the NS2/3 protease has recently been solved [10]. The enzyme is a dimeric cysteine protease with the cysteine contributed by one

monomer and the catalytic histidine and glutamate residues by the other. NS2/3 protease may serve as an attractive antiviral target.

NS3 consists of 2 domains, an N-terminal 180 amino acid serine protease domain and residues 181–631 which act as a helicase [6]. NS4A serves as a protease cofactor. The NS3/NS4A protease makes an internal cleavage to liberate the NS3 and 4A proteins. The protease subsequently makes *trans*-cleavages resulting in individual NS4B, NS5A and NS5B proteins. The NS3/NS4A protease and the NS3 helicase have been the subject of extensive antiviral screening efforts. Although the NS3 helicase activity is thought to be an excellent antiviral target, there has not been extensive success at finding small-molecule inhibitors of this function. One reason that has been offered is that helicase domain 3 moves to only transiently form the NTP binding pocket, thus it may be difficult to find small-molecule inhibitors [11]. Antiviral discovery and resistance studies for the NS3/4A protease will be discussed in detail below.

The NS4B protein serves in part to localize the viral replication complex to a membrane compartment. Infected cells may appear to display a cytoplasmic web structure and the expression of NS4B alone may induce the formation of the membraneous web [12]. Other HCV proteins associate with these structures and they may be the sites of viral replication in the cytoplasm.

The NS5A protein, which also inserts into membranes, is a serine phosphoprotein and in cells NS5A can be found as hypophosphorylated (56k) and hyperphosphorylated (58k) forms [6]. Numerous investigations have addressed the interaction between different NS5A domains and various cellular proteins. These may all play a role in influencing the cellular response to HCV infection, but it remains to be determined whether any of these interactions serve as attractive antiviral targets. Recent structural analyses of NS5A will likely aid in the evaluation of antivirals directed at NS5A [6].

When one looks across HCV genotypes, the NS5A protein shows the level of amino acid variability as in other viral proteins but also a second level of variability, namely insertion of amino acids resulting in proteins of slightly different lengths. The NS5A protein of the original HCV-1 isolate is 448 amino acids in length. The NS5A protein of the related genotype 1b is 1 amino acid smaller and this is reflected in a genotype 1b polyprotein that is also 1 amino acid smaller (3,011 as compared with 3,010 amino acids for 1a and 1b respectively). The prototypes for genotypes 2a and 2b have the largest NS5A proteins, while genotype 4a has the smallest. Amino acid alignments of NS5A across multiple genotypes suggest that there is not a single site of insertion/deletion variability but rather scattered insertions relative to the smallest NS5A. Much of this variability is found in the C-terminal half of NS5A [6]. The significance of the variability of NS5A to the biology of HCV has not been defined.

The HCV NS5B protein is an RNA-dependent RNA polymerase. This protein has been the subject of extensive biochemical characterization and antiviral discovery efforts and these will be described in detail below.

Many attempts to introduce the original HCV-1 isolate and subsequent isolates from other sources into either established hepatocyte lines or primary hepatocytes in cell culture yielded equivocal results. It was difficult to consistently demonstrate viral replication or viral spread in cell culture. It had long been noted that serial passage of infectious plasma from chimp to chimp could propagate infection in that animal. There were 3 major developments which have furthered HCV molecular virology to the point where we now have defined systems that demonstrate HCV replication. The first was the description of a chimp infectious molecular clone for genotype 1a. The second development was the description of the HCV-1b replicon and the description of permissive hepatocyte cell lines. The third development was the demonstration that genotype 2a JFH-1 strain produced virus in permissive cell lines and that released virus could be serially passaged in cell culture.

Chimp Studies

The first demonstration of a truly infectious cloned viral isolate came in 1997 with the genotype 1a H77 strain [13]. The H77 strain arose from the inoculation of human plasma into a chimp and had been serially passaged in chimp for approximately 20 years. An analysis of 6 cloned full-length genomes was used to construct a consensus molecular clone. The introduction of in vitro transcribed RNA into chimps led to viral persistence, typical hepatic pathogenesis and reisolation of the same H77 strain which was also infectious in subsequent recipient chimps.

Replicon Studies

Prior to the description of the HCV replicon, there were no reliable cell culture systems to study HCV replication. The first description of an HCV replicon came from Lohmann et al. [14] in 1999. The HCV replicon was a subgenomic RNA species capable of replication and persistence in cell culture. Unlike a true virus infection, however, it did not produce infectious virus and could not spread cell to cell. Rather, the introduction of RNA into cells led to the maintenance of replicon genomes over multiple cell divisions demonstrating the replication competence of the input RNA. The replicon genome and its comparison to the full-length HCV genome is shown in figure 1. Since the HCV IRES extends into the core protein coding region, this was cloned in frame with a neomycin phosphotransferase selectable marker (neo), such that translation gave rise to a core/neo fusion protein. Following the neo protein termination codon, there is a second IRES derived from encephalomyocarditis virus, a picornavirus. The encephalomyocarditis virus IRES is not related to the HCV IRES at either the sequence or structural level but serves the function to allow bicistronic translation of a second protein. The second polyprotein consisted of either the NS2 through NS5B or NS3 through NS5B followed by the 3′ nontranslated region. The viral strain used was a consensus 1b sequence termed Con1. The replicons were transcribed

and RNA was electroporated into the human hepatocellular carcinoma Huh-7 cell line. The electroporated cells were subsequently cultured in the presence of geneticin (G418), which killed cells that did not express the neo gene. After about 3 weeks, colonies began to appear which survived antibiotic selection. These colonies maintained the HCV replicon indefinitely. In the cells, HCV proteins could be detected and as an indication of persistent RNA replication, (−) strand RNA could be found.

The successful description of an HCV replicon was followed by 3 important developments. The first was the reisolation of the input RNA followed by analysis of the replicon sequence. Since the RNA polymerase does not have proofreading capability, over time the input Con1 sequence was outgrown by replicons that contained cell culture adaptive substitutions which increased replicative fitness. Different adaptive substitutions were scattered throughout the nonstructural proteins. Mutagenesis of the parental Con1 sequence to contain cell culture adaptive substitutions followed by transfection and colony formation assays showed that cell culture adaptive substitutions may increase colony formation by several orders of magnitude [15]. One interesting study has demonstrated an apparent inverse correlation between cell culture adaptation and chimp infectivity [16].

In parallel with the description of cell culture adaptive substitutions, it was also reasoned that perhaps the cells which were able to maintain genomes over extended periods of time displayed enhanced permissivity relative to the parental Huh-7 cell line. Replicon-containing cells could be cured of replicons by treatment with interferon (interferon-α, 100 IU/ml, for 4 passes). Reintroduction of replicon RNA into cured cells followed by neo selection gave rise to greater colony numbers (up to 33-fold), demonstrating that these cured cells had enhanced permissivity [17].

Despite these 2 major developments, the colony formation assay still took about 3 weeks. The use of a reporter rather than a selectable marker allows for indirect quantitation of viral replication by following reporter activity. These transient assays typically take <1 week. This third development led to cell culture systems suitable for high-throughput screening for the discovery of inhibitors of HCV replication. Using some of the newer developments in the replicon system, one can simultaneously assess both reduction of HCV replication by inhibitors and compound cytotoxicity. Both selectable and reporter replicons play an important role in assessing compound inhibition and resistance selection in cell culture and this will be described in detail below.

Genotype 2a Strain JFH-1 Virus Studies

The replicon system has been extended from the original Huh-7 cell line and genotype 1b Con1 isolate to other cells and genotypes including genotype 2a JFH-1 [6]. The JFH-1 strain was isolated from a case of fulminant hepatitis. Following transfection of full-length JFH-1 viral RNA Huh-7 cells, virions could be found in the cell culture supernate 4 days after transfection [18, 19]. Furthermore these virions could be passed sequentially in cell culture. Initially, virus titers were reported to approach 10^5

focus-forming units per milliliter. Subsequent modifications resulted in JFH-1 chimeras with replicative capacity sufficiently high that one can demonstrate antiviral efficacy in cell culture [20]. The discovery of a true infectious virus/cell culture system has tremendous implications by providing tools to examine important questions regarding viral infectivity and assembly that cannot be answered with the replicon system.

Current Antiviral Therapy

Even before the discovery of HCV, attempts were made to treat non-A, non-B hepatitis with interferon [reviewed in 21]. More recently, recombinant interferon has been conjugated to polyethylene glycol to increase the half-life, resulting in less frequent injections. Currently, FDA-approved monotherapy consists of either interferon-α 2a (Roche) or 2b (Schering) and their pegylated versions. Consensus interferon (Intermune) is also licensed for treatment.

Interferon binds to a specific receptor, and this is followed by internalization and induction of expression of a number of interferon-stimulated genes. It is the combined effect of expression of these interferon-stimulated genes that leads to the induction of the intracellular antiviral environment that is not conducive to HCV replication. Although cells may produce endogenous interferon in response to viral infection, it is likely that the exogenous introduction of interferon achieves higher levels than those produced normally. Double-stranded RNA can induce the expression of signal transduction pathways via interaction with Toll-like receptor 3 (TLR 3) and this may induce an antiviral state as well. A complete description of the signal transduction pathways induced by activation of TLRs and the interferon pathway is beyond the scope of this review and for recent reviews of the interferon signal transduction pathway see Gale and Foy [22]. In addition, it has been demonstrated that the HCV NS3/4A protease may interfere with the interferon and TLR 3 signal transduction pathways by interaction or processing of RIG-1 and TRIF, which are essential cell proteins involved it these signal transduction pathways [reviewed in 22].

More recently combination therapy of interferon coupled with oral ribavirin has been authorized and current FDA-approved combination therapy consists of both interferon α 2a and 2b or their pegylated versions in conjunction with oral ribavirin. Together with interferon, ribavirin may double the treatment success rate over interferon monotherapy. As a nucleoside analog, ribavirin is phosphorylated to the monophosphate, the diphosphate and ultimately the triphosphate. The monophosphate may inhibit inosine monophosphate dehydrogenase, an enzyme within the GTP metabolic pathway, thus reducing cellular GTP pools. Further comments on ribavirin as a nucleoside analog will be discussed below.

One can examine viral dynamics in patients by determining the block in virus production, the rate of viral clearance and the death of virus-infected cells. For the

patients who show a sustained virological response (undetectable circulating virus at time points well beyond the cessation of treatment), it has been noted that viral clearance follows a biphasic decline curve [5]. The first phase represents a rapid decline which is attributed to the clearance of free virus. The second phase is shallow and of longer duration and represents clearance of virus-infected cells. Analysis of the second-phase clearance rate may serve as a useful predictor of undetectable serum virus at 3 months after treatment. The effectiveness of interferon in blocking the production of new virus may contribute to both phases. Therefore, one can examine viral dynamics in patients by determining the block in virus production, the rate of viral clearance and the death of virus-infected cells. It has been noted that for genotype 1 or genotype 2 patients receiving 10 million units of interferon-α 2b for 14 days, the genotype 2 patients showed greater responses for all 3 facets [23]. This analysis of HCV dynamics may explain why genotype 2 viruses respond better to interferon-based therapies. Separately, some studies have noted the presence of an interferon sensitivity determining region in NS5A, but this may be patient specific [21].

Given concerns of interferon and ribavirin side effects and the lack of broad genotypic efficacy, there is clearly a need for additional antivirals including mechanistic inhibitors of specific viral functions. The viral functions currently targeted for antiviral discovery include the viral NS3/4A protease and the viral NS5B RNA polymerase. One hope for protease inhibitors, in addition to the suppression of viral replication, is a more rapid restoration of the endogenous cellular signal transduction pathways which contribute to the establishment of a cellular environment less favorable to HCV replication. That is, by blocking protease function, essential proteins in these pathways will not be cleaved by protease, allowing the signal transduction pathways to proceed normally.

Hepatitis C Virus NS3/4A Protease

Protease Inhibitor Discovery

Even before the development of cell culture systems to measure virus replication and to screen for inhibitors of HCV replication, the 3 viral enzymes, NS3/4A protease, NS3 helicase and NS5B RNA polymerase were the focus of biochemical assays to screen for HCV inhibitors [reviewed in 24]. Largely due to the availability of reagents, most of the basic biochemistry and screening efforts used enzymes from genotype 1b. Similarly after the description of robust HCV genotype 1b replicons, most of the cell culture activity and resistance selection studies have also used genotype 1b. As noted above, there may be a greater need for effective therapy for genotype 1 isolates as these respond less favorably to current interferon/ribavirin treatment.

The NS3/4A protease is a chymotrypsin-like serine protease with an unusual structural zinc ion [6]. The first 180 amino acids of NS3 are the protease domain and contain the catalytic triad consisting of histidine 57, aspartic acid 81 and serine 139

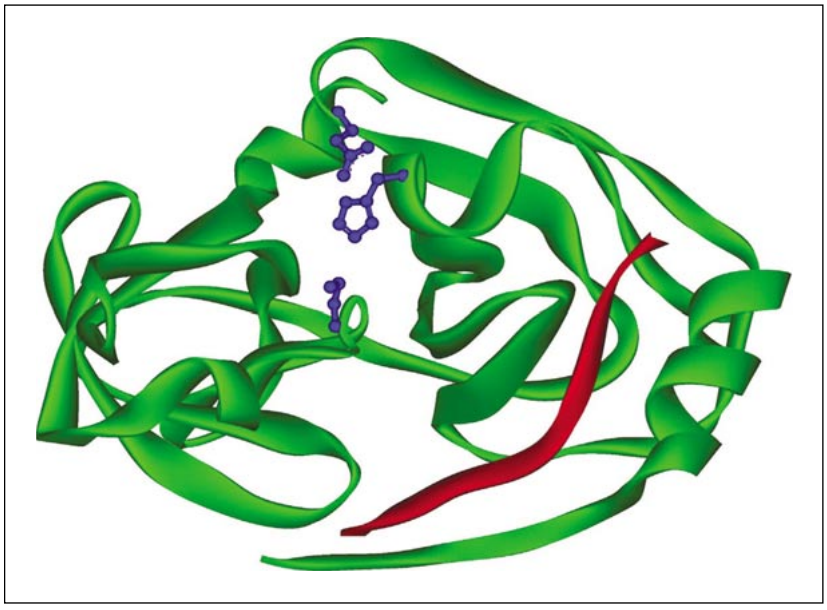


Fig. 2. The structure of the NS3 protease domain, amino acids 1–180, is shown in green. The blue ball and stick structures represent the catalytic triad of D81, H57 and S136 respectively from top to bottom. The NS4A protease cofactor is shown in red.

(fig. 2). Given the importance for catalysis, these residues are conserved across all genotypes. The NS4A protease cofactor is 54 amino acids in length, however, in biochemical assays, truncated versions are also functional.

The NS3/4A protease first makes a *cis*-cleavage between NS3 and NS4A. The protease then makes 3 *trans*-cleavages to liberate separate NS4B, NS5A and NS5B proteins. Figure 3 [redrawn and modified from 25] shows the 6 amino acids upstream to the cleavage site (termed P6–P1 as one approaches the cleavage site) and 1 amino acid downstream from the cleavage site (termed P1'). When one examines the protease cleavage sites, one can see 2 levels of variation. Within any isolate, the 4 NS3/4A sites differ from one another. For example, the NS3/4A cleavage site of genotype 1a is DLEVVT/S (cleavage between P1 threonine and P1' serine), while the NS4A/4B cleavage site of genotype 1a is DEMEEC/S. The second level of variation is across genotypes. For example, the NS4B/5A cleavage site of genotype 1a is ECTTPC/S, while the same site for genotype 1b is DCSTPC/S. There are, however, some similarities across both cleavage sites and genotypes. The P6 residue is generally acidic, either aspartic acid (D) or glutamic acid (E); the exception being the genotype 3a NS5A/5B site, where P6 is glutamine (Q). The second generalization is that the P1' site is preferentially alanine (A) or serine (S).

As seen in the structure of the NS3/4A protease, the active site generally appears as 'flat', seemingly lacking in a specificity-binding pocket. This, coupled with the length

Genotype and strain	NS3/4A							NS4A/4B						
1a 1	D	L	E	V	V	T	S	D	E	M	E	E	C	S
1a H77	D	L	E	V	V	T	S	D	E	M	E	E	C	S
1b Con1	D	L	E	V	V	T	S	D	E	M	E	E	C	A
1b BK	D	L	E	V	V	T	S	D	E	M	E	E	C	A
1b J	D	L	E	V	V	T	S	D	E	M	E	E	C	A
1b N	D	L	E	V	V	T	S	D	E	M	E	E	C	A
2a J6	D	L	E	V	M	T	S	D	E	M	E	E	C	A
2a JFH-1	D	L	E	V	M	T	S	D	E	M	E	E	C	A
2b J8	D	L	E	I	M	T	S	D	E	M	E	E	C	A
3a NZL1	D	L	E	V	T	T	S	D	E	M	E	E	C	S
4a ED43	D	L	E	V	V	T	S	D	E	M	E	E	C	S
5a EVH1480	D	L	E	V	I	T	S	D	E	M	E	E	C	S
6a EUHK2	D	L	E	V	I	T	S	D	E	M	E	E	C	S
P residue	6	5	4	3	2	1	1'	6	5	4	3	2	1	1'

	NS4B/5A							NS5A/5B						
1a 1	E	C	T	T	P	C	S	E	D	V	V	C	C	S
1a H77	E	C	T	T	P	C	S	E	D	V	V	C	C	S
1b Con1	D	C	S	T	P	C	S	E	D	V	V	C	C	S
1b BK	D	C	S	T	P	C	S	E	D	V	V	C	C	S
1b J	D	C	S	T	P	C	S	E	D	V	V	C	C	S
1b N	D	C	S	T	P	C	S	E	S	V	V	C	C	S
2a J6	D	C	P	I	P	C	S	D	S	V	V	C	C	S
2a JFH-1	D	C	P	I	P	C	S	D	T	T	V	C	C	S
2b J8	D	C	P	V	P	C	S	D	S	V	I	C	C	S
3a NZL1	D	Y	P	S	P	C	S	Q	S	V	V	C	C	S
4a ED43	D	C	S	T	P	C	A	E	D	V	V	C	C	S
5a EVH1480	D	Y	S	T	P	C	D	D	N	V	V	C	C	S
6a EUHK2	D	T	A	T	P	C	A	D	D	V	V	C	C	S
P residue	6	5	4	3	2	1	1'	6	5	4	3	2	1	1'

Fig. 3. The HCV NS3/4A protease cleavage sites are shown. Representative sequences from the major HCV genotypes 1a, 1b, 2a, 2b, 3a, 4a, 5a and 6a are listed with the HCV strain name following the genotype designation. The vertical bar represents the cleavage sites between NS3/4A, NS4A/4B, NS4B/5A and NS5A/5B. P residue depicts the order of amino acids from 6 amino acids before the cleavage site (P6) through 1 amino acid after the cleavage site (P1′). The standard 1-letter amino acid code is used throughout. The Genbank accession numbers for the isolates shown are AF009606, 1a H77; M62321, 1a 1; AJ238799, 1b Con1; D90208, 1b J; M58335, 1b BK; AF139594, 1b N; D00944, 2a J4; AB047639, 2a JFH-1; D10988, 2b J8; D17763, 3a NZL1; Y11604, 4a ED43; Y13184, 5a EVH1480; Y12083, 6a EUHK2.

of the peptide substrate as described above, suggested that the search for specific NS3/4A inhibitors displaying high-affinity binding may be difficult. One way around this problem was the discovery of macrocyclic protease inhibitors such as BILN 2061 (Ciluprevir, Boehringer Ingelheim) [26]. BILN 2061 displays a P1-P3 linkage and the

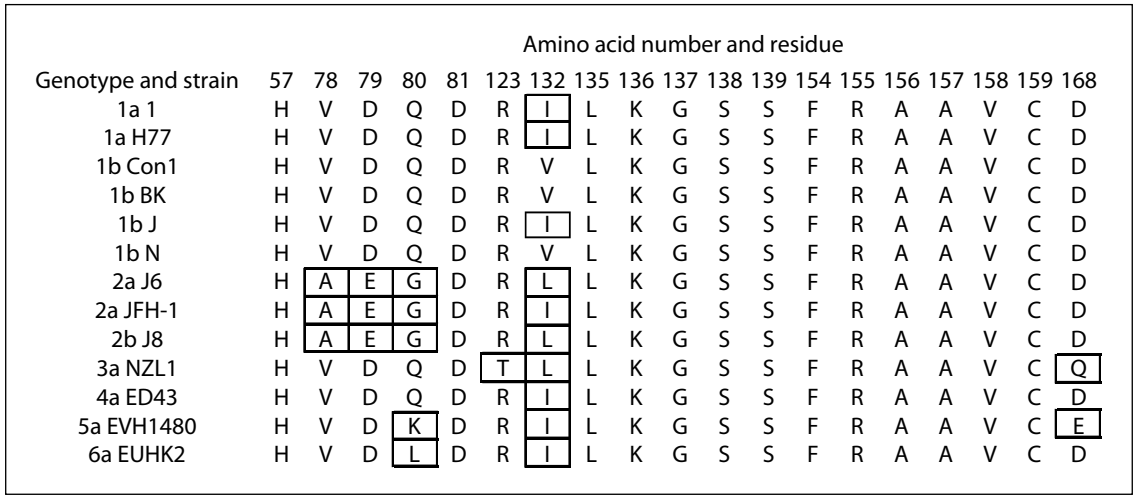


Fig. 4. Amino acids within 5 Å of the binding site of BILN 2061 (Boehringer Ingelheim) are taken and extended from Thibeault et al. [27]. The amino acid number is shown along with the amino acid residue in that position for the major HCV genotypes. Genotype designation, strain name and accession number are as in the legend to figure 3. Boxed amino acids are those which differ from the sequence for 1b Con1. Con1 is chosen as the reference sequence as this is the isolate most commonly used for replicon studies.

size of the inhibitor may provide for additional interactions with the enzyme (fig. 4). BILN 2061 is a reversible inhibitor and does not form a covalent interaction with the enzyme. Using the full-length NS3/4A protease, BILN 2061 has a reported Ki of 1.5–1.6 nM for genotype 1a and 1b, but $>$80 nM for the enzymes of genotypes 2a, 2b or 3 [27]. The compound is active against the 1b replicon with an EC_{50} of 3–6 nM [26, 28, 29] and also against the JFH-1 virus in cell culture with an EC_{50} of 246 nM [20]. BILN 2061 was taken into genotype 1 patients and 2-day treatment (25, 200 or 500 mg, twice daily) resulted in $\geq$2-log drop in virus titer in selected patients [26]. Subsequent trials showed up to 3-log drop in titers in genotype 1 patients receiving the 500-mg dose [30]. The viral levels rebounded to baseline 1–7 days following cessation of treatment. Unfortunately, development of BILN 2061 was terminated due to adverse heart effects during animal safety studies [30]. A second macrocycle, ITMN-191 (Intermune), is also shown in figure 4.

Another approach to the discovery of NS3/4A protease inhibitors is the use of linear peptidomimetics exemplified by VX-950 (Telaprevir, Vertex). With the linear compounds it is more straightforward to observe the peptide backbone and for VX-950, a 4 amino acid scaffold is shown in figure 4. Due to the presence of its C-terminal α-ketoamide, VX-950 forms a covalent interaction with the protease with slow binding and slow dissociation kinetics [31]. The authors reported inhibition constants of 7, 30–50 and 300 nM for protease for genotypes 1a, 2a and 3a respectively.

The EC_{50} for the genotype 1b replicon is submicromolar [32]. In the replicon system, 7 μM of compound for 9 days resulted in a >4-log drop in viral genomes. Treatment of replicon cells for 13 days with 17.5 μM of VX-950 eliminated the replicon to the extent that when the protease inhibitor was removed and the neo selectable compound G418 was added, no replicon harboring colonies developed. VX-950 was reported to be additive to moderately synergistic with interferon-α in replicon cells. When VX-950 was taken to the clinic for proof of concept phase I trials, in the VX-950 monotherapy arm, the greatest reduction in virus titer of up to 4.4 log decrease was seen in the group receiving 750 mg every 12 h for 14 days [33].

SCH 503034 (Boceprevir, Schering) is a linear peptidomimetic ketoamide which also has the ability to form a covalent adduct with the enzyme active site serine resulting in a long half-life of approximately 23 h [34]. The Ki binding constant for adduct formation is 14 nM and the EC_{50} in the replicon system is 200 nM. SCH 503034 shows additivity with interferon when both are used to treat replicon cells. In phase Ib trials, 400 mg of SCH 503034 3 times daily resulted in a 1.61-log decrease in virus titer in genotype patients who were previous nonresponders to interferon-α 2b +/− ribavirin therapy [35].

It was noted above that the NS3/4A protease may interfere with the innate immunity pathways by cleaving cell proteins within signal transduction pathways [reviewed in 22]. This is an important issue, but at this point, it remains an open question as to what degree protease inhibitors will impact the restoration of innate immunity pathways in patients.

Protease Inhibitor Resistance

Resistance selection using the neomycin colony selection protocol usually starts at some fold excess over the EC_{50}, for example $5 \times EC_{50}$, and may involve increasing selective pressure over time. Colony selection may take 3–4 weeks and this is followed by sequence analysis of the protease to determine the substitutions which may engender resistance. These substitutions may be reintroduced into a wild-type replicon bearing a reporter in transient transfection assays. These studies provide both a level of resistance and a measure of replication fitness. The caveat to these studies is that they generally employ only the genotype 1b replicon so that although the same amino acid substitution may engender resistance in another genotypic background, the EC_{50} shift and fitness values may not directly translate to other genotypes. For biochemical analysis, one can always generate recombinant protease carrying amino acid substitutions and measure biochemical resistance to inhibitors.

A comparison of genotype 1b protease with that from genotypes 2 and 3 with reference to the BILN 2061 binding site examined variation in the 19 amino acids within 5 Å of the binding site [27]. Figure 5 is redrawn and extended to include additional genotypes and shows the variation across NS3 protease for the 6 major genotypes at those 19 positions. Although there may be better conservation of residues close to the active site, across the rest of the protein, one sees the standard level of genetic variability

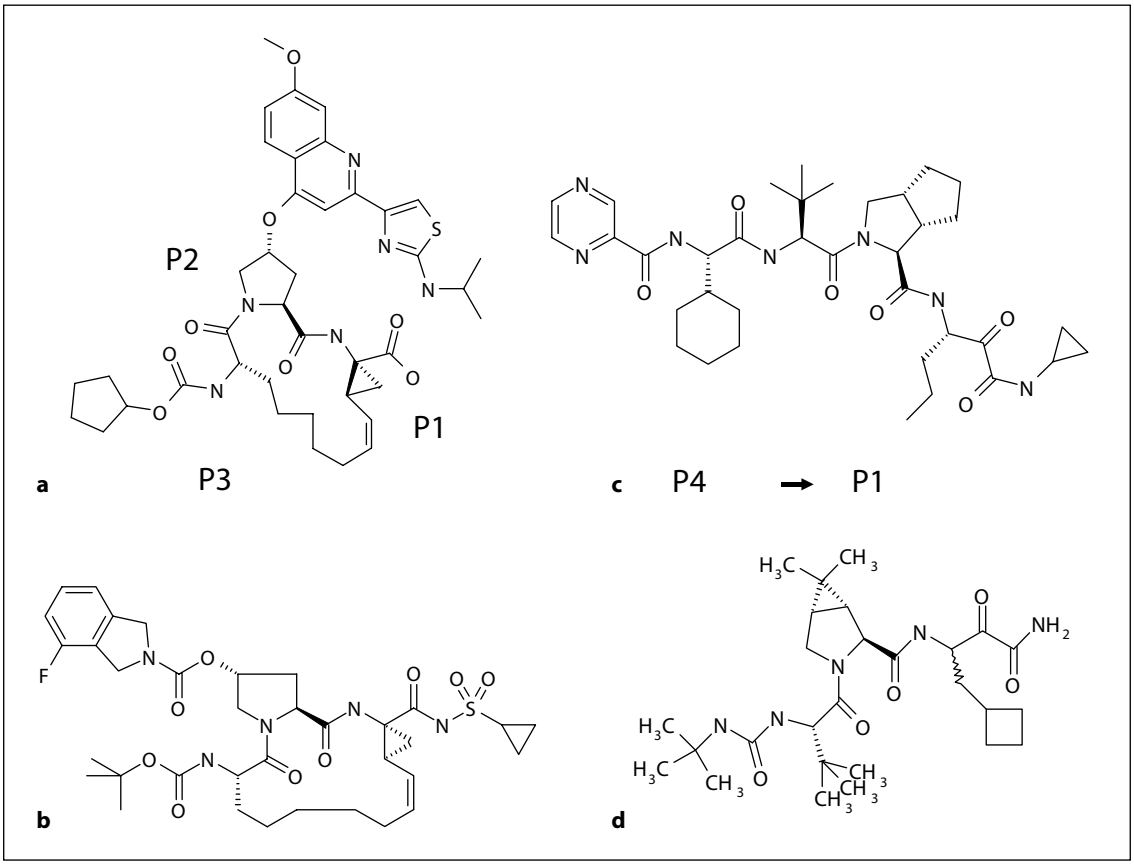


Fig. 5. Chemical structures of NS3/4A protease inhibitors. **a** BILN 2061 (Boehringer Ingelheim). **b** ITMN-191 (Intermune). **c** VX-950 (Vertex). **d** SCH 503034 (Schering). Structures are taken from references as described in the text. P numbers are provided for alignment with the protease cleavage site residues as described in the legend to figure 3.

across genotypes at other residues. One potentially important distinction amongst genotype active site residues is that all genotypes except 3a show arginine at position 123 (R123) and aspartic acid at position 168 (D168). Genotype 3a shows a pattern of threonine 123 (T123) and glutamine 168 (Q168). One may suppose that resistance is likely to develop in residues close to the active site to interfere with inhibitor binding. This does not eliminate the possibility that resistance may develop in distal residues resulting in a distortion of the inhibitor binding site. Finally, there is the possibility that since the protease domain is part of the larger NS3 protein, substitutions in the helicase domain may also influence inhibitor binding. Figure 5 focuses on the prototypic sequences for each genotype, major subtype and biologically active isolates. One

can also examine variation across all published sequences for these positions within a given genotype or subtype to address intertypic variation. For example, in October 2005, prior to the deposition of protease-resistant variants into Genbank, there were 145 full-length NS3/4A sequences for genotype 1b. Within that dataset, the major 2 variable positions amongst these 19 residues were 23 Q80L and 33 V132I. There were 2 minor variable positions and these were 1 example of R123K and 2 examples of D168E [R. L., unpubl. obs.].

A number of studies using genotype 1b replicons have demonstrated that substitutions at A156 can give rise to resistance against BILN 2061 (A156T/V), VX-950 (A156S/T/V), SCH 503034 (A156S/T) and ITMN-191 (A156S/V) [28, 29, 36–38]. Substitutions at residue D168 may also cause resistance to multiple compounds, for example BILN 2061 (D168A/V) and ITMN-191 (D168A/V/E) [28, 29, 38]. For individual compounds, the third active site residue involved in resistance to BILN 2061 is R155Q [29]. A number of other residues have been implicated in resistance to ITMN-191 and these include the nonactive site residue Q41R, the active site residue S138T, a helicase residue S489L and an NS4A residue V23A [38]. Nonactive site residues T54A and V170A may contribute to resistance to SCH 503034 [37]. In clinical testing, VX-950 monotherapy, the major resistant variants were V36A/M, T54A, R155K/T or A156S/T/V in genotype 1 patients, while R155G/I/M/S were less frequently observed [39, 40].

For the confirmation that these amino acid substitutions confer resistance, one can introduce the substitution into the recombinant enzyme for biochemical verification. One can also introduce the amino acid change(s) into the replicon to measure both replicative fitness and resistance. Without detailing every substitution, 2 generalizations can be made. The introduction of single substitutions into the replicon, especially for the predominant residues R155, A156 and D168, resulted in diminished replication capacity compared to wild-type replicons and the resistant replicons retained sensitivity to interferon [29, 36, 40].

A summary of the distribution across genotypes for the residues implicated in resistance to $\geq$1 protease inhibitors is shown in figure 6. This figure also summarizes which substitutions have been found to confer resistance to the 4 inhibitors discussed above. The examination of variation in positions implicated in protease inhibitor resistance suggests that the frequency of naturally resistant isolates is low [41]. Resistance determining substitutions may arise randomly in the absence of selective pressure due to the fidelity error rate of the viral polymerase. However, if these variants suffer a diminished replicative fitness, they will likely be outgrown except under selective pressure. It is also possible that under selective pressure, the resistant variant may develop second-site compensatory substitutions to restore some level of fitness. With the advancement of compounds into clinical trials, an analysis of the patient viral titers before, during and after treatment and amino acid substitutions over time in response to treatment provides important information regarding the development of resistance.

	Amino acid number and residue									
	NS3 protease domain								Helicase	NS4A
Genotype and strain	36	41	54	138	155	156	168	170	489	23
1a 1	V	Q	T	S	R	A	D	I	S	V
1a H77	V	Q	T	S	R	A	D	I	S	V
1b Con1	V	Q	T	S	R	A	D	V	S	V
1b BK	V	Q	T	S	R	A	D	V	S	V
1b J	L	Q	T	S	R	A	D	I	S	V
1b N	V	Q	T	S	R	A	D	V	S	V
2a J6	L	Q	T	S	R	A	D	I	V	V
2a JFH-1	L	Q	T	S	R	A	D	I	V	V
2b J8	L	Q	T	S	R	A	D	I	V	I
3a NZL1	L	Q	T	S	R	A	Q	I	V	V
4a ED43	L	Q	T	S	R	A	D	V	A	V
5a EVH1480	L	Q	T	S	R	A	E	V	V	V
6a EUHK2	V	Q	T	S	R	A	D	I	V	V
Compound	Amino acid substitution									
BILN 2061					Q	TV	AV			
ITMN-191		R		T		SV	AVE		L	A
SCH 503034			A			ST		A		
VX-950	AM		A		KT	STV				
Amino acid number	36	41	54	138	155	156	168	170	489	23

Fig. 6. Residues implicated in resistance to protease inhibitors. Genotype and strain are as in the legend to figure 3. Amino acid number and protein domain are shown as NS3 protease 1–180 (NS3 protease), NS3 helicase domain amino acids 181–631 (helicase) and NS4A protease cofactor (NS4A). Amino acids conferring resistance are presented for the 4 compounds described in the text; these compounds are BILN 2061 (Boehringer Ingelheim), ITMN-191 (Intermune), SCH 503034 (Schering) and VX-950 (Vertex). References for amino acids substitutions are given in the text.

Hepatitis C Virus NS5B RNA-Dependent RNA Polymerase

HCV NS5B is the viral RNA polymerase and uses the viral RNA genome as template. By analogy with other related viruses, the polymerase first produces a (−) strand copy of the genome followed by the asymmetric production of more (+) RNA than (−) RNA. Some of the (+) RNA enters the replication pool to drive the production of even more (−) and (+) RNA, some of the (+) RNA is translated to produce viral proteins, and some of the (+) RNA is packaged as progeny virus [6]. Cellular RNA polymerases use DNA as a template, so the description of viral RNA-dependent RNA polymerases suggests that these may be promising as antiviral targets [24].

Although the full-length NS5B from all genotypes consists of 591 amino acids, the production of soluble enzyme for biochemical, screening and crystallographic studies initially relied on C-terminal truncated versions of the viral polymerase. These

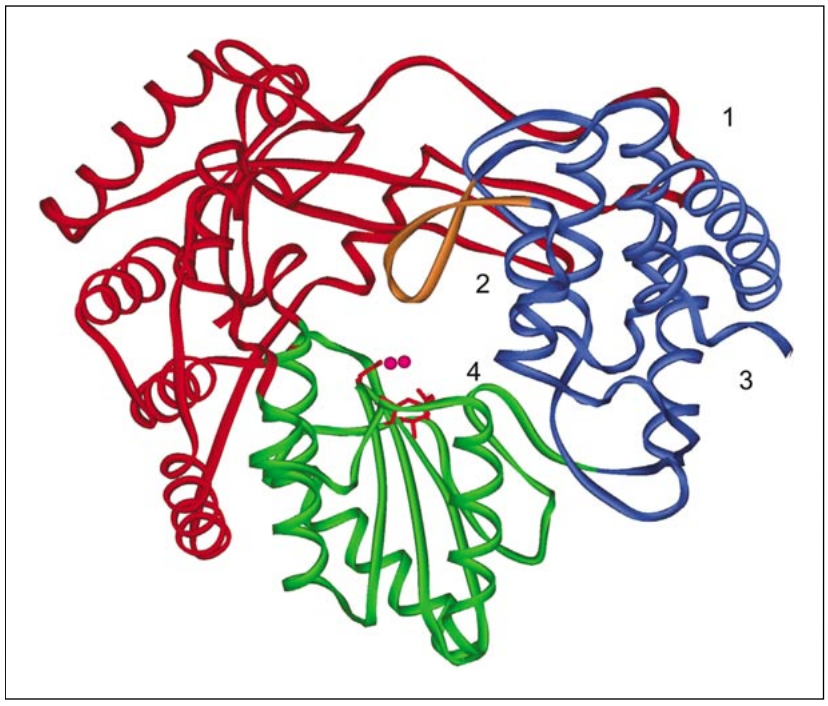


Fig. 7. Structure of HCV NS5B δ-55 RNA polymerase. The finger domain is in red, the palm domain in green, the thumb domain in blue and the β-loop in yellow. The ball and stick amino acids towards the center of the figure represent the catalytic aspartic acid residues. The 2 metal ions are depicted as purple spheres. General locations of nonnucleoside inhibitor binding sites are indicated by 1, 2, 3 and 4.

enzymes consisted of a C-terminal 55 amino acid truncated version and a similar C-terminal 21 amino acid truncated enzyme and these are termed δ-55 and δ-21 respectively. X-ray crystallography of the HCV NS5B RNA polymerase shows that like other replicative polymerases, it can be viewed as a right hand having fingers, palm and thumb domains [42–44]. The perspective of figure 7 is shown with the thumb on the right and the finger domain on the left, and the palm is centered. NS5B has 2 loops which connect the finger and thumb domains (δ-1 and δ-2 loops). Finally, NS5B has a loop, termed the β-loop, that extends off of the thumb in towards the palm of the protein. The NS5B palm contributes to the active site and contains a GDD motif (amino acids 317–319) characteristic of other viral RNA-dependent RNA polymerases. Active site aspartic acid residues coordinate 2 metal ions to interact with the phosphates in the primer and incoming NTP. This allows a nucleophilic attack by the primer ribose 3′-OH on the α-phosphate of the NTP to release pyrophosphate and extend the primer by 1 base. The overall biochemistry of the extension reaction is thus generally well conserved amongst polymerases and this is obviously not surprising.

Most other polymerases utilize a primer longer than a single nucleotide. However, HCV NS5B can use a single nucleotide, preferentially GTP, as a primer in a reaction

termed de novo initiation [45]. This observation impacts inhibitor discovery efforts and the way in which the antiviral screens for inhibitors are performed. One can set up biochemical assays with differential ordering of addition of reaction components; the reaction components being enzyme, primer/template, NTPs and inhibitor. For example, one can incubate enzyme, primer/template plus inhibitor and then initiate the elongation reaction by the addition of NTPs. One can also screen for inhibitors of de novo initiation using enzyme, template and inhibitor and then add high levels of GTP to serve as the primer and other NTPs to allow processivity. One could find different classes of inhibitor depending upon the assay configuration.

As for NS3/4A protease, much of the basic biochemistry and antiviral screening was performed using the polymerase from genotype 1b. So again, another important question is specificity and as with protease, this falls on 2 levels, selectivity for the viral polymerase over host cell polymerases and broad activity across multiple genotypes.

Nucleoside Analogs

One lesson learned from inhibitor studies using other viruses is that nucleoside analogs can be discovered which have selectivity for viral polymerases over cellular polymerases. In addition to host cell polymerases, nucleoside analogs useful as antivirals should not inhibit other cellular enzymes involved in the metabolic pathways associated with nucleotide biochemistry or enzymes such as NTPases including helicases and kinases. Nucleoside analogs act as chain terminators, thus by definition, they must act at the viral polymerase active site. Another feature of nucleoside analogs is that they are inherently prodrugs. The nucleoside must be taken into the cell and phosphorylated by kinases to the NTP. The active inhibitor is the triphosphate. There are thus 2 separate processes essential for nucleoside analog activity, namely uptake and phosphorylation. The converse of this is that nucleoside analogs would not be active in biochemical assays using purified polymerase. Biochemical screening assays require the NTP to demonstrate inhibition of elongation. For this reason, it is often more direct to screen nucleoside analogs using the cell-based HCV replicon system, where inhibitors of replication must have shown sufficient uptake and phosphorylation to serve as effective inhibitors of replication. Multiplexing the cell-based assay to simultaneously measure cytotoxicity serves as a general counterscreen to eliminate inhibitors of cellular enzymes. Nucleoside analogs can be chemically triphosphorylated for use in polymerase assays to demonstrate biochemical confirmation of inhibition. Figure 8 shows nucleoside analogs demonstrated as inhibitors of HCV replication.

A demonstration of the importance of uptake and phosphorylation comes from the use of ribose 2′-C-methyl-purine-based nucleoside analogs [46]. The biochemical IC_{50} (inhibitory concentration necessary to achieve 50% inhibition in a biochemical assay where the exact concentration of the compound added to the reaction is known) value for 2′-C-methyl-7-deaza-GTP is 100 nM, while that for the related 2′-C-methyl-7-deaza-ATP is essentially the same at 108 nM. However, in the replicon

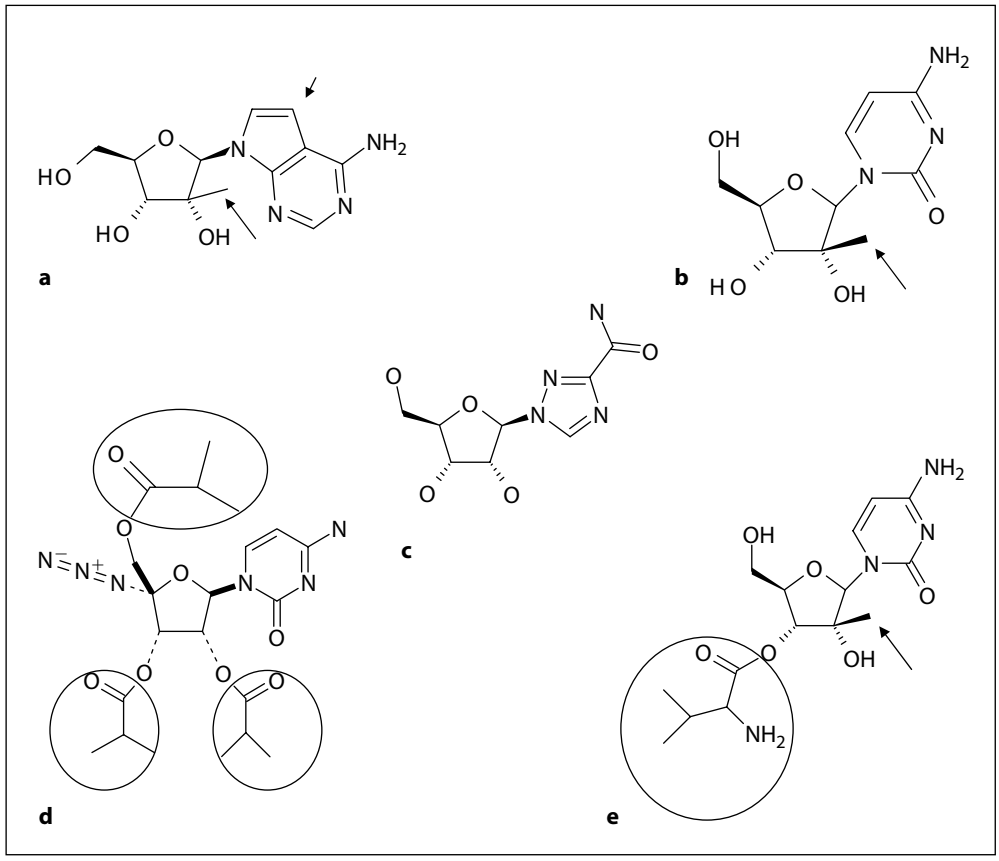


Fig. 8. Nucleoside analog inhibitors of HCV NS5B polymerase. **a** 2′- C-methyl-7-deaza-adenosine (MK-0608, Merck). The short arrow depicts the 7-deaza site. **b** 2′-C-methylcytidine (NM107, Idenix). **c** Ribavirin. **d** 4′-azidothymidine prodrug (R1626, Roche). **e** 2′-C-methylcytidine prodrug (NM283, Idenix). **a**, **b**, **e** The long arrow indicates the 2′-C-methyl ribose modification. **d**, **e** The circled elements indicate the prodrug modifications. References for structures are given in the text.

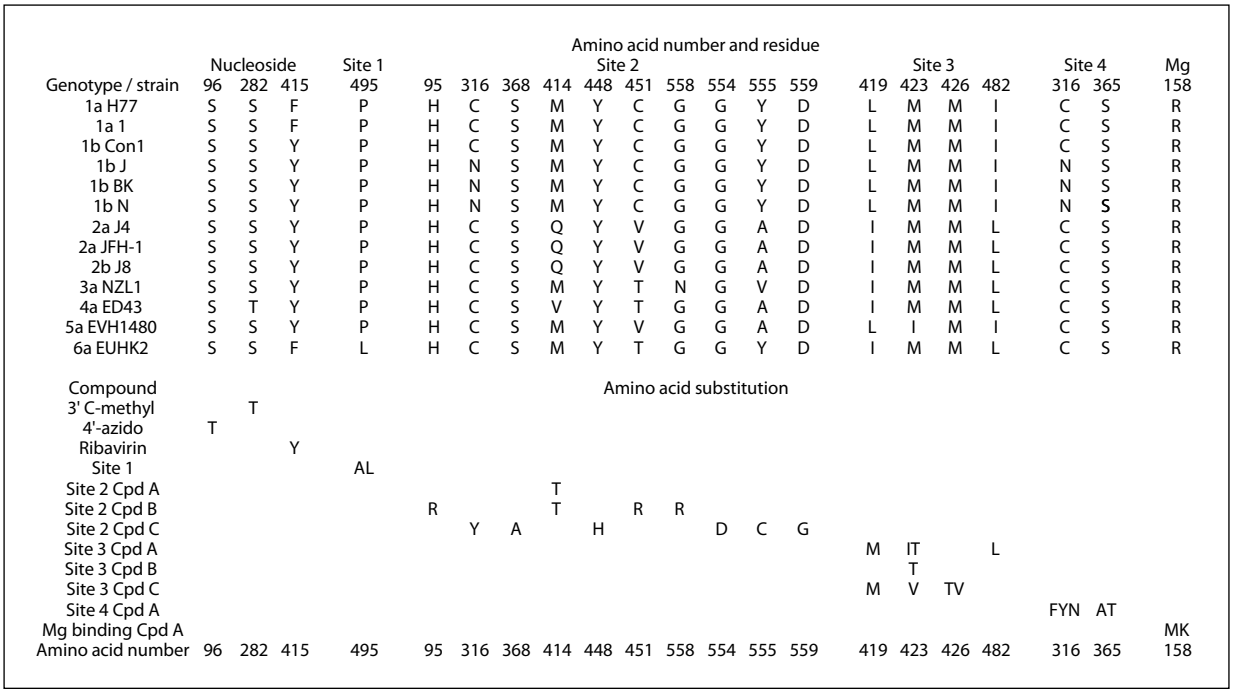
system 2′-C-methyl-7-deaza-guanoosine is essentially inactive at >50 μM, while 2′-C-methyl-7-deaza-adenosine (fig. 8, MK-0608, Merck) shows quite good inhibition of HCV replication with an EC_{50} of 300 nM. One can analytically measure the degree of uptake and phosphorylation of radio-labeled nucleoside and for 2′-C-methyl-7-deaza-adenosine >50% of the intracellular compound is phosphorylated to the triphosphate within 3 h in replicon cells following the addition of 2 μM of compound to the media. This compound shows similar activity against the related pestivirus bovine viral diarrhea virus in cell culture but starts to lose activity against other (+) strand RNA viruses such as flaviviruses and picornaviruses. Compounds with a blocked 3′ hydroxyl or lacking a 3′ hydroxyl would be expected to be efficient chain terminators in that once incorporated, they cannot be extended. The 2′-C-methyl

compounds are not obligate chain terminators but extension of an incorporated 2′-C-methyl compound is inefficient [46]. Molecular modeling suggests that the presence of the incorporated 2′-C-methyl group at the primer terminus may distort the positioning of incoming NTP, thus severely reducing elongation [46].

Although nucleoside analogs are inherently prodrugs, additional modifications especially to the ribose hydroxyls leads to compounds which are themselves prodrugs of the active nucleoside analog. These modifications are usually synthesized to increase oral bioavailability. Selected modifications may also serve to prevent degradation, increase cell uptake or allow for specific organ targeting. One prodrug found to be active against HCV is shown in figure 8 as NM283 (valopicitabine, Idenix), a 3′ ribose O-valine ester, in comparison with the parental NM107, 2′-C-methylcytidine, a pyrimidine nucleoside analog [47]. Another prodrug, R1626 (Roche), with modifications to the 2', 3′ and 5′ ribose hydroxyls is also shown. R1626 is not a 2′-C-methyl compound but rather a 4′-azido modified compound [48]. The active nucleosides, 4′-azidocytidine (R1479, Roche) and 2′-C-methylcytidine, show similar activity in the replicon with EC_{50} values of 1.28 and 1.13 μM respectively and R1479-TP is active against genotype 1b NS5B with an IC_{50} of 300 nM [48]. R1479-TP is incorporated into RNA by NS5B, but once incorporated, further elongation was blocked, demonstrating that its mechanism of action is via chain termination [49].

Resistance to Nucleoside Analogs

The methodology for the selection of resistant replicons generally follows that described above for protease inhibitors. Cells containing the selectable replicon are cultured in the presence of inhibitor, surviving colonies are analyzed for the presence of amino acid substitution, and these substitutions are introduced into both the wild-type replicon and the recombinant enzyme to demonstrate resistance. In the replicon system, 2′-C-methyl-7-deaza-adenosine selects resistant replicons carrying the amino acid substitution S282T in the NS5B polymerase (see fig. 9 for amino acid variation in all positions contributing to NS5B resistance to polymerase inhibitors) [46]. A colony selected for resistance to the deaza compound shows about a 30-fold shift in EC_{50} relative to the parental replicon cell line. This value may reflect inherent resistance, but different cell lines may show altered uptake and phosphorylation efficiencies. The NS5B enzyme carrying the S282T substitution is also resistant to 2′-C-methyl-7-deaza-ATP with IC_{50} of 0.07 and 25 μM for the parental and S282T enzymes respectively [46]. The introduction of S282T into the 1b replicon or a 1a polymerase in an otherwise 1b replicon background demonstrates that the replicon has reduced fitness relative to the parental replicon showing about 10–20% of the reporter activity at 6 days after transfection [50]. The S282T substitution also confers resistance to 2′-C-methylcytidine [51]. It is interesting that the one carbon addition of the methyl group to the ribose is counteracted by the substitution of serine to threonine, which also has one carbon addition. S282 is conserved across all genotypes with the exception of genotype 4a, where the prototype ED43 strain has threonine at position 282 (Genbank



	Amino acid number and residue																				
	Nucleoside			Site 1	Site 2										Site 3				Site 4		Mg
Genotype / strain	96	282	415	495	95	316	368	414	448	451	558	554	555	559	419	423	426	482	316	365	158
1a H77	S	S	F	P	H	C	S	M	Y	C	G	G	Y	D	L	M	M	I	C	S	R
1a 1	S	S	F	P	H	C	S	M	Y	C	G	G	Y	D	L	M	M	I	C	S	R
1b Con1	S	S	Y	P	H	C	S	M	Y	C	G	G	Y	D	L	M	M	I	C	S	R
1b J	S	S	Y	P	H	N	S	M	Y	C	G	G	Y	D	L	M	M	I	N	S	R
1b BK	S	S	Y	P	H	N	S	M	Y	C	G	G	Y	D	L	M	M	I	N	S	R
1b N	S	S	Y	P	H	N	S	M	Y	C	G	G	Y	D	L	M	M	I	N	S	R
2a J4	S	S	Y	P	H	C	S	Q	Y	V	G	G	A	D	I	M	M	L	C	S	R
2a JFH-1	S	S	Y	P	H	C	S	Q	Y	V	G	G	A	D	I	M	M	L	C	S	R
2b J8	S	S	Y	P	H	C	S	Q	Y	V	G	G	A	D	I	M	M	L	C	S	R
3a NZL1	S	S	Y	P	H	C	S	M	Y	T	N	G	V	D	I	M	M	L	C	S	R
4a ED43	S	T	Y	P	H	C	S	V	Y	T	G	G	A	D	I	M	M	L	C	S	R
5a EVH1480	S	S	Y	P	H	C	S	M	Y	V	G	G	A	D	L	I	M	I	C	S	R
6a EUHK2	S	S	F	L	H	C	S	M	Y	T	G	G	Y	D	I	M	M	L	C	S	R
Compound					Amino acid substitution																
3' C-methyl		T																			
4'-azido	T																				
Ribavirin			Y																		
Site 1				AL																	
Site 2 Cpd A								T													
Site 2 Cpd B					R			T		R	R										
Site 2 Cpd C						Y	A		H			D	C	G							
Site 3 Cpd A															M	IT		L			
Site 3 Cpd B																T					
Site 3 Cpd C															M	V	TV				
Site 4 Cpd A																			FYN	AT	
Mg binding Cpd A																					MK
Amino acid number	96	282	415	495	95	316	368	414	448	451	558	554	555	559	419	423	426	482	316	365	158

Fig. 9. Amino acids implicated in resistance to HCV NS5B polymerase inhibitors. The top part of the figure shows genetic variation for genotypic prototypes and biologically relevant isolates. Genotype designation, strain name and accession number are as in the legend to figure 3. The amino acid number is given along with the amino acid residue shown in that position for these strains. The bottom part of the figure shows amino acid substitutions which confer resistance to polymerase inhibitors. Nucleoside analog resistance determining substitutions are shown under the nucleoside column; 2′-C-methyl shows resistance to both MK-0608 and NM283; 4′-azido shows resistance to R1479 (compound structures in fig. 8). Genetic variation for the indicated isolates within nonnucleoside sites 1, 2, 3 and 4 and the Mg-binding site are given under their respective columns. Amino acid substitutions shown to confer resistance to compounds listed are provided for those positions along the compound name line. Nonnucleoside structures are illustrated in figure 10.

accession No. Y11604). However, T282 may not be an indicator of all genotype 4a sequences as additional sequence analysis of this same strain passed through chimps showed the presence of S282 [50]. MK-0608 is active in infected chimps and may result in up to a 5-log decrease in viral titers; at 1 postcompound dosing time point, in 1 chimp, there was detection of S282T, but this disappeared upon further posttreatment follow-up [52]. The S282T replicon retains sensitivity to R1479 [49].

For the 4′-azido compound prodrug, R1426, in phase Ib trials up to a 3.7-log drop in viral titers was observed and no resistance substitutions were observed during the 14-day dosage period [48]. In the Con1 replicon system, R1479 selects for resistance at S96T, but these replicons are not cross-resistant to the 2′-C-methylcytidine inhibitor [51]. S96 is conserved across all genotypes.

Ribavirin as a Nucleoside Analog

The error catastrophe model suggests that the mechanism of action of ribavirin (fig. 8) is to increase the frequency of mutations to the point that genomes accumulate sufficient mutations such that they cannot replicate [53]. This implies that as a triphosphate, ribavirin triphosphate acts directly on the viral polymerase. There is some biochemical evidence using a β-loop deletion of HCV NS5B to support the error catastrophe model [54]. There has been 1 report that genotype 1 patients treated with ribavirin showed a change in polymerase of F415Y [55]. When both variants were tested in the replicon, the Y415 replicon was resistant to ribavirin, while the F415 replicon was sensitive to ribavirin. When one looks across genotypes F415 is found in genotypes 1a and 6a. All other genotypes are Y415. Ribavirin is not very effective as an inhibitor in the 1b replicon system with an EC_{50} of approximately 40 μM [36, 40]. Attempts to select ribavirin resistance in the replicon system showed the presence of 2 substitutions in NS5A, G404S and E442G which may reduce the sensitivity to ribavirin [56]. Changes in NS5B which reduced the sensitivity to ribavirin were not detected. A direct comparison of replicons engineered to contain either Y415 or F415 showed that the F415 replicon was the more sensitive to inhibition by ribavirin.

Nonnucleoside Polymerase Inhibitors

As with nucleoside analogs and protease inhibitors much of the screening efforts for nonnucleoside inhibitors relied on the use of the NS5B from genotype 1b. Nonnucleoside inhibitors need not bind at the active site and may act as allosteric inhibitors. For HCV NS5B, there are multiple well-defined nonnucleoside binding pockets as indicated in figure 7.

Nonnucleoside Site 1

Inhibitors targeting nonnucleoside site 1 include the benzimadizoles (fig. 10, site 1, Cpd A, Japan Tobacco) [57] and related indoles (fig. 10, site 1, Cpd B, Merck) [58]. These compounds bind near the upper, outer surface of the thumb domain close to the region contacted by the first finger thumb loop. The benzimidazole compound shows an IC_{50} of 280 nM for the NS5B enzyme [58]. The benzimidazole is active against the 1b replicon with an EC of 300–350 nM. The indole compound shown is active in NS5B assays with an IC_{50} of 26 nM and is active against the 1b replicon with an EC_{50} of 800 nM [59]. Site 1 inhibitors may show distinct profiles in the order of addition experiments [58]. Although the IC_{50} does not shift, the residual enzyme activity remains when the inhibitor is added to a preformed enzyme template complex. In contrast, there is little residual enzyme activity when the enzyme is preincubated with inhibitor prior to the addition of template. These observations are consistent with a conformational change in the enzyme form between open (inactive) and closed (active) forms of the enzyme [59]. Site 1 inhibitors may lock the enzyme in the open conformation.

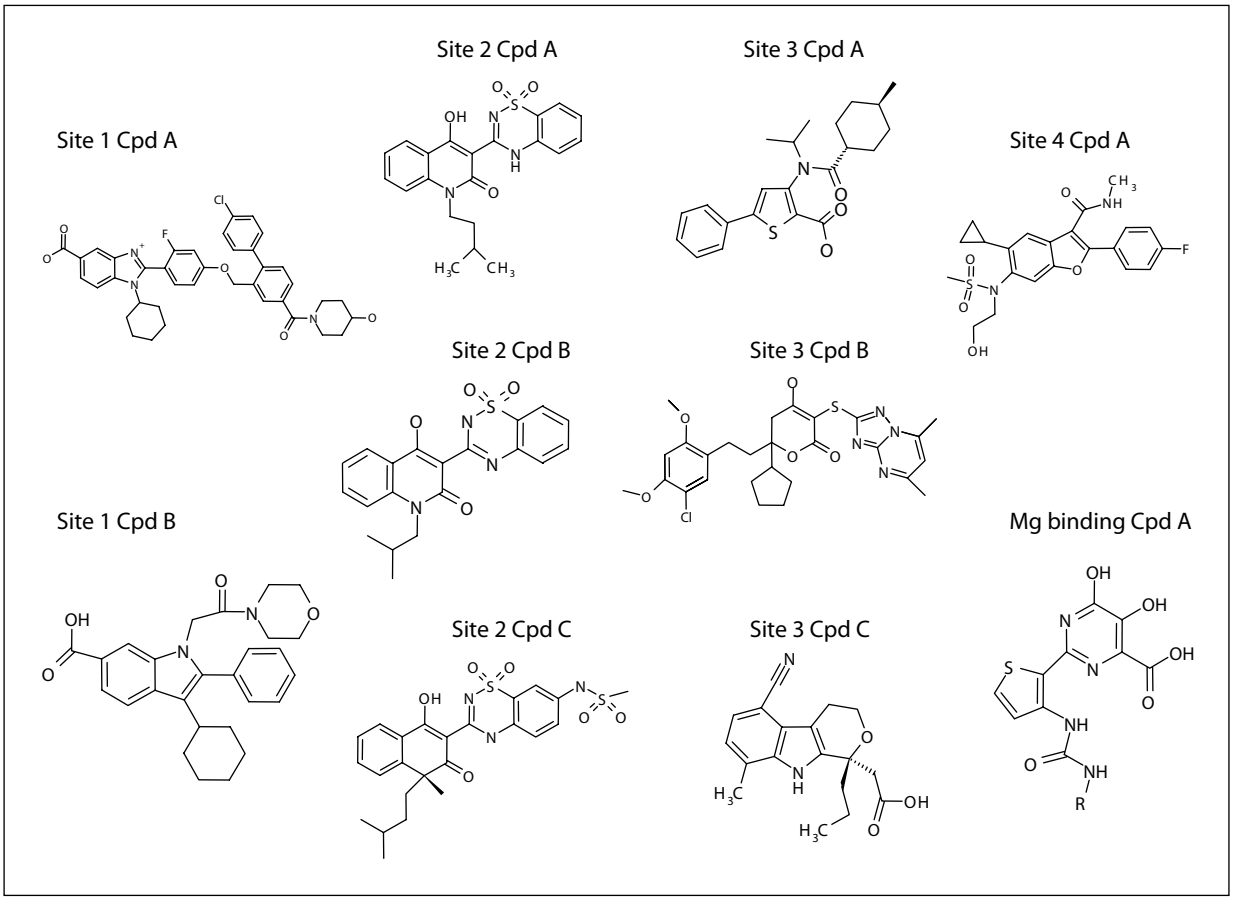


Fig. 10. Nonnucleoside inhibitors of HCV NS5B. Site 1 compound (Cpd) A is a benzimidazole (Japan Tobacco), and Cpd B is an indole (compound 1, Merck). Site 2 benzothiadiazines are Cpd A (compound 4, GSK), Cpd B (compound 2, Merck) and Cpd C (A-837093, Abbott). Site 3 inhibitors are Cpd A (thiophene, Shire), Cpd B (dihydropyrone, AG-021541, Pfizer) and Cpd C (pyronoindole, HCV-371, Wyeth/Viropharma). Site 4 Cpd A is HCV-796, a benzofuran (Wyeth/Viropharma). Mg binding Cpd A (compound 34, Merck) has an R group of CH2(2-Cl-Ph). Structures and company designations are taken from references described in the text.

Substitutions at residue 495 confer resistance to site 1 inhibitors [58]. In addition, replicons carrying NS5B P495L or P495A show reduced replicative capacity. P495 is conserved across HCV genotypes except for the genotype 6a prototype, EUHK2 strain (Genbank accession No. Y12083), which is L495.

Nonnucleoside Site 2

Nonnucleoside inhibitor site 2 is in the vicinity of the β-loop toward the internal part of the thumb. Site 2 inhibitors include the benzothiadiazines and 3 examples of these

molecules are shown in figure 7. The first compound (fig. 10, site 2, Cpd A, Glaxo) shown is active against the enzyme with an IC_{50} of 80 nM and has a replicon EC_{50} of 500 nM [60]. Replicon resistance selection for this benzothiadiazine was reported to arise predominantly by the M414T substitution and this change shifts the replicon EC_{50} to >10 μM; the enzyme carrying M414T also has an IC_{50} >10 μM [60]. M414 is generally conserved across all genotypes except for genotype 2 isolates, which have Q414.

The second compound (fig. 10, site 2, Cpd B, Merck) is active against NS5B with an IC_{50} of 300 nM, but the cell culture activity against the 1b Con1 replicon is relatively high at 10 μM [61]. Resistance selection pointed to residues H95R, M414T, C451R and G558R. These 4 substitutions each shifted the EC_{50} to >50 μM. In biochemical assays the enzyme containing M414T showed the greatest IC_{50} shift in both elongation and de novo initiation assays. For the elongation assay, the IC_{50} shifted from 300 nM to 5.9 μM (20-fold), while in the de novo reaction the wild-type enzyme had an IC_{50} of 60 nM, whereas the M414T enzyme had an IC_{50} of 3 μM (50-fold). The distribution of these 4 residues across HCV genotypes shows that H95 is generally conserved across all genotypes, M414T as noted above, is Q414 in genotype 2, and G558 is conserved across all genotypes except for genotype 3a, which carries N558. Residue 451 is variable in genotype 1b; C predominates over T, but Y, I, V and H have also been found. For the other genotypes, 1a carries C451, 2 and 5a carry V451, while genotypes 3, 4 and 6 show generally T451.

The third compound, A-837093 (fig. 10, site 2, Cpd C, Abbott), has a reported potency of 11 nM against the genotype 1a H77 replicon and 6 nM against the genotype 1b strain N replicon [62]. For A-837093, resistance selection for the 1b replicon tracks to residues S368A (173-fold EC_{50} shift), Y448H (19-fold shift), G554D (244-fold shift), D559G (288-fold shift) and Y555C. All replicons were debilitated for replicative capacity and the Y555C replicon was the most debilitated such that a shift in EC_{50} could not be determined [63]. In a genotype 1b infected chimp a 2.5-log drop in viral titer was observed by 2 days after treatment and subsequently 2 amino acid substitutions were reported, C316Y and G554D [64]. S368, Y448, G554 and G559 are generally well conserved across all genotypes. C316, which is close to the active site, is heterogeneous in genotype 1b strains with an approximate 1:1 split between C316 and N316. This residue is generally C316 across all other genotypes. Y555 is conserved in genotypes 1a and 1b (and also the 6a prototype), while A555 is generally found across genotypes 2a, 2b, 4a and 5a. Genotype 3a generally carries V555.

One important question for all antiviral resistance studies is whether given the high virus loads in patients and the mutation frequency of the viral polymerase, variants carrying amino acid substitutions which confer resistance exist in patients prior to the initiation of therapy. Another benzothiadiazine, A-782759 (not shown), selects for M414T. During the characterization of this inhibitor, it was noted that in the Con1 replicon, M414T exists in 0.22% of the population. In the genotype 1b strain N replicon cells, the M414T substitution exists in 0.18% of the population. Finally, 15 genotype 1b sera samples from compound naïve patients were examined for the presence

of M414T. Nine samples were below the limit of detection of 0.1%, while the other 6 patients had frequencies of M414T ranging from 0.11 to 0.6% [65].

Nonnucleoside Site 3

Nonnucleoside inhibitor site 3 is on the external side of the thumb domain, close to what one could consider as the base of the thumb. A number of inhibitors have been found to interact at this site and 3 examples are shown in figure 9. The first structure is a thiophene, while the second is dihydropyrone, and the third is a pyranoindole. Despite the structural differences, all 3 compounds interact at the same site.

The thiophene (fig. 10, site 3, Cpd A, Shire) [66] has a polymerase IC_{50} of 140 nM using the HCV IRES as template and 290 nM using poly-A-RNA as template and is active against the 1b Con1 replicon with a cell culture activity of 150 nM [67]. Resistance was tracked to substitutions L419M, M423T/I or I482L in NS5B. The 4 substitutions individually displayed an EC_{50} shift in the replicon assay and where tested the enzyme was also resistant to the thiophene. M423T, while displaying approximately 100-fold biochemical and almost 30-fold cell culture shifts in activity, was not debilitated for replication. In 1b Con1, M423I showed a replicative fitness of only 25% relative to the parental Con1 or the M423T replicons, while displaying a 15-fold EC_{50} shift in cell culture. M423 is conserved across all HCV genotypes except for genotype 5a, which is I423. L419 is found only in genotypes 1a, 1b and 5a and residue 419 is predominantly I in other genotypes. Residue 482 is I in genotypes 1a, 1b and 5a but L in all other HCV genotypes.

The dihydropyrone (fig. 10, site 3, Cpd B, Pfizer) has a cell culture EC_{50} of 2.3 μM against a novel Con1 replicon having both a quantifiable luciferase reporter and a selectable neo marker [68]. High-level resistance selection predominantly selects for replicons having M423T and the resistant replicons have an EC_{50} of 201 μM demonstrating an 87-fold shift in EC_{50}. The conservation of M423 across all HCV genotypes except for 5a was noted above.

The third nonnucleoside site 3 inhibitor shown is a pyranoindole (fig. 10, site 3, Cpd C, Wyeth/Viropharma). This compound is the R-enantiomer (HCV-371) purified from an earlier lead compound HCV-570, which contained a racemic mix of R- and S-enantiomers [69]. The parental replicon displayed an EC_{50} of 2.3 μM. Resistance selection found 4 single amino acid substitutions and these were L419M (10-fold EC_{50} shift), M423V (8-fold), M426T (4-fold) and M426V (3-fold). The distribution of L419 and M423 was described above for the thiophene. M426 is generally conserved across all HCV genotypes.

For all of the mentioned nucleoside analogs and nonnucleoside site 1, 2 and 3 inhibitors discussed above, a summary of genotypic variation and amino acids which confer resistance are shown in figure 9. Beside the primary discovery and resistance citations referenced above, additional analysis using purified enzymes from genotypes 1a, 1b, 2a, 2b, 3a, 4a, 5a and 6a and selected nonnucleosides from different classes of inhibitor has been published [70]. The authors also perform molecular

modeling and mutagenesis studies which may extend our knowledge of the residues which may impart loss of sensitivity to selected compounds.

Near Active Site Nonnucleoside

A fourth binding site which has recently been described as the interaction site for HCV-796 (fig. 10, site 4, Cpd A, Wyeth/Viropharma) is located closer to the active site. Resistance selection showed the presence of C316F/Y/N and S365A/T [71]. C316F or Y showed a >100-fold EC_{50} shift, while C316N demonstrated a 26-fold shift over the parental 1b EC_{50} of 3 nM. As noted above, residue 316 is heterogeneous in genotype 1b isolates. S365 is conserved across all HCV genotypes.

Compounds which interact with the active site Mg ions such as dihydroxypyrimidine carboxylates may mimic pyrophosphates (fig. 10, Mg binding, Cpd A, Merck). The compound shown has an R group of CH2(2-Cl-Ph) and is active in the replicon system with an EC_{50} of 9.3 μM [72]. This compound is active against the δ-21 form of the 1b, 2a and 3a polymerases with IC_{50} values of 45, 44 and 48 nM respectively. Resistant replicons have not been selected, but modeling and mutagenesis studies demonstrate the importance of R158 for binding of related compounds. Mutagenesis to R158M or R158K results in a decrease in potency. R158 is conserved across all genotypes.

Summary and Concluding Comments

Although this review concentrated on protease and polymerase inhibitors, there is a need to assess novel HCV targets. Among the possibilities which have not been covered are the interactions of the viral envelope glycoproteins with cell receptors. Studies along these lines may be aided by the recent description of an infectious viral isolate capable of recapitulating all of the steps in the virus replication cycle. Another novel approach not discussed in this review is the use of RNA interference; siRNA homologous to the viral genome followed by the degradation of the viral genome. Such siRNA molecules have been described as active in the replicon system. However, a complete illustration of this process falls outside of the scope of a review on mechanism-based inhibitors. Both of these approaches target the virus. As we gain more knowledge about the involvement of cell pathways linked to HCV replication, it may become more feasible to approach potential cellular targets. Along these lines, one may consider the use of small molecules to activate innate immunity. One example of this may be the activation of the TLR 7 pathway by small molecules. Again, efforts to define cell targets fall outside the main focus of this review.

This chapter has traced the development of biochemical and biological systems useful for the discovery of mechanism-based inhibitors to the HCV NS3/4A protease and NS5B polymerase. Special emphasis was put on the biochemical characterization and mechanism of action of these inhibitors and the selection for amino acid substitutions

which confer resistance to these inhibitors. Developmental aspects of these inhibitors leading up to clinical trials including lead optimization, pharmacokinetics and safety testing are generally compound specific and were not covered in this review. It is important to note that for any compound and any dosing regimen, sufficient compound levels even at trough should be sufficiently high to prevent viral replication thus impeding the development of antiviral resistance. Some of the compounds discussed are currently in clinical testing and some have already fallen out in clinical trials. The failure of compounds in clinical trials especially for reasons of safety issues accentuates the difficulties in licensure of effective antivirals. Given the proven need for inhibitors of HCV, it is obviously hoped that one or more of these compounds will survive clinical trials and prove to be effective inhibitors of HCV replication. Should this come to fruition, it is highly likely that these inhibitors will be co-dosed at least with interferon. When tested, usually using the replicon system, replicons resistant to protease or polymerase inhibitors retained sensitivity to interferon and it is thought that co-treatment with interferon may impede the selection of virus resistant to these inhibitors. If and when mechanism-based inhibitors targeting different viral functions ever replace interferon with and without ribavirin, is an open question. There may come a time when an analysis of a patient's circulating HCV may allow the clinician to tailor treatment to the patient.

Note Added in Proof

Two recent articles appeared which are relevant to the discussion of mechanism based inhibitors of HCV. β-D-2′-Deoxy-2′-fluoro-2′-*C*-methylcytidine (PSI-6130, Pharmasset/Roche) is a nucleoside analog inhibitor of NS5B polymerase [73]. Cellular uptake and metabolism leads to the formation of the monophosphate some of which undergoes deamination to β-D-2′-Deoxy-2′-fluoro-2′-*C*-methyluridine (RO2433-MP). Both the cytidine and the uridine compounds become triphosphorylated, and both triphosphates can inhibit NS5B polymerase although the cytidine compound is more active. Resistance is conferred by the S282T substitution in NS5B. For HCV NS3/4A protease, Merck recently described novel P2-P4 cyclic macrocyclic inhibitors. The best compound, 25a, showed 4.5 nM activity against the Con1 replicon and 0.07 nM activity against the enzyme [74].

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Role of the Hepatitis B Virus in Hepatocellular Carcinoma

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Abstract

Hepatocellular carcinoma (HCC) remains one of the leading causes of cancer death in the world due to late symptom manifestations and limited treatment options. The hepatitis B virus (HBV) has been epidemiologically linked to the development of HCC as chronic carriers of HBV have a significantly increased risk for HCC development later in life. HBV may play a role in HCC either through inducing recurring cycles of necrosis and regeneration or through integrating itself into the genome. When HBV is integrated into the host genome, it can alter the host genome (*'cis'* role) through insertional mutagenesis and/or its own genome. During integration, the HBV genome is often mutated, deleted, inverse duplicated and rearranged, and only versions of HBV genes like the PreS2 activators and HBV X protein (HBx) are retained and expressed. These proteins are implicated to play a *'trans'* role in hepatocarcinogenesis. HBx, a pleiotropic transactivator protein, interacts with numerous cellular proteins deregulating various cellular processes including signal transduction, DNA damage repair network, subcellular localization of proteins as well as centrosome integrity. HBx has also recently been implicated in the deregulation of the cellular epigenetic process.

Hepatocellular Carcinoma

Liver cancer is amongst one of the most common cancers in the world. Hepatocellular carcinoma (HCC) represents the most frequent subtype of liver cancer. Based on the GLOBOCAN 2002 database, the world standardized incidence rate of liver cancer per 100,000 is 15.8 worldwide, 36.9 in Eastern Asia, 27.3 in Middle Africa and 18.3 in Southeast Asia (http://www-dep.iarc.fr/). It is the third leading cause of cancer death in the world with a world standardized mortality rate per 100,000 at 14.9, which is similar to its incidence rate. This high mortality results from the poor prognosis due to the late manifestations of symptoms and its unresponsiveness to treatment [1]. Currently, the most effective treatment strategies for HCC include surgical resection and liver transplantation, while other treatments, e.g. chemotherapy, are primarily palliative [2–4].

However, therapies that can prolong life for up to a few years are only available in developed countries, while most people with HCC in developing countries die within months of diagnosis. Nonetheless, even with the availability of these life-prolonging treatment strategies, the number of resectable cases remains small, due to the multifocality of the tumor, early vascular invasion and concurrent liver cirrhosis that complicate the treatment.

The risk factors for the development of HCC include hepatitis B and C infection, aflatoxin exposure, excessive alcohol consumption and rare genetic disorder, e.g. hemochromatosis [5]. Of these, viral hepatitis [hepatitis B virus (HBV) and hepatitis C virus] infection emerged as the most epidemiologically associated risk factor as it accounts for 75–80% of all HCC cases in the world [6]. Worldwide, 50–55% of all HCC cases have been associated with HBV [1, 6], while 25–30% have been related to hepatitis C virus [6]. In fact, in some countries, e.g. Taiwan, HBV is implicated to account for ~80% of all HCC patients [6, 7]. Chronic carriers of HBV have a 20- to 100-fold higher risk for the development of HCC compared to noncarriers [5, 7–9].

Hepatitis B Virus

The HBV virus represents one of the first viruses to be causally linked to a human tumor [7]. It belongs to the family of hepadnaviruses, which are small enveloped DNA viruses [10] characterized by their pronounced tropism to the liver [11]. Four open reading frames, which encode for the surface proteins, core protein, polymerase protein and the X protein, have so far been identified in the small 3.2-kb DNA genome of HBV [12], which is circular and partially double-stranded (fig. 1).

Infection with the HBV represents a serious global public health problem as chronically infected individuals are at a higher risk of death from liver cirrhosis and cancer. According to the World Health Organization, of the 2 billion people who have been infected with HBV, more than 350 million people have chronic (lifelong) infection (http://www.who.int/mediacentre/factsheets/fs204/en/).

The availability of safe and effective vaccines against HBV makes this a preventable disease. However, poor compliance with immunization programs in some countries due to ignorance, anxiety or poverty has limited the effective eradication or prevention of HBV infection. Hence, the elucidation of the mechanism of HBV-associated HCC and the identification of diagnostic/prognostic markers or therapeutic targets remain important to address HBV-related clinical consequences.

While the epidemiological association between HBV and HCC is well established, the molecular mechanism by which the HBV virus causes HCC remains less understood.

Two major mechanisms have been proposed for the role of HBV in the development of HCC. The first one involves chronic inflammation, degeneration and regeneration of the liver induced by HBV infection [13]. The second mechanism consists in the HBV genome integrating entirely or partially into the host chromosome causing

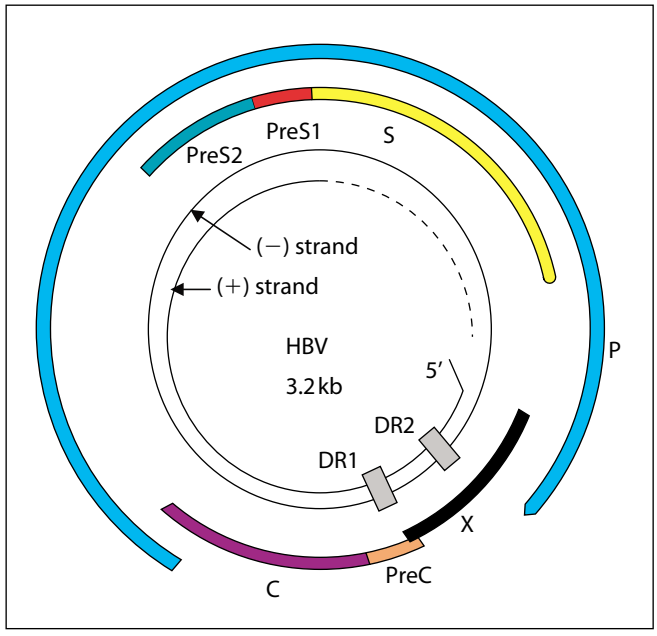

Fig. 1. Genomic organization of the HBV. Partially double-stranded DNA with the positions of direct repeats (DR) 1 and 2 are shown. Four open reading frames, S, C, P and X, encoding the surface proteins, core protein, polymerase protein and the X protein, are indicated.

either *cis* effects including the activation of tumor-promoting genes or the inhibition of tumor suppressor genes or *trans* effects involving the expression of various HBV proteins that modulates gene expression and altering intracellular signaling pathways [1, 14]. Here, we will describe the HBV virus in more detail and also present an updated account of both the roles HBV may play in the development of HCC.

Roles of Hepatitis B Virus in Hepatocarcinogenesis

Like other cancers, HBV-associated hepatocellular carcinogenesis is a multistep process [15]. Although the peak age for the development of HCC is 35–65 years old [6] (http://www.who.int/mediacentre/factsheets/fs204/en/), neonates, infants or very young children infected with HBV have ~80–90% chance of becoming chronic carriers of the virus and these early carriers are often at increased risk for the development of HCC much later in life [7]. Hence, harboring of HBV precedes the development of HCC by several years, suggesting that multiple events occurred before the development of HCC in a chronically infected HBV individual. This time gap between HBV infection and HCC development could potentially provide the mitogenic and mutagenic environment to precipitate genetic and chromosomal changes pertinent to the development of HCC.

HCC is often also associated with cirrhosis, which is observed in ~70–80% of the HCC patients [16]. The majority of the individuals acutely infected with HBV remain asymptomatic with little evidence of liver disease. However, 10–30% of these individuals develop chronic hepatitis which progresses to cirrhosis and finally HCC [17, 18]. Hence, chronic HBV infection is often accompanied by chronic inflammation of the liver, which is then followed by cirrhosis, finally leading to HCC. Evidence has shown that HBV-specific chronic immune-mediated liver cell injury was able to initiate and sustain the process of hepatocarcinogenesis in a HBV-transgenic mouse model system [13].

Cirrhosis was proposed to contribute to hepatocarcinogenesis via several ways.

Recurring cycles of hepatocyte necrosis and regeneration, which is a characteristic of viral-induced chronic necroinflammation, markedly increase the hepatocyte turnover rates and the chance of genomic alteration, which plays an important role in the development of many cancers, including liver cancer [19, 20]. A high cellular turnover can facilitate clones of cells to reactivate telomerase activity and become immortalized. These cells are then more prone to additional genetic and epigenetic changes that could lead to malignant transformation. Spontaneous mutations and damage to DNA are more likely to occur when quiescent hepatocytes enter the cell cycle [21]. As multiple mutations have been strongly associated with the establishment of cancers, this recurring cycle of necrosis and regeneration caused by HBV infection might be one of the mechanisms leading to HCC. In addition, an accelerated rate of cell division prevents some of the mutated or damaged DNA to be repaired before the cell divides again, thereby allowing the damages to be passed on to the daughter cells. If these cells escape elimination by apoptosis, the number of mutations accumulates over time, leading to tumorigenesis. Furthermore, an increase in cell division rate also provides an opportunity for the selective growth advantage of these initiated cells, providing a crucial step in tumor promotion and progression [15]. Additionally, hepatic inflammation itself causes the release of proinflammatory cytokines, which results in the local production of reactive oxygen species that have been implicated in oncogenesis [22].

The role of the immune system in hepatocarcinogenesis is also highlighted by the observations that liver cancer occurs in the background of necrosis, inflammation and regeneration (cirrhosis) in several human liver diseases other than HBV-associated, including chronic hepatitis C, alcoholism [23], hemochromatosis [24], glycogen storage disease [25] and α1-antitrypsin deficiency [26]. Hence, an ineffective immune response during a chronic HBV infection could be pro-oncogenic. Antiviral T cells are implicated to play a role in controlling HBV infection [27]. During an acute phase HBV infection, T cell response was found to be polyclonal, vigorous and multispecific, resulting in the ultimate eradication of the virus from the patient. However,

except during acute exacerbations, chronic HBV carriers generally have relatively weak, narrowly focused T cell responses, which result in the elimination of some but not all the infected cells, thus setting up a cycle of liver cell destruction and regeneration in the background of continuous intrahepatic inflammation, often terminating in HCC. Interestingly, this same T cell response that can eradicate HBV from the liver when it is strong can become pro-oncogenic when it is unable to eradicate the virus by triggering a chronic necroinflammatory liver disease.

Hepatitis B Virus Integration into Host Genome

In addition to the role of HBV-specific chronic immune-mediated liver cell injury in HCC, HBV was also implicated in HCC by its integration into the host genome during chronic HBV infection. The role of HBV integration in HCC development remains unclear as HBV integration per se does not seem to be absolutely required for HCC development. However, integration of HBV into the host genome was observed in 85–90% of the HBV-related HCC and this event precedes the development of HCC [28]. HBV was thought to integrate into the host genome in the period of enhanced DNA damage and replication that occurs during chronic inflammation with cycles of cell death and regeneration that will increase the availability of DNA ends in the host genome and promote the process of viral integration [28]. The integration of HBV into the host genome has 2 potential consequences: (1) the host genome becomes altered and (2) the HBV genome becomes altered.

Cis *Role of Hepatitis B Virus in Hepatocellular Carcinoma Development*

A potential role in hepatocarcinogenesis that the integration of HBV DNA into the host genome can play is via *cis* effect caused by insertional mutagenesis. A potential *cis* role in HCC development that HBV can play is to integrate its DNA adjacent to oncogenes or tumor suppressor genes, resulting in the separation of the gene's upstream regulatory elements from its coding sequences. It has been suggested that the HBV sequence could act as an insertional *cis*-acting promoter/enhancer that activates nearby cellular genes such as cancer-related genes [29]. This would result in the deregulation of host gene expression and thus deregulated production of proteins that may be involved in cell proliferation and viability. Another potential c*is* role of HBV in hepatocellular carcinogenesis may be the disruption of gene function through the integration of its DNA into the coding region of the gene.

Although it is clear that HBV does not integrate into a single site in the host genome, it remains unclear if HBV integrates into the host genome randomly or via preferred site(s). Animal studies with woodchucks have shown that in some tumors the viral DNA integrates at or close to the cellular proto-oncogene *c-myc* and disrupts its expression [30, 31]. HBV was also found to be integrated into an intron of the *cyclin A* gene in a human HCC sample. This integration resulted in the deletion

of the N-terminus of *cyclin A* including the signals for cyclin degradation and replacement of this region of *cyclin A* by viral *PreS2/S* sequences which were transcribed by the HBV *PreS2/S* promoter [32]. This resulted in strong expression of the chimeric HBV-*cyclin A* transcripts encoding an unusually stable, undegradable *cyclin A* [32]. Constitutive activation of *cyclin A* may result in an unregulated cell cycle and contribute to tumorigenesis. In another report, the HBV genome was found to integrate next to the retinoic acid receptor (*RAR*) [33, 34]. As retinoic acid is known to regulate the transcription of genes crucial for cellular growth and differentiation, the integration of HBV into the *RAR* causes it to be inappropriately expressed as chimeric RAR and contributing to cellular transformation [34]. It was reported that HBV-RAR chimeric but not wild-type RAR protein can cause transformation of erythroid progenitor cells [35]. HBV was also found to integrate into the vicinity of *p53* on chromosome 17p [36]. This region of chromosome 17p was reported to be commonly altered in hepatomas and loss of 1 allele of *p53* was also observed in HCC patients from the same region of China [37]. As p53 is known to be a tumor suppressor, the functional loss of this gene may contribute significantly to the development of a subset of HCCs.

In various HCC patients and hepatoma-derived cell lines, HBV was also reported to integrate in the vicinity of the human telomerase reverse transcriptase gene, which plays an important role in cell immortalization [38–41].

Studies thus far have shown that HBV integrates primarily into intronic or intergenic regions of the host genome [42]. Several isolated studies have suggested that the preferred host sites of HBV integration include chromosomal fragile sites, scaffold/matrix attachment regions and a repeat/satellite-sequence-rich region of the genome [28].

Although the available data suggest that the majority of HBV integration occurs in regions that do not disrupt the structure of the gene or change its expression, it has been proposed that the process of viral integration may lead to more generalized genomic instability and cause secondary effects that may not be near the site of integration [28].

Trans *Role of Hepatitis B Virus in Hepatocellular Carcinoma Development*

The integrated HBV genome was found to be altered in several ways including having portions of the HBV genome being deleted, inverse duplicated or rearranged [28]. Nonetheless, in HCC tissues and cell lines in which the HBV genome is integrated into the host genome, the coding regions of the PreS2 regulatory protein and the HBV X protein (HBx) protein were found to be conserved and can be transcribed [43]. Hence, these 2 HBV proteins have been implicated to play a *trans* role in hepatocarcinogenesis.

PreS2 Activators

The HBV surface gene open reading frame encodes 3 PreS2 activators, namely, PreS1, PreS2 and S. The large hepatitis B surface protein (LHBs) is encoded by all 3 PreS1,

PreS2 and S transcripts, while the mid-size hepatitis B surface protein (MHBs) is derived from PreS2 and S [44–46]. However, only the LHBs and a truncated form of MHBs, MHBst, were found to display transactivation properties. Functional MHBst were shown to occur in HBV-associated HCC samples in which the HBV has been integrated into the host genome. These MHBst are derived from MHBs following deletion of the 3′ end of the HBV surface gene [43, 47]. MHBst PreS2 activators were reported to be encoded by the 3′-truncated *preS2/S* sequences of integrated HBV DNA but not by the intact viral gene itself [48]. MHBs and MHBst display different characteristics. While the amino terminal of the MHBs protein faces the lumen of the endoplasmic reticulum, preventing it from interacting with cytosolic proteins [49], the same domain of the MHBst is oriented towards the cytoplasm, facilitating interaction with cytosolic proteins [50]. Hence, MHBst was found to be able to activate protein kinase C, resulting in protein-kinase-C-dependent activation of c-Raf-1/Erk2 signaling and subsequently the activation of activator protein AP-1 and nuclear factor NF-κB [51].

LHBs proteins have similar pleiotropic transcriptional activities as the MHBst activator [45]. The ability to activate key players in cell proliferation regulation suggests that PreS2 activators may deregulate cell proliferation resulting in tumor formation. Notably, transgenic mice expressing MHBst specifically in the liver showed an increased hepatocyte proliferation rate as well as increased incidence of liver tumors [51]. Although the roles of these viral products in tumorigenesis remain inconclusive, observations thus far suggest that HBV integrants may produce factors capable of modulating cellular pathways.

HBV X protein

The HBx represents a more hotly pursued and interesting transactivator that is implicated in the hepatocarcinogenesis process. This is primarily because during the integration of the HBV genome into the host, a significant percentage of viral-host junctions are found to be localized at the carboxyterminal part of the X gene conserving the HBx function [52, 53]. Furthermore, HBx, but not other HBV transcripts, are regularly detected in the tumors of HBV-associated HCC patients [54, 55]. The integrated HBx usually exists as incomplete sequences but conserves their transactivation function modulating the transcription of cellular genes [7, 43]. The role of HBx in hepatocarcinogenesis is also inferred by the observation that duck HBV [56] and heron hepatitis virus [57] that do not have an X gene do not develop HCC. In animal models, HBx transgenic mice exhibit a positive correlation between HBx expression level and HCC development [58, 59].

Importantly, DNA fragments containing the HBx gene derived from HCC patients were found to produce functional HBx protein [53], transform nontumorigenic liver cells in soft agar assays and form tumors in mice [60]. The development of hepatic neoplasia in HBx transgenic mice is dependent on the level of HBx gene expression [61]. However, certain lineages of HBx transgenic mice do not show evidence of

tumor development unless a second event occurred, e.g. induction of the *c-myc* gene expression [62] or exposure to the hepatocarcinogen diethylnitrosamine [63]. HBx has also been implicated as a promoting factor in hepatocarcinogenesis by deregulating DNA repair mechanisms during DNA damage [64]. Hence, HBx may contribute to tumorigenesis by being a promoter or cofactor. Although the pleiotropic nature of the HBx protein poses a challenge in the unraveling of its role in tumorigenesis, it remains important to understand how HBx may contribute to HCC as this may provide important leads for future treatment.

HBx is a *trans*-activating protein that contributes to cell growth and survival by constitutively activating cytoplasmic signal transduction pathways such as NF-κB, src, ras, AP-1, AP-2, PI3K/Akt, Jak/STAT, Smad and Wnt, and by binding to nuclear transcription factors such as CREB, ATF-2, Oct-1 and TBP [48, 65]. Cytoplasmic HBx was reported to activate transcription factors AP-1 and NF-κB via cytoplasmic pathways including ras-MAP kinase; whereas, nuclear HBx is thought to activate the transcriptional machinery directly [48]. Furthermore, HBx may enhance HBV-related carcinogenesis by inactivating the tumor suppressor p53 [48]. Hence, HBx may contribute to the pathogenesis of chronic infection and development of HCC in a variety of ways [65]. Some of the cellular pathways that HBx deregulates are shown in figure 2.

In addition to activating various signal transduction pathways through its pleiotropic transactivation function, HBx was also found to interact with various cellular proteins in deregulating other cellular processes including cellular DNA damage repair network, subcellular localization of these proteins, centrosome integrity and cellular epigenetic process. These will be discussed in the following sections.

HBx Deregulates Cellular DNA Damage Repair Network. In addition to its transactivating functions, HBx has also been implicated to interact with cellular proteins in the DNA damage repair network to deregulate their functions. HBx has been reported to bind and inactivate the UV-damaged DNA binding protein compromising the nucleotide excision repair pathway [66]. HBx interaction with p53 was found to deregulate the transcription of various downstream genes affecting both apoptosis and cellular DNA repair mechanisms [67]. HBx was shown to inhibit p53 sequence-specific DNA binding, thus deregulating p53-mediated transcriptional activation [67]. Additionally, HBx interferes with the binding of p53 to factors involved in nucleotide excision repair including ERCC3, a general transcription factor [67], as well as TFIIH-associated transcription factors XPB or XPD [68, 69], thus deregulating normal cellular response to DNA damage. HBx was also found to bind directly to XPB or XPD DNA helicase, suggesting that HBx may interfere with the nucleotide excision repair pathway through both p53-dependent and p53-independent mechanisms [70].

HBx Deregulates Subcellular Localization of Proteins and Centrosome Integrity. The interaction of HBx with cellular proteins has been reported to interfere with the normal subcellular localization of cellular protein resulting in the deregulation of the protein's function.

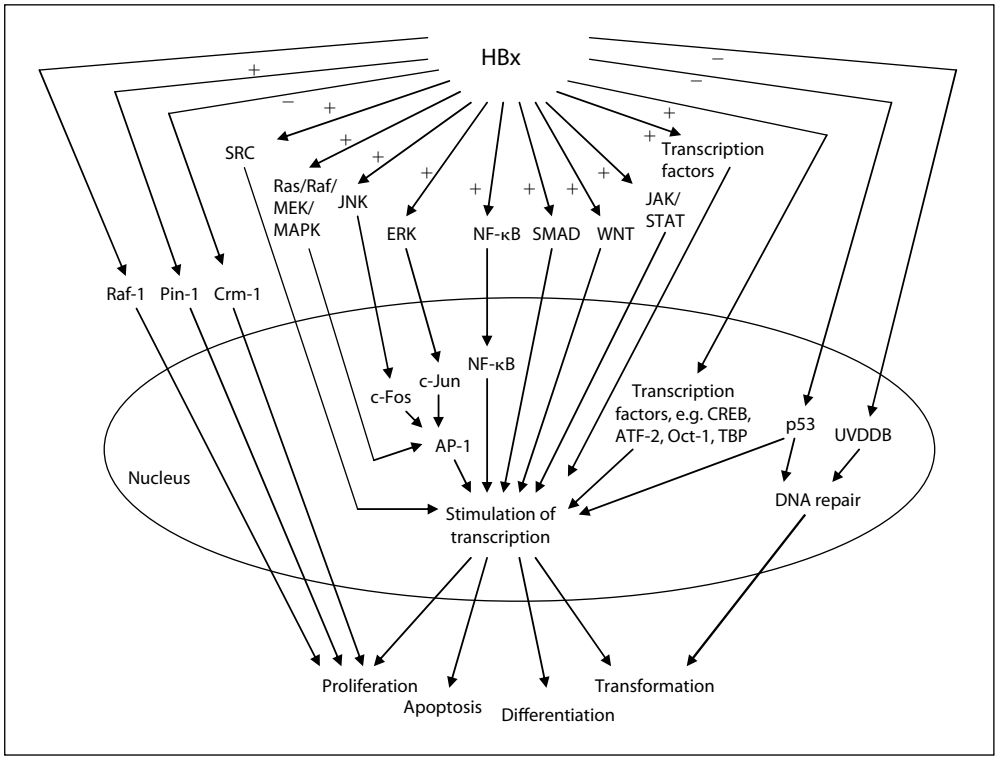

Fig. 2. Various putative mechanisms by which HBx contributes to tumorigenesis. Putative signal transduction pathways and cellular factors affected by HBX. HBx may cause increased proliferation, cell differentiation, disrupted apoptotic responses and/or malignant transformation by either activating or inhibiting various signal transduction pathways and/or by direct binding to transcription factors/other cellular factors. UVDDB = UV-damaged DNA binding protein.

The nuclear export receptor, Crm1, and its cofactor Ran GTPase play important roles in the transport of large cellular proteins from the nucleus to the cytoplasm by recognizing the leucine-rich nuclear export signal [71, 72]. Crm1 has also been implicated in maintaining chromosome integrity [73]. HBx contains a functional nuclear export signal and utilizes the Crm1/Ran GTPase-mediated pathway [74]. HBx was also reported to interact with Crm1 to disrupt the normal function of Crm1 in the cell. The binding of HBx to Crm1 altered the Crm1/Ran GTPase-dependent nuclear export of NF-κB/IκB-α complex [74], resulting in the accumulation of these proteins in the nucleus and promoting deregulated cell growth. Additionally HBx was reported to disrupt Crm1 normal cellular function of maintaining centrosome integrity leading to supernumerary centrosomes and multipolar spindles [75], thereby contributing to hepatocarcinogenesis.

Recently, HBx was also shown to induce the translocation of cytoplasmic Raf-1 kinase to the mitochondria through HBx-induced oxidative stress via the phosphorylation

of Raf-1 at the serine338/339 and Y340/341 residues by p21-activated protein kinase 1 and Src kinase, respectively [76]. The Raf serine/threonine kinases, which are activated in many cancers, act downstream of Ras and are involved in the Ras-induced mitogen-activated protein kinase pathway [77]. Raf-1, when it is localized in the mitochondria, was reported to protect cells from stress-mediated apoptosis [78]. HBx was observed to interact with Raf-1 in the mitochondria and hence implicated to disrupt the anti-apoptosis function of Raf-1 [76].

In another recent study, the peptidyl-prolyl isomerase, Pin1, which is frequently overexpressed in HBV-associated HCC, was reported to interact with specific serine-proline motifs of the phosphorylated HBx protein to stabilize the HBx protein and enhance its transactivation function, leading to increased cellular proliferation and augmented tumor growth in nude mouse transplantation models [79].

Interestingly, in addition to regulating protein stability, Pin1 has also been implicated in the alteration of the subcellular distribution of its interacting partners [80] as well as the regulation of centrosome duplication [81]. It would be interesting to evaluate if Pin1 could influence the intracellular distribution of HBx and if Pin1-HBx interaction could induce centrosome amplification, chromosome instability leading to tumorigenesis.

HBx Deregulates Cellular Epigenetic Processes. In addition to modulating cellular gene expression by being a cotransactivator, the HBx protein was recently also reported to deregulate cellular gene expression via the alteration of the cellular epigenetic process. HBx was found to upregulate the activities of DNA methyltransferases (DNMT) including *DNMT*1, *DNMT3A*1 and *DNMT3A*2. This resulted in the regional hypermethylation and the repression of gene expression of specific tumor suppressor genes including insulin-like growth factor-3. HBx was also found to induce global hypomethylation through the downregulation of *DNMT*3*B*. These specific methylation abnormalities by HBx were shown to be significantly correlated with HBx expression in HBV-infected HCC patients [82]. Another recent study found that in livers of fine-needle biopsied chronic hepatitis B patients as well as the peritumoral tissues of HCC patients, high HBx expression was correlated with greater methylation at the p16INK4A promoter, implicating the role of HBx in the deregulation of epigenetic events in early HBV-related HCC [83].

Conclusion

Chronic infection with the HBV has been associated with increased risk for the development of HCC later in life. The long time difference between the infection with HBV and the development of HCC provides opportunities for multiple oncogenic events to occur. HBV may play a role in hepatocarcinogenesis through 2 major mechanisms. (1) HBV may induce chronic inflammation of the liver resulting in cirrhosis and finally leading to HCC through the recurring cycles of necrosis and regeneration. (2) HBV

was also implicated in HCC via its integration into the host genome during chronic HBV infection. The integration of HBV into the host genome has 2 potential consequences, namely altering the host genome and altering its own genome. HBV can thus play a *cis* role in hepatocarcinogenesis by altering the host genome through insertional mutagenesis. HBV can also alter its own genome during the integration process through deletion, inverse duplication and rearrangements of its genome retaining only versions of genes which are implicated to play *trans* roles in hepatocarcinogenesis. Two such proteins include the PreS2 activators and the HBx protein. Greater emphasis has been placed on the role of HBx protein in hepatocarcinogenesis. HBx is a pleiotropic transactivator protein that binds to various cellular proteins and deregulates various cellular processes including signal transduction, DNA damage repair network, subcellular localization of proteins as well as centrosome integrity. It has also recently been implicated in the deregulation of the cellular epigenetic process.

Nonetheless, it remains unclear if the integration of the HBV genome into the host genome is completely random or if there are preferred sites. Additionally, the extent of mutations, deletions and rearrangements of the HBV genome in the integrated form remains unknown. It is also not known which of the viral proteins are expressed after integration into the host or if any chimeric host HBx proteins are expressed and what their consequences are. With the recent advent of high-throughput genomic strategies, it may now be possible to get a glimpse of the entire spectrum of host integration sites, mutations, deletions, rearrangements and duplications of the HBV genome when it is integrated and perhaps begin to address which of the viral proteins are more commonly expressed either in a mutated, wild-type or chimeric form. Additionally, although the HBx protein has been implicated in hepatocarcinogenesis, there are several contradictory reports about its exact role. Hopefully, with the current and emerging high-throughput technologies, we may perhaps begin to understand more about its pleiotropic roles in hepatocarcinogenesis. Only then can we design better diagnostic and prognostic tools as well as better therapeutic strategies to overcome this disease.

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Carcinogenesis Induced by Hepatitis B Virus

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Abstract

Hepatitis B virus (HBV) is a small enveloped DNA virus, which primarily infects hepatocytes and causes acute and persistent liver disease. Chronic HBV infection is a major risk factor for the development of hepatocarcinoma. The role of HBV in carcinogenesis appears to be complex and may involve both direct and indirect mechanisms. Chronic liver inflammation and hepatic regeneration induced by cellular immune responses may favor the accumulation of genetic alterations. Also important is the role of integration of HBV DNA into host cellular DNA, which could disrupt or promote the expression of cellular genes that are important in cell growth and differentiation. In addition, prolonged expression of HBx protein and PreS2 activators may contribute to deregulating the control of the cellular transcriptional program and proliferation, and sensitize cells to carcinogens. Recent genetic studies have provided insight into the mechanisms underlying viral-associated hepatocarcinogenesis showing that the rate of chromosomal alterations is significantly increased in HBV-related tumors compared with tumors associated with other risk factors. HBV might therefore play a role in enhancing genomic instability. Together, these data strongly support the notion that chronic HBV infection triggers oncogenic pathways, thus playing a role beyond stimulation of host immune responses and chronic necroinflammatory liver disease.

Despite the existence of effective vaccines, it has been estimated that 2 billion people have been infected with the human hepatitis B virus (HBV), with more than 350 million chronically infected individuals worldwide. Epidemiological studies have established that persistent HBV infection is a major risk factor for the development of hepatocellular carcinoma (HCC) and HBV is now thought to be one of the most important environmental carcinogens for humans [1, 2]. Depending on the region of the world, 10% (North America, Northern and Western Europe) to at least 70% of HCC (sub-Saharan Africa, China and South East Asia) will be attributable to HBV infection [1, 3]. A recent prospective study demonstrated that, at least in Asia, there is a viral-load-related risk of HCC [4]. Moreover, other studies have shown that the lifetime risk of HCC is also increased in patients with occult infection and after hepatitis B surface antigen clearance [5–7].

HCC is the most frequent form of liver cancer in most areas in the world and its incidence, which is estimated to have been more than 500,000 in 2000, is rising in many countries [1, 8]. Because of its very poor prognosis, HCC represents the third most common cause of death from cancer [1]. Beside chronic HBV infection, other factors, such as chronic HCV infection, exposure to aflatoxin B1, alcohol abuse, hemochromatosis, obesity, diabetes and hormonal factors, seem to increase the risk of HCC [9]. Some of these risk factors could have synergistic effects with HBV infection to increase the incidence of HCC [10–13]. Regardless of etiology, the development of HCC is, in the large majority of cases if not all, associated with necroinflammatory liver disease including necrosis, inflammation and regeneration of hepatocytes, which ultimately leads to cirrhosis [9, 10, 14]. In the case of HBV-induced HCC, malignant transformation will be the result of both viral and host factors. Indeed, HCC occurs after a long period of chronic liver disease, frequently associated with cirrhosis, suggesting that continuous cell death and subsequent cell proliferation will provide a mutagenic environment increasing the frequency of genetic alterations. HBV-associated chronic inflammatory disease and the subsequent development of HCC could be the consequence of the host immune response but may also result from the direct cytotoxic activity of viral proteins such as the large surface protein or the viral transactivator HBx, which could sensitize cells to apoptosis [15–19]. Alternatively, the virus might play a direct role in oncogenesis by acting as an insertional mutagen. The integration of the virus in the cellular genome might cause direct activation of cancer-related genes or secondary chromosomal alterations such as genomic rearrangements or microdeletions. Finally, viral proteins may act in *trans* to alter cellular metabolism and growth. Thus HBV-associated carcinogenesis appears as a multifactorial process, which will lead to the accumulation of cellular dysfunction and alterations favoring cell transformation.

This chapter reviews the different mechanisms that may be implicated in the development of HBV-related HCC.

Hepatitis B Virus Genome and Replication

Human HBV is the prototype member of a family of small, enveloped DNA viruses called hepadnaviruses. These viruses can infect both mammals and birds and they all share the same narrow host range and infect hepatocytes preferentially. Hepadnaviruses share a similar virion structure, a relaxed circular, partially double-stranded DNA genome that is replicated via an RNA intermediate [20]. Under this conformation, both strands of the duplex are held together by base-pairing between 250 and 300 nucleotides at the 5′ extremities of the 2 strands. Both strands are modified at their 5′ ends. The strand with unit length called minus strand is covalently linked to the viral polymerase. Additionally, the minus strand bears a 9-nucleotide redundant sequence at its extremities. The complementary strand, called plus strand, carries a capped

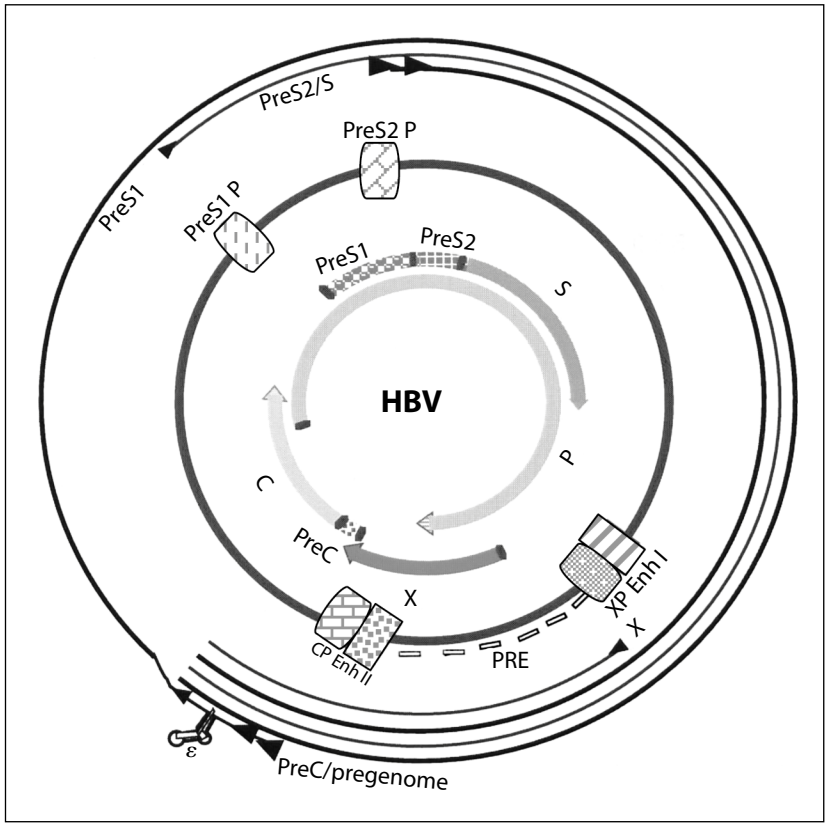

Fig. 1. The HBV genome and transcripts. The 4 ORF encoding 7 proteins are indicated by large arrows. The *cis* elements that regulate HBV transcription are represented by oval and rectangular symbols. PreS1 promoter (PreS1 P), PreS2 promoter (PreS2 P), core promoter (CP) and X promoter (XP), enhancer I (Enh I) and enhancer II (Enh II) are shown. The viral transcripts are represented in the outer layers, with arrows indicating the direction of transcription.

oligonucleotide at its 5′ end [20]. The plus strand is less than unit length and terminates at different positions, resulting in the presence of a single-stranded region of variable length. Two 11-bp repeats, DR1 and DR2, located at the 5′ ends of the minus and plus strands respectively, appear to play a critical role in viral DNA replication [20, 21]. The HBV genome is approximately 3.2 kb in length and presents a highly compact genetic organization. Four open reading frames (ORF) have been identified on the minus strand DNA: the ORF pre-S/S encodes the 3 viral surface proteins (the large, the medium and the small proteins); the ORF pre-C/C encodes the hepatitis B e antigen and the structural protein of the core: hepatitis B core antigen (HBcAg); the P gene encodes the viral polymerase that possesses DNA polymerase, reverse transcriptase and RNaseH activities, and the X gene encodes a small protein that is essential for virus replication but whose function remains partially understood (fig. 1). Because of

the compact organization of the genome, coding sequences overlap with regulatory sequences. Four promoters and 2 enhancers regulate the transcription of viral genomic and subgenomic RNA: the pre-S1, S, pre-C and X promoters, enhancer I and enhancer II (fig. 1).

HBV replication occurs mainly in hepatocytes, the primary site of infection, however, the mechanisms underlying virus attachment, uncoating and entry are not yet fully defined and a cell surface receptor has not been identified yet. After the virus has entered the cell and released its nucleocapsid, the genomic DNA is transported to the nucleus and the relaxed circular partially duplexed genome is converted into a covalently closed circular molecule: cccDNA [22–24]. This process is probably mediated in part by host cell proteins [22]. cccDNA is the template for the transcription of both subgenomic RNAs as well as pregenomic RNA. Pregenomic RNA is then selectively packaged in the cytoplasm into progeny capsids and reverse-transcribed by the viral polymerase into relaxed circular DNA. Capsids containing mature relaxed circular DNA will be either used for intracellular cccDNA amplification or will assemble with hepatitis B surface antigen in the endoplasmic reticulum (ER) to form the viral particles that will be released from the cell [20] (fig. 2).

Interestingly, the integration of viral DNA in cellular DNA is not necessary for virus replication, and persistence of the viral genome is allowed in part through amplification of the cccDNA. Thus, hepadnavirus-infected hepatocytes contain up to 50 copies of cccDNA [25]. However, HBV DNA integrates into hepatocyte DNA and this event probably takes place during liver cell regeneration associated with the necroinflammatory process. Indeed, it has been observed that different conditions, such as exposure to oxidative stress or to DNA-damaging agents, loss of DNA repair capacity or viral infection, increase the frequency of HBV insertion [26–28]. Integrated DNA is observed in chronic HBV carriers with no evidence of HCC as well as in the acute stage of HBV infection [10, 29–33]. Finally, up to 85–90% of HBV-associated HCC show HBV genome integrations [34–36]. The majority of HCC present viral integration with a clonal pattern [9, 37, 38]. HBV-induced HCCs usually show single or multiple discrete HBV integration events within the host genome. Hepadnavirus does not encode an integrase and different mechanisms, associated with minimal to major disruption of the cellular DNA, seem to be involved in HBV integration. Integration occurs during a recombination event and there is evidence to suggest that linear DNA appears to be the preferred substrate for integration and that cellular topoisomerase 1 could be involved in the integration of viral replication intermediates [34]. The integration of HBV DNA into the cellular genome leads to the deletion of some viral sequences, DNA mutation ranging from small to large deletions, chromosomal translocation, and head-to-tail duplication of viral and cellular elements, as well as DNA amplifications. Studies of HBV DNA integration have revealed a high frequency of integration into repeat regions, such as long interspersed nuclear element 1 and short interspersed nuclear elements like Alu sequences or satellite-sequence-rich regions [34, 39–44]. Chromosomal fragile sites, large regions

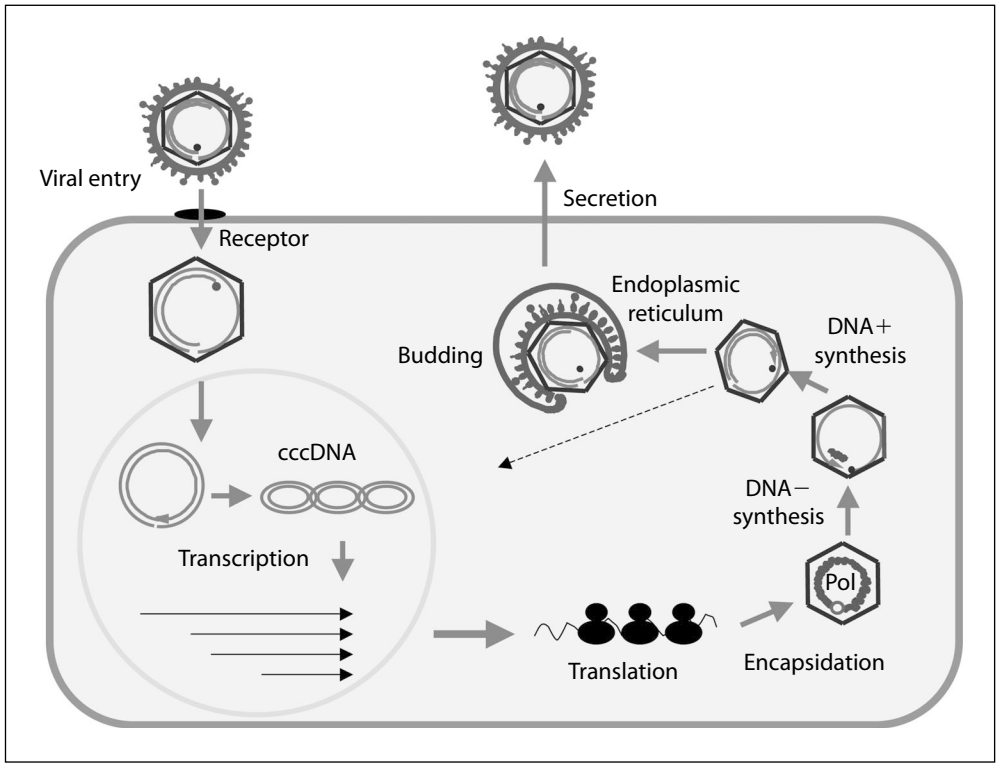

Fig. 2. The life cycle of HBV. After attachment, the nucleocapsid is released into the cytosol and the viral genomic DNA is transported to the nucleus, where the partially double-stranded DNA genome is converted to cccDNA. The cccDNA is the template for transcription of all viral RNAs. The pregenome RNA is encapsidated into core particles, along with the HBV polymerase. The polymerase synthesizes a negative-strand DNA copy and degrades the RNA template. Positive-strand DNA synthesis begins within the intact core but is only partially completed. With completion of 50% or more of the plus strand, nucleocapsids are packaged into envelopes by budding into the endoplasmic reticulum. Alternatively, nucleocapsids may also migrate to the nucleus to facilitate production of additional cccDNA.

of the genome prone to breakage and recombination events, also appear to be preferential targets of oncogenic viruses, including HBV [45]. Finally, woodchuck hepatitis virus (WHV) has been shown to integrate at or adjacent to scaffold/matrix attachment regions [46].

The main question is whether viral integration might play a part in the transformation process. Whereas WHV-induced HCC is commonly due to insertional activation of proto-oncogenes, such a mechanism seems to be less obvious in HBV-associated human HCC, where different functions have been proposed for integrated HBV sequences.

Consequences of Hepatitis B Virus DNA Integration

The integration of viral DNA may induce the disruption or rearrangement of the viral DNA sequences but also disrupt or rearrange host genomic DNA. The integration could have multiple consequences and might either induce chromosomal modification associated with changes in the structure and function of the genes within the cell, or modify the regulation of the expression of the genes at or near the integration site (i.e. *cis*-activation). The integration of viral DNA might thus confer a selective growth advantage on target cells, leading to the emergence of preneoplastic nodules, or providing an additional step in tumor progression. In HCCs developing in woodchucks infected by WHV, it has been shown that the virus acts as a potent insertional mutagen, activating *myc* family genes [47–50]. Insertional activation of *myc* genes by WHV DNA occurs in more than 90% of the woodchuck HCCs, with the majority of integration events targeting the N-*myc*2 oncogene. The integration occurs either upstream of the N-*myc*2 gene or in a short sequence of the 3′ untranslated region. Activation of N-*myc2* could also result from the integration of WHV DNA in *win* and *b3n* loci located 200 and 10 kb away from the N-*myc2* gene [47, 51, 52]. Evidence for a direct role of WHV integration into *myc* genes in HCC development has been demonstrated in mouse transgenic models bearing WHV and *myc* sequences from mutated alleles of woodchuck HCCs. Such transgenic mice develop liver tumors with high incidence [53, 54]. In contrast, to date HBV integration is believed to be random and HBV-induced insertional mutagenesis is viewed as a rare event. However, recent reports using a PCR-based approach and studying a large number of HBV-related HCCs suggest that targeting of cellular genes by HBV is a more frequent event than suspected before (around 70%). Moreover, these studies showed that HBV integration often targets cellular pathways involved in cell survival, proliferation and immortalization [55–58]. HBV targets genes that are involved in cell cycle control, such as the nuclear matrix protein p84 gene and the cyclin-A2-encoding gene [56, 59]. Studies have shown that the integration of HBV in the cyclin A2 gene results in the production of a hybrid HBV-cyclin A2 transcript, encoding a preS2/S-cyclin A2 fusion protein that has been found to possess transforming activities [60, 61]. HBV integration could also deregulate the expression of genes involved in transcriptional regulation, such as the retinoic acid receptor β (RARβ) gene [62, 63]. The integration of HBV in the coding region of the RARB gene led to the production of a fusion protein, HBV-RARβ, that could be involved in cell transformation. Indeed, subsequent studies showed that the HBV-RARβ fusion protein was able to transform erythrocytic progenitors [64]. Pathways that control Ras signaling [58, 65] as well as calcium signaling are also targeted by HBV integrations [55, 58]. Interestingly, integration in genes controlling DNA replication and senescence has been described by several independent groups. Thus, human telomerase gene is frequently targeted by HBV [57, 58, 66, 67]. HBV integrations do not alter the human telomerase gene coding sequence and it has been shown that its expression is activated by enhancer I in the

Huh4 cell line [67]. Indeed the HBV genome contains strong enhancers that are still active after integration [39, 67–69]. Finally the mixed lineage leukemia gene, a gene coding for a transcriptional activator and found amplified in some solid tumors [70, 71], has been identified as a target of HBV by 2 different groups [57, 65]. All together these data suggest that viral integration might be nonrandom and might provide a growth advantage that will select for clones of transformed hepatocytes.

Beside acting directly by *cis*-activation, HBV DNA integration is associated with major genetic alterations within the cell genome, including deletions, duplications and chromosomal translocations [9, 34, 43, 72–75]. In addition, upon active cellular growth, HBV integration may promote homologous recombination at a distance from the insertion site [76]. Different genetic alterations have been described in human HCC, yielding a very heterogeneous profile of alterations [77]. Allelic losses on chromosomes 1p, 4q, 6q, 8p, 9p, 13q, 16p, 16q and 17q are frequently observed in livers tumors, and essentially similar chromosomal regions harbor changes in DNA copy numbers, as seen by comparative hybridization. These regions of the human genome may contain genes that play an important role in hepatocellular carcinogenesis. The accumulation of large-scale chromosomal alterations probably reflects the fact that control mechanisms that safeguard chromosomal integrity are abrogated. Although the majority of abnormalities are similarly found in HBV-associated HCC and in non-HBV-associated HCC, it is important to point out that HBV-related tumors generally harbor a higher rate of chromosomal abnormalities than HCC linked to other risk factors [78, 79]. However, it cannot be completely excluded that the accumulation of genetic alterations is a consequence of a synergistic interaction between HBV infection and environmental carcinogens such as aflatoxin [Pineau et al., unpubl. data]. Finally, although integrated viral sequences, made of linear subgenomic fragments or rearranged fragments in different orientations, are defective for replication, they might also contribute in *trans* to tumorigenesis through the production of HBx or preS2/S proteins. These proteins may act on HCC development either by favoring viral replication and/or by disrupting cellular gene regulation or signaling pathways.

Oncogenic Properties of Viral Proteins

HBx: A Potential Candidate in HCC Development

The x gene was the last to be characterized and was named X because it shares no homology with any known gene. It is the smallest ORF present in the HBV genome and is highly conserved among all mammalian hepadnaviruses. The x gene encodes a 154-amino-acid polypeptide called HBx that is produced at a very low level during acute and chronic hepatitis and induces humoral and cellular immune responses [80–83]. It was first suggested that HBx was essential for virus replication in vivo, since WHV deficient for the expression of WHx cannot replicate in the animal host [84, 85].

Using a similar model, another group found that such mutant viruses are still able to replicate, albeit at a low level. However, WHV revertants expressing a wild-type WHx protein eventually emerged, pointing out the importance of a wild-type WHx for full replication. This observation is supported by a recent study showing that an HBx-deficient HBV genome is strongly compromised for HBV replication. The expression of HBx in this model is able to restore virus replication and viremia to wild-type levels [86]. The role of HBx in virus replication is difficult to assess, since depending on the cellular model, HBx expression will be more or less essential. Indeed, it has been shown that HBx-deficient HBV genomes are still able to replicate in the Huh7 cell line, while in HepG2 cells reduction of viral replication has been observed using the same construct [86–89]. Altogether, these studies support the importance of HBx in the virus life cycle. Yet, the functions supplied by HBx in virus replication still need to be fully elucidated.

HBx has first been suspected to play a role in the development of liver cancer because anti-HBx antibodies are frequently detected in chronic hepatitis B surface antigen carriers, showing markers of active viral replication and chronic liver disease and in 70% of the HCC patients [90, 91]. Moreover, the x gene is usually conserved and transcribed in most integrated HBV subviral DNA and HBx expression is preferentially maintained in HCC [92–94]. Interestingly, recent studies report that HCCs are frequently associated with deletion in the C-terminal portion of HBx sequences [74, 95–97]. Although these HBx mutants derived from the integrated sequences have lost most of the activities associated with wild-type HBx, they can enhance the transforming activity of Ras and Myc [97]. It remains unclear, however, whether these mutants play a role in HCC development during HBV infection. It will be interesting to determine if they are involved in the first stage of oncogenesis or if they emerge later on during tumor progression in order to allow full cellular transformation or to favor an additional step in the transformation process.

HBx and Tumorigenesis

Different models have been used to study the oncogenic property of HBx but the results remain controversial. It has been shown that HBx is able to transform several cell lines such as the NIH3T3 and Rev-2 as well as a fetal mouse hepatocyte cell line harboring simian virus 40 large tumor antigen [98–100]. In agreement with these reports, HBx has been found to cooperate with Ras in the transformation of NIH3T3 and immortalized rodent cells [101]. In contrast, other laboratories have reported that HBx can suppress the transformation of primary rat embryo fibroblasts or of NIH3T3 cells transformed by different oncogenes due to induction of apoptosis [102, 103]. The oncogenic potential of HBx has also been studied in transgenic mice, again giving rise to conflicting results. These studies have been carried out in transgenic mice generated from different strains, carrying the HBx gene under the control of its natural HBV enhancer/promoter sequences or under the control of heterologous liver-specific promoters. Development of HCC associated with HBx expression was

essentially described for a transgenic mouse line generated in the outbred CD-1 background and expressing a high level of HBx in the liver [101, 104]. In other transgenic mice, the expression of HBx by itself does not lead to HCC development. However, it cooperates with c-*myc* or chemical carcinogens in hepatocarcinogenesis [105, 106]. It has also been reported that HBx expression induces the development of HCC in p21-deficient mice [107]. These data suggest that HBx alone does not behave as a strong carcinogen but rather acts as a cofactor during hepatocarcinogenesis. The exact mechanisms remain, however, not elucidated. HBx is a multifunctional protein exhibiting numerous activities affecting gene transcription, intracellular signal transduction, cell proliferation and apoptotic cell death. Any or all of these multiple activities could contribute to hepatocarcinogenesis.

Transactivation Mechanism of HBx

Among the different activities of HBx, its transactivation function is believed to be crucial in the development of liver cancer because it is involved in HBV transcription/replication as well as in the upregulation of a large number of cellular genes involved in oncogenesis, proliferation, inflammation and immune response [89, 108–121]. Thus, HBx has been shown to activate the expression of genes involved in cellular proliferation, such as c-*jun,* c-*fos,* PCNA and cyclin D1, or in angiogenesis, such as the vascular endothelial growth factor and IL-8 [108, 110, 113, 116, 117, 122, 123]. HBx upregulates DNA methyltransferase (DNMT): DNMT1, DNMT3A1 and DNMT3A2, leading to an increase in DNMT enzymatic activity. HBx thus acts at the epigenetic level, inducing regional hypermethylation causing inactivation of tumor suppressor genes such as the tumor suppressor $p16^{INK4A}$ [124, 125].

HBx is a weak transactivator [38] but is capable of activating a wide range of cellular and viral promoters including HBV promoter and enhancer [120, 126]. HBx activates transcription via several DNA-binding sites such as those for NF-κB, AP-1, C/EBP, ATF/CREB, Sp1, HIF-α and NF-AT [113, 122, 127–133]. HBx does not directly bind DNA and its transcriptional activity is mediated by different mechanisms, including direct interaction with nuclear transcriptional regulators and activation of cytosolic signal transduction pathways. HBx has been shown to interact with components of the basal transcriptional machinery (TFIIB, TFIIH, RPB5 and TBP) [134–137] or with transcription factors (CREB/ATF, ATF2, C/EBPα, ATF3, NF-IL-6, Oct1) [101, 132, 138–140], as well as coactivators [110] (fig. 3). The activation of CREB/ATF transactivation function by HBx appears dual, since HBx has been shown to increase the CREB/ATF DNA-binding affinity and to enhance the recruitment of CBP/p300 to CREB/ATF bound to endogenous cellular DNA [110, 141]. The modulation of CREB/ATF activity by HBx might represent an important aspect of HBx activities, since the CREB/ATF family members play an essential role in liver metabolism and proliferation. Recently CREB has also been implicated in hepatocarcinogenesis [142]. Moreover, this activity could be involved in the activation of HBV transcription mediated by HBx, since a CREB-binding site-like sequence (CRE) is

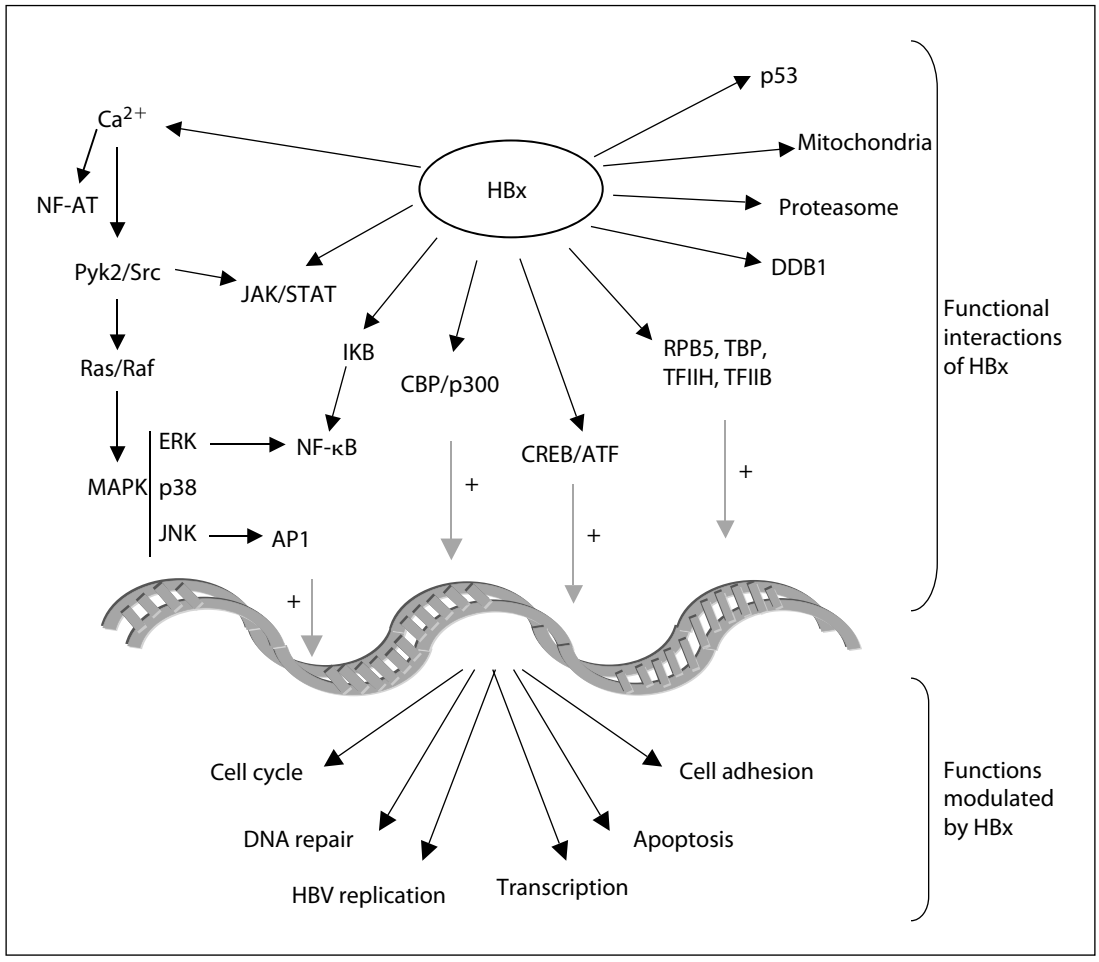

Fig. 3. Functional interaction of HBx with cellular partners. The figure illustrates the complexity of the biological actions of HBx. HBx activates transcription through direct binding to transcription factors, coactivators and components of the basal transcription machinery. HBx transcriptional activity is also linked to its ability to stimulate MAPKs and JAK/STAT signaling pathways. Activation of these pathways is indirect and HBx is thought to trigger the release of calcium into the cytosol, which in turn activates the proline-rich tyrosine kinase/focal adhesion kinase (Pyk2/FAK) and Src kinase families. Activated Src kinases in turn stimulate a variety of signaling pathways, leading for example to the activation of transcription factors. HBx interacts with different cellular partners such as CRM1, p53, mitochondria, proteasome and DDB1 that are involved in HBx activities and could be relevant to cell transformation.

present in the HBV enhancer I and in PreS2 [143, 144]. In recent studies from our laboratory, it appears that HBx enhances the CREB transcriptional activity through its interaction with the CBP/p300 coactivators. Further studies will be needed to investigate the respective role of CREB and of coactivators such as CBP/p300 in the activation of HBV replication by HBx. Finally, CBP/p300 are known to bind and

activate a large variety of cellular transcription factors [145]. Some of these factors, such as c-Jun, c-Fos and NF-κB, are also activated by HBx. It will be interesting to determine whether the interaction between HBx and transcriptional coactivators participates in the activation of these transcription factors. This interaction could thus partially explain the broad effect of HBx on transcription.

A second important mechanism for HBx transcriptional activity is linked to its capacity to activate signal transduction pathways (fig. 3). This function is mediated by the cytoplasmic pool of HBx [146]. HBx has been shown to activate mitogen-activated protein kinase pathways including the extracellular signal-regulated kinases (ERKs), the stress-activated protein kinases/NH2-terminal jun kinases (SAPK/JNKs) and the p38 kinases, and Janus family of tyrosine kinase/signal transducers and activators of transcription (JAK/STAT) pathways [147–156]. The activation of these pathways by HBx is dependent on the activation of nonreceptor tyrosine kinases of the Src family, since the inhibition of Src kinases prevents the activation of the Ras-Raf-MAP kinase, JNK, p38 MAPK or JAK/STAT pathways [151, 156]. However, alternative activation of the JAK/STAT signaling pathway mediated through a direct interaction between HBx and JAK1 has also been described [152]. HBx does not interact directly with Src kinases and recent studies from Bouchard et al. [157, 158] made a significant contribution to our understanding of Src activation by HBx. They showed that HBx induces the activation of upstream activators of Src kinases, the focal adhesion kinase (FAK) and the proline-rich tyrosine kinase (PyKa), through the modulation of cytosolic calcium. Direct measurement of cytosolic calcium in HBx-expressing cells confirmed that HBx expression correlates with an increase in cytosolic calcium [159, 160]. HBx might mediate this activity through its association with mitochondria [160]. The role of calcium as a mediator of HBx activities has been confirmed for the activation of the MAPK pathways but also in the activation of transcription factors, such as nuclear factor of activated T cells, and in virus replication [156, 161–163]. Some studies have reported that the activation of diacylglyerol-dependent protein kinase C (PKC) is responsible for HBx induction of AP-1 and NF-κB activity [164, 165]. However, other studies have not confirmed this finding [131].

Among the factors or the functions modulated by HBx through the activation of the MAPK pathways, it has been shown that HBx induced NF-κB, an important mediator of the cellular stress responses that control the expression of several acute-phase response proteins, cytokines and adhesion molecules [150, 166, 167]. The activation of NF-κB is Src- and Ras-dependent and acts through the degradation of the NF-κB inhibitor IκB-α [154, 157]. Ras-independent pathways are, however, suspected to be involved in NF-κB activation, such as sequestration of newly synthesized IκB-α by HBx leading to the sustained activation of NF-κB [168, 169]. Importantly, the activation of Src kinases by HBx has also been shown to stimulate HBV replication at the level of DNA replication and to destabilize cellular adherent junctions [157, 162, 170, 171]. HBx also causes activation of the transcription factor AP-1 though the activation of the Ras-RAF-MAPK and JNK pathways [147–149, 153, 157]. Finally, Ras signaling

is also involved in the stimulation of RNA-polymerase-I- and polymerase-III-dependent transcription [172–174]. Importantly, HBx activation of MAPKs and JNKs has been demonstrated in the liver of HBx-transduced mice. Such constitutive activation is associated with increased activity of AP-1 [175]. Finally, HBx has been shown to activate the Wnt/β-catenin signaling pathway through the activation of Src kinase or ERK [176, 177]. This finding could be of significant importance for hepatocarcinogenesis. Indeed, abnormal activation of the Wnt signaling pathway is associated with the development of different tumors such as HCC [178, 179].

The transactivation activity of HBx could thus lead to the modulation of a large number of functions, such as virus replication, cell cycle regulation, angiogenesis, apoptosis and DNA repair that could be relevant for cellular transformation (fig. 3).

Other Partners and Functions of HBx

Beside its interaction with transcription factors or components of the basal transcription machinery, a myriad of HBx partners have been described that can be relevant for virus replication or oncogenesis or both (fig. 3).

Among them, the tumor suppressor protein p53 has been shown to interact in vitro and in vivo with HBx [180]. Although this interaction remains controversial, it is thought to be involved in the inactivation of several critical p53-dependent activities. HBx has been reported to inhibit p53-mediated transactivation and p53 sequence-specific DNA binding [181, 182]. Recently, Chung et al. [183] reported the downregulation of the tumor suppressor PTEN by HBx through the repression of p53 transcriptional activity. It has been proposed that by interacting with p53, HBx is able to sequester p53 in the cytoplasm, leading to its functional inactivation [184, 185], but other studies have failed to detect any colocalization between p53 and HBx [186]. Functional inhibition of the tumor suppressor gene p53 is a common abnormality detected in human cancer cells. It is tempting then to hypothesize that HBx, through p53 inactivation, contributes to the high chromosomal instability of HBV-related tumors and to HCC development.

It has also been reported that HBx interacts with components of the proteasome, such as the PSMA7 subunit [187–189]. However, it remains unclear whether HBx inhibits proteasome activity or whether proteasome is needed for HBx activity. One study reported that the inhibition of the proteasome impairs HBx transcriptional activity, whereas a second study suggested that HBx might enhance HBV replication through proteasome inhibition [187, 190]. Finally, HBx has been shown to interfere with the ubiquitin degradation pathway and to block the degradation of c-Myc through a direct interaction with the F box region of Skp2 [191]. Interestingly, dysregulation of protein degradation pathways is a common strategy used by viruses to provide a favorable environment for their replication and to escape protective mechanisms developed by the host cell [192]. The interaction with DDB1, a core subunit of the Cul4A-based ubiquitin E3 ligase complex, has been very well documented. It has been shown that the HBx/DDB1 interaction is essential for virus replication and for

the maintenance of HBx activities [87, 193–197]. The role of DDB1 in HBx activities remains unknown, however. DDB1 was first described as a protein involved in DNA repair [198]. Thus, it was proposed that HBx impairs DNA repair through its interaction with DDB1. In vitro as well as in vivo studies led to conflicting results and the role of DDB1 in the inhibition of DNA repair by HBx has not been confirmed [199–202]. Further studies will be needed to determine the function of HBx/DDB1 interaction in virus replication and in HBx activities at the molecular level.

HBx has been shown to interact with and to sequester the nuclear export receptor CRM1, leading to the nuclear localization of NF-κB and to the aberrant centriole replication as well as formation of multipolar spindles [203, 204]. Deregulation of mitotic spindle assembly by HBx is associated with aneuploidy, which can lead to genomic instability and contribute to cancer development [203].

Through these multiple activities and interactions, the HBx protein subverts cellular functions, such as cell cycle regulation, apoptosis and DNA repair. This will induce the accumulation of dysfunctions and alterations that may ultimately lead to the development of liver cancer.

HBx and Regulation of Cell Cycle

Dysregulation of the cell cycle is a common feature of transformed cells. In this regard, many viral oncoproteins, such as adenovirus E1A, HTLV-I Tax and HPV-16E6, deregulate cell cycle phase progression. Actively replicating cells are believed to provide a favorable environment for the replication of the viruses [205, 206]. Many studies have focused on the impact of HBx gene expression on the cell cycle. It was found that the activation of signal transduction pathways (described above) such as MAPK, JNK and Src kinases by HBx stimulates cell cycle progression, accelerating the progression of quiescent G_0 cells through the G_1 to S phase, as well as from the G_2 to M phase [16, 207]. The consequences of HBx expression on the cell cycle depend on the presence of stimulatory factors. Indeed, Bouchard et al. [161] have demonstrated that serum-starved HBx-expressing cells exited G_0 but stalled at the G_1/S boundary. Similar findings have been reported by Chirillo et al. [208]. They observed that, in serum-starved cells, HBx induces DNA synthesis followed by apoptosis. The question remains open as to whether HBx stimulates cell cycle progression or apoptosis. Similarly, some studies have shown that HBx induces the expression of the cell cycle regulators p21 and p27 and the subsequent arrest of the cell at the G_1/S boundary [209, 210]. Others have reported a repression of p21 expression leading to cellular growth [211, 212]. These conflicting results on HBx activity might stem from the model used or/and from the expression level of HBx. It has been shown that HBx differentially regulates cell cycle progression depending on the differentiated or dedifferentiated state of a hepatocytic cell line [213]. Studies performed with HBx transgenic mice reflect the ex vivo conflicting results. Madden et al. [214] reported that the expression of HBx is associated with a significant increase in S-phase hepatocytes in the liver of young animals but not in adult mice. Another study found

increased apoptosis in the liver of HBx transgenic mice. However, using the same model it was shown that HBx cooperates with *myc* in oncogenesis, arguing that HBx behaves differentially depending of the cellular context [19, 106]. Finally, HBx impairs hepatocyte regeneration induced by hepatectomy [215, 216]. Interestingly, one study reported the same complicated pattern as observed in cell culture: HBx promoted the transition of quiescent hepatocytes from G_0 to G_1, but the cells stalled at the G_1/S boundary and underwent apoptosis [216].

HBx and Apoptosis

As mentioned before, several studies have shown that HBx can modulate both cellular proliferation and viability. HBx has been found to either mediate apoptosis, sensitize cells to proapoptotic stimuli or to prevent apoptosis. In chronic HBV infection, liver cell injury is thought to be mediated mostly by the cellular immune response. However, several studies suggest that HBx might contribute to liver disease by modulating pathways controlling apoptosis. HBx exerts a spontaneous proapoptotic effect in cultured primary hepatocytes and in the liver of HBx transgenic mice [16–19]. The induction of cell death by HBx has been described to be both p53-dependent as well as -independent and could be mediated through interaction with c-FLIP or by causing loss of mitochondrial membrane potential [19, 184, 208, 217–219]. The role of mitochondria in HBx-induced apoptosis is supported by the fact that direct interaction has been reported between HBx and the mitochondria [218, 220]. However, it is important to note that replication of HBV in the livers of transgenic mice is not associated with pathological killing of hepatocytes [221]. Furthermore, HBx expressed from a replicating HBV genome does not induce apoptosis but acts as a 'sensitizer' to other proapoptotic stimuli and provides hypersensitivity to killing by tumor necrosis factor-α (TNF-α). This hypersensitivity required a particular set of conditions involving activation by HBx of JNK and Myc pathways [222, 223]. This finding has been confirmed by different groups [217, 224]. In striking contrast, HBx has been found to inhibit apoptosis induced by p53, transforming growth factor-β (TGF-β) or Fas [225–227]. The antiapoptotic activity of HBx could be mediated through its interaction with the survivin-HBXIP complex or through the activation of the phosphatidylinositol-3-kinase (PI-3-K) signaling pathway or NF-κB [223, 227, 228]. From the study of Su et al. [223], it seems that the effect of HBx on cell viability might be highly dependent on the cellular context. To date, there is no direct evidence that HBV can modulate the apoptotic pathways, especially under in vivo conditions, nor that apoptosis could provide any advantage to virus replication. A reasonable hypothesis is that HBx would inhibit apoptosis during early hepatocyte infection, favoring viral replication, and that it would activate apoptosis at later stages to facilitate viral spread and immune evasion. A result of HBx-induced apoptosis could be the enhancement of the regeneration process providing a larger reservoir of hepatocytes for virus spreading. Alternatively, apoptosis could be a consequence linked to other activities of HBx that are deleterious for the cell, such as the deregulation of the cell cycle, leading to the accumulation of the cells at the G_1/S junction.

HBx and DNA Repair

Active mechanisms protect the genome of human cells from endogenous or exogenous substances that damage cellular DNA. The DNA repair enzymes constantly scan the global genome to detect and remove DNA damage. Five DNA repair pathways have been identified, such as homologous recombinational repair, nonhomologous end joining, mismatch repair, nucleotide excision repair (NER) and base excision repair (for review see Bernstein et al. [229]. NER affects the repair of different types of lesions. In particular, it eliminates highly promutagenic DNA lesions induced by UV irradiation or by DNA-adducting carcinogens such as aflatoxin B1 (a liver-specific carcinogen), lesions that are known to block transcription. A dysregulation of this function leads to the accumulation of mutations that predisposes cells to transformation. Several groups have investigated whether HBx could interfere with this process. It has been described that HBx inhibits the repair of DNA damage in cell culture [199, 230–232]. The mechanism by which HBx inhibits NER is unknown but is thought to occur through the interaction of HBx with proteins or protein complexes involved in DNA repair such as TFIIH and p53 [180, 182, 231–233]. HBx could also modulate NER activity through downregulation of the XPB and XPD components of TFIIH [234]. Madden et al. [202] developed a transgenic mouse model allowing them to measure the effect of HBx on DNA repair in vivo. They showed that HBx did not significantly increase the accumulation of spontaneous mutations, suggesting that the inhibition of NER by HBx may lead to an increase in mutation frequency only after exposure to exogenous mutagenic agents. Interference of HBx with the cellular DNA repair system provides yet another potential mechanism by which HBx contributes to liver carcinogenesis. In support of this hypothesis, a study from the same group showed an increase in mutations in the liver of HBx transgenic mice treated with the hepatocarcinogen diethylnitrosamine [214].

PreS2/S Activators

The HBV genome encodes 2 types of transactivator: the HBx protein (see above) and the PreS2 activators. This second family of HBV regulatory proteins is composed of the truncated form of the PreS2/S gene product: the truncated middle surface protein (MHBs^t) and the PreS1/PreS2/S gene product: the large surface protein (LHBs) [235, 236]. LHBs and MHBs^t genes are thought to be involved in HCC development, since epidemiological studies have shown that their expression is maintained in the tumors and cell lines analyzed [237, 238].

MHBs^t Protein

The MHBs secondary structure is determined by 3 hydrophobic transmembrane regions (I, II and III), the first serving as insertion signal for the ER. MHBs can be glycosylated in the PreS2 region and can be detected in 3 forms: unglycosylated, monoglycosylated or biglycosylated. MHBs is synthesized as an integral membrane protein that is secreted after modifications in the ER and Golgi complex. Full-length MHBs display no transcriptional transactivation function. The generation of a functional

MHBs^{t} requires deletion of the 3′ end of the PreS2/S gene corresponding of the hydrophobic region III.

The truncated form of the MHBs protein has been isolated for the first time in the hepatoma Huh4 cell line, which contains an integrated HBV genome and is positive for HBV surface antigen [236]. To date, different MHBs^{t} proteins have been identified and allow the cartography of the transactivating region, which encompasses deletion of nt_{HBV} 573–221 corresponding to MHBs$^{t194–76}$ [239]. It was then speculated that the functional difference between full-length MHBs and MHBs^{t} resides in their secondary structure. Hildt et al. [240] found that the major difference between these proteins is that the truncated form is not glycosylated, although the glycosylation site is still present, suggesting that it is inaccessible to the glycosyltransferase residing in the lumen of the ER-Golgi network. It was then shown that in the MHBs^{t} protein, the PreS2 domain faces the cytoplasm, whereas in the case of the MHBs, the PreS2 domain faces the lumen of the ER.

LHBs Protein

Although the LHBs protein harbors 2 glycosylation sites: one in the S region and the other in the PreS2 region, LHBs is only monoglycosylated in the S region. This is due to the cytoplasmic orientation of the PreS1/PreS2 domain. In fact, the PreS1/PreS2 domain of the LHBs protein displays a dual membrane orientation. In one fraction of the LHBs protein, the first transmembrane region is used, in this case the PreS1/PreS2 region faces the lumen of the ER. In the other fraction, this first transmembrane domain is not used, resulting in a PreS1/PreS2 domain oriented toward the cytoplasm [241, 242]. In contrast to the 2 other surface proteins, the LHBs, when it is expressed in the absence of MHBs and SHBs, is not secreted and accumulates in the cytoplasm [243]. Moreover, the production of roughly equimolar ratios of LHBs with respect to MHBs and SHBs leads to the intracellular accumulation of all proteins and induces the development of 'ground-glass' hepatocytes [243, 244]. Different types of ground-glass hepatocytes have been characterized, leading to the identification of PreS-LHBs mutants [245].

Transactivation Function of the Surface Proteins and HCC

The formation of the truncated form of MHBs requires deletions of 3′ sequences of the PreS2/PreS gene that were frequently observed in HCC. In contrast to MHBs^{t}, LHBs is constitutively present in the HBV genome. The transactivation function of the surface proteins requires the cytoplasmic orientation of the PreS2 domain that occurs in MHBs^{t} or in a fraction of LHBs [241, 246]. Studies on the transactivation function of MHBs^{t} and LHBs reveal that these proteins share the same mechanism for transcriptional activation and could be included in the PreS2 family of activators [235]. It has been shown that their transactivating effects are mediated by the modulation of PKC signal transduction [235, 236]. Moreover, 1 study reported that MHBs^{t} has DNA-binding activity [247].

Due to its cytoplasmic orientation, the PreS2 domain interacts with cytosolic binding partners, triggering intracellular signal transduction pathways. It has been described that the PreS2 activators bind PKC α/β, leading to a Ras-independent, PKC-dependent activation of the c-Raf-1/MEK signal transduction cascade. Hildt et al. [248] demonstrated that this activation required phosphorylation of Ser27/28 of MHBs^t by PKC. The authors proposed the following model: the PreS2 domain binds PKC, this interaction causes DAG-dependent activation of PKC and phosphorylation of the PreS2 domain. The activation of PKC is transduced by the c-Raf-1/MEK/ERK signaling cascade, leading to the activation of AP-1, AP-2 or NF-κB-dependent transcription [249] and disruption of cellular gene regulation. Moreover, the activation of these pathways has been described to be involved in HBV replication [250]. Finally, a potentially oncogenic transcriptional effect of MHBs^t includes the stimulation of c-*myc* or c-*fos* promoter activity [251, 252].

Transgenic Mouse Models

Studies with transgenic mice that express the PreS2 activators support the oncogenic properties of these proteins. In transgenic mice producing MHBs^t, a significant induction of c-Raf-1/MAP2 kinase activity was detected. Permanent activation of this pathway results in increased proliferation of hepatocytes, demonstrated by an increase in PCNA level. Furthermore, Hildt et al. [248] found that in these MHBs^t transgenic mice, the accumulation of mutations is increased by MHBs^t-dependent inactivation of p53. Moreover, a proapoptotic activity of the PreS2 MHBs^t has been suspected. Indeed, the increase in cellular proliferation in these mice was not reflected by a significantly enlarged liver, suggesting the existence of a compensatory mechanism [248]. Furthermore, an increased sensitivity to tumor-necrosis-factor-α-dependent apoptosis was also observed [248, 253].

The overexpression of LHBs in transgenic mice has been shown to be cytopathic and could lead to liver injury and eventually progression to HCC [254, 255]. Wang et al. [256] have demonstrated that in transgenic mice expressing PreS-LHBs mutants, the activation of the NF-κB pathway leads to higher expression levels of COX-2. Moreover, the authors showed that in these mice, cyclin A expression is enhanced, which may contribute to aberrant centrosome duplication.

These findings support the hypothesis that the expression of truncated preS2 proteins or LHBs could be involved in HBV-associated HCC.

Immune Pathogenesis of Hepatocellular Carcinoma

All cases of HBV-associated HCC develop after many years of chronic hepatitis mediated by the host immune response and characterized by liver cell necrosis, inflammation and regeneration [257]. Chronic hepatitis is believed to be the consequence of an inefficient immune response destroying some but not all of the infected cells, thus

inducing the turnover of hepatocytes in the context of intrahepatic inflammation. This inflammatory environment would then create favorable conditions for the development of HCC. The role of chronic hepatitis as a procarcinogenic risk factor was first demonstrated using a transgenic mouse model producing hepatotoxic amounts of the HBV large envelope polypeptide. These mice develop a chronic necroinflammatory liver disease associated with Kupffer cell activation, oxygen radical production, oxidative DNA damage and increased hepatocellular proliferation that leads to HCC development [15, 254, 258, 259]. The role of the virus-specific immune response in the development of HCC was next assessed using a mouse transgenic model. Adoptive transfer of cytotoxic T lymphocytes specific for hepatitis B surface antigen into these transgenic mice, thymectomized, irradiated and bone-marrow-reconstituted, induces the development of chronic immune-mediated liver disease ultimately leading to HCC [260]. This model highlights the importance of the immune response in the development of chronic hepatitis. Yet, the transgenic mice contain the entire HBV envelope-coding region and develop ground-glass hepatocytes. In another model of HBV-associated chronic liver disease, after adoptive transfer of syngenic unprimed splenocytes, the mice cleared virus from the liver and serum and developed a chronic disease, but without the appearance of HCC [261]. While the immune response is largely involved in the development of liver disease, HBV itself can have cytopathic effects and contribute to the inflammatory process. Indeed, severe combined immune-deficient mice harboring human hepatocytes infected by HBV develop liver disease, with the presence of ground-glass hepatocytes [262].

Conclusion

Cancer arises from a stepwise accumulation of genetic changes that confer on transformed cells unlimited self-sufficient growth and resistance to normal homeostatic regulatory mechanisms [263]. As in other human cancers, the exponential relationship between HCC incidence and age reveals a multistaged mechanism, involving independent genetic lesions due to a synergistic effect between immune response and HBV. Although it has been shown that HBV transgenic mice are tolerant to the transgene and do not develop liver injury [264–266], HBV DNA is able to transform immortalized cell lines ex vivo, pointing out a transforming activity associated with HBV [267]. These data suggest that HBV integration and/or expression of viral proteins are able to provide an additional step in the transformation process. Thus, during HBV infection, the establishment of a suboptimal cellular immune response unable to clear the virus will trigger the development of necroinflammatory liver disease, initiating the first stage for the development of HCC. In this context, long-term toxic effects of viral gene products or the occurrence of a decisive HBV integration event that would promote genetic instability or lead to *cis*-activation will bring additional mutagenic steps that lead to the fully transformed phenotype of hepatocytes. In

this model, HBV infection will also potentiate the action of exogenous carcinogenic factors, such as aflatoxin and alcohol.

Given the high incidence of HBV infection in spite of effective vaccination, understanding the exact mechanisms of HBV-associated hepatocarcinogenesis, especially the role of viral proteins such as HBx or the PreS2/S activators, is of major importance in order to develop novel therapeutic strategies against this lethal disease.

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Disease and Pathogenesis Associated with Epstein-Barr Virus

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Abstract

Epstein-Barr virus is a ubiquitous virus. Primary infection is usually asymptomatic but may be associated with the syndrome of infectious mononucleosis. In unusual situations, infection may lead to chronic active infection or lymphoproliferative disorder, especially in patients with underlying immune deficiencies. The virus is also related to a variety of malignancies. These include lymphoid, epithelial and smooth-muscle malignancies. Virus is generally present in tumors as a multicopy episome. The patterns of viral gene expression differ among the malignancies. The only viral genes that appear to be universally expressed are Epstein-Barr virus nuclear antigen 1 and the EBERs 1 and 2. There is a relationship to immunodeficiency in some but not all of the malignancies. Associations with infectious, genetic and environmental cofactors are also recognized but are variable among the malignancies.

Epstein-Barr virus (EBV) is a ubiquitous γ-herpesvirus that infects most of the adult population in the world [1]. Infection has few if any recognized consequences in most people. However, EBV is associated with illnesses ranging from pharyngitis to cancer. This chapter will review these diseases and their pathogenesis with a special focus on malignancy.

The virus is transmitted in saliva. Depending on the living conditions, cultural practices, particularly those related to eating and childcare, primary infection may occur in infancy, childhood, adolescence or adulthood [2]. Maternal chewing of food for young infants such as occurs in some cultures, the sharing of eating utensils such as chopsticks and kissing are all likely to be important modes of transmission [3]. Transmission may also occur through other sexual activities, blood transfusion, and organ or bone marrow transplantation [4].

Primary infection in any age group may be associated with symptoms but is most commonly related to symptoms in adolescents and adults [5]. The disease associated with primary infection is infectious mononucleosis. Pharyngitis and lymphadenopathy are accompanied by 'mononucleosis', i.e. an increase in mononuclear cells. Pharyngitis

entails an inflammatory response to viral infection. Following infection of B cells in the oropharyngeal mucosa, there is a proliferation of virus-infected B cells. At its peak, several percent of B cells may harbor virus. This in turn provokes innate and adaptive cellular immune responses. These account for the lymphocytosis (T cells and natural killer cells) characteristic of the disease [6, 7]. Lymphadenopathy is generally most prominent in cervical nodes but lymphoid hyperplasia also occurs in other lymphoid tissues including tonsils and spleen. Virus-infected cells in lymph nodes or tonsils from patients with infectious mononucleosis include B and T cells and are found predominantly in the interfollicular areas rather than in the germinal centers [8].

Other characteristic manifestations are fever, malaise and fatigue. These are presumed to relate to inflammation and inflammatory cytokines. Fever, pharyngitis and lymphadenopathy usually resolve in a few weeks but malaise and fatigue may persist for many weeks or months [9]. Infection is generally lifelong as evidenced by persistence of antibody responses to some latent and lytic antigens, intermittent shedding of virus in saliva and persistence of viral DNA in peripheral blood lymphocytes [10, 11]. Persistent infection appears to require B cells as it is not established in patients with a congenital absence of B cells (X-linked agammaglobulinemia) [12].

Primary infection is associated with polyclonal B cell activation and hypergammaglobulinemia. Among the antibodies produced are IgM antibodies that lead to the agglutination of horse, sheep or cow erythrocytes [13]. Referred to as heterophile antibodies, they appear at about the same time as symptoms and resolve several months later. This time course and the ease of testing for such antibodies has made them the standard for the diagnosis of infectious mononucleosis. The explanation for their appearance is poorly understood but there is a consensus that they are not directed against viral antigens nor cross-reactive with viral antigens [13]. EBV infection may be associated with many other manifestations including maculopapular rash, hepatitis, autoimmune hemolytic anemia, genital ulcers, tonsillar enlargement, aplastic anemia, encephalitis, aseptic meningitis, transverse myelitis and others [14].

In patients with congenital immunodeficiencies such as the severe combined immunodeficiencies, primary EBV infection may lead to hemophagocytosis (erythrocytes are phagocytosed by activated monocytes), dysgammaglobulinemia and EBV-driven lymphoproliferative diseases [15, 16]. Most of the immunodeficiences are associated with increased susceptibility to a variety of infections. However, X-linked lymphoproliferative disease is distinctive in that global immunodeficiency is not characteristic [17]. These patients typically first come to medical attention due to primary EBV infection, which is very commonly fatal. Genetic defects in these patients consistently map to SLAM-associated protein gene. This protein is thought to be involved in the coordination of the cellular immune response to EBV and some other viral infections [18, 19]. It is expressed on natural killer cells, CD4+ T cells and CD8+ T cells.

Primary infection may evolve into chronic active infection, a severe progressive illness characterized by major organ involvement, such as hepatitis, lymphadenitis and hemophagocytosis, and extreme elevations of EBV antibody titers, with evidence of

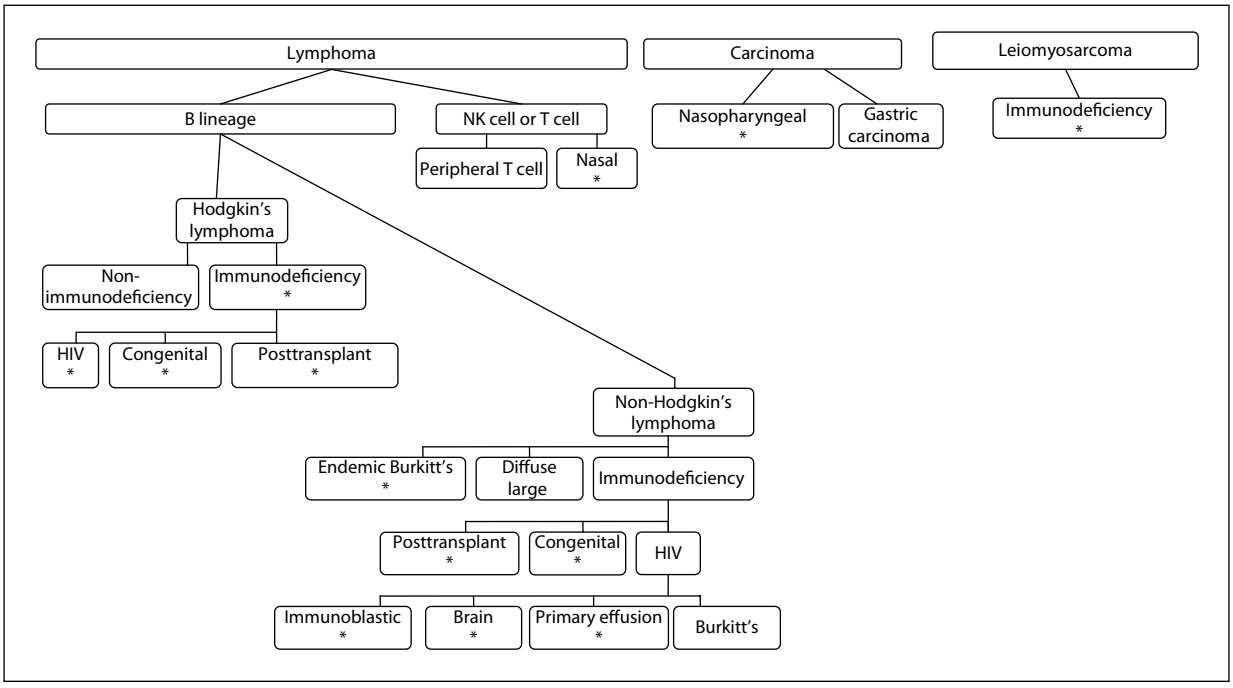

Fig. 1. EBV-associated malignancies divided by the malignant tissue type and then by the presence of immunodeficiency in the host. These may be subdivided further to indicate the particular type of immunodeficiency (HIV, transplant, congenital). Malignancies associated with EBV in at least 80% of the cases are indicated by an asterisk.

very high viral burden as is detected by in situ hybridization, immunohistochemistry or PCR [20]. Most reports come from Japan, Taiwan, Korea and China. The disease is likely heterogeneous in etiology but a genetic defect in both alleles of the gene encoding perforin (present in the granules of cytotoxic T lymphocytes and natural killer cells and required for their cytotoxic activity) has been recognized in 1 case [20].

Patients with HIV infection typically have increased EBV DNA in peripheral blood mononuclear cells and increased viral shedding in oropharyngeal secretions [21, 22]. EBV is associated with a benign disorder, oral hairy leukoplakia [23]. This is a hyperplastic lesion of the oral mucosa, especially the tongue. Lesions show lytic EBV gene expression. The condition is occasionally seen in other immunosuppressed populations such as organ transplant recipients. Oral hairy leukoplakia resolves or is suppressed with antiviral therapy like acyclovir [23–26].

Tumors: General Considerations

EBV is associated with a variety of malignancies (fig. 1). These include lymphoid, epithelial and connective tissue malignancies. Some occur in the setting of immunodeficiency,

others in hosts with apparently normal immune function. Some develop rapidly, others arise decades after primary infection. Some show striking geographic or ethnic variation in their degree of association with the virus, others are consistently linked to the virus. Some have genetic underpinnings in their hosts.

The spectrum of EBV-associated tumors with regard to tissue types is poorly understood. In vitro and in vivo, EBV is a B lymphotropic virus. CD21, a complement receptor, is well characterized as important for the infection of B cells by virus [27, 28]. However, CD21 expression is not an absolute requirement for EBV infection in vitro, and the cell types of EBV-associated tumors could not be predicted from patterns of CD21 expression. Thus CD21 is not expressed on Burkitt's or Hodgkin's lymphoma (both of which may involve EBV), while it is expressed on follicular lymphoma (which is never related to EBV). In B cell tumors the argument may be made that CD21 provides the gateway for entry and subsequently is downregulated. However, a similar argument with regard to nasopharyngeal carcinoma, gastric carcinoma or smooth-muscle sarcoma is much more difficult to make.

The anatomic distribution of EBV-associated tumors is also poorly understood. Almost all arise in the head, neck or upper aerodigestive system. Thus endemic Burkitt's lymphoma typically presents in the mandible. EBV-associated Hodgkin's lymphoma typically occurs in the cervical lymph nodes. The aerodigestive localization of other tumors is defined in the name (nasal lymphoma, nasopharyngeal carcinoma and gastric carcinoma). However, there is no anatomic site that is uniquely associated with these viral tumors. Thus oral and esophageal cancers are never EBV-related (although oral lymphoma in AIDS patients and gastric carcinoma in general are associated). Primary central nervous system lymphoma in AIDS patients is consistently linked to EBV, although this lymphoma in nonimmunocompromised populations is not associated with the virus and other central nervous system malignancies are not related to the virus.

Immunodeficiency plays a role in a substantial subset of these patients (fig. 1). Organ and hematopoietic stem cell transplant recipients, patients with congenital immunodeficiencies and HIV-infected patients are at increased risk for EBV-associated B cell malignancies including Hodgkin's lymphoma. Transplant and HIV recipients are also at risk for the much rarer EBV-related leiomyosarcoma [29–31]. However, not all malignancies caused by EBV involve immunodeficiency. There has been no reported increase in the risk of EBV-associated nasal lymphoma, nasopharyngeal carcinoma or gastric carcinoma in these populations [16, 29, 32–34]. Furthermore, the impact of immunodeficiency is complex. Thus while endemic Burkitt's lymphoma is consistently EBV-associated, AIDS Burkitt's lymphoma is usually not.

The time from infection to the clinical appearance of tumor varies among tumor types [29, 35, 36]. Posttransplant lymphoma may develop in weeks after primary infection in the setting of immunosuppression. Hodgkin's lymphoma may appear in 6 months to 10 years after primary symptomatic EBV infection in the normal host. In

patients who have undergone organ transplantation, non-Hodgkin's B cell lymphomas typically arise in the first year after transplantation, whereas Hodgkin's lymphoma occurs several years after transplantation. In HIV patients, non-Hodgkin's lymphomas also appear to arise earlier in the course of HIV disease than Hodgkin's lymphoma. Indeed several years of moderate immunodeficiency (with CD4 T cell counts $>200/mm^2$) may be required for the development of Hodgkin's lymphoma. Nasoparhyngeal and gastric carcinomas typically arise in the fourth, fifth and six decades.

Geographic variation is also well recognized, with endemic Burkitt's lymphoma being the best example. Malarial infection is widely believed to explain the geographic differences, with the EBV association strongest in regions with holoendemic malaria. The rare occurrence of EBV-related Burkitt's lymphoma in North America and Western Europe even in African populations suggests that the geographic discrepancies cannot be ascribed to genetic variation in any simple way [34]. Nasopharyngeal and gastric carcinoma also show marked geographic variation in the incidence of disease, but in contrast to Burkitt's lymphoma, the geographic differences in incidence do not translate into geographic variation in the degree of EBV association. Thus in all parts of the world undifferentiated nasopharyngeal carcinoma is predominantly EBV-related, as is approximately 10% of the gastric carcinoma.

Genetic factors are well recognized in congenital immunodeficiencies, whereas a mix of genetic and environmental factors is implicated in nasopharyngeal carcinoma, which is particularly common in southern Chinese and Eskimo populations [37]. Twin studies confirm a genetic contribution to nasopharyngeal carcinoma and high-risk families are recognized [38]. The risk falls when high-risk Cantonese emigrate, but not to the North American/Western European Caucasian baseline.

Tumor genetics are very different among EBV-associated tumor types even in the same cell type. Thus in B cell tumors, chromosomal translocations that juxtapose immunoglobulin loci, most commonly the heavy-chain locus on chromosome 14 and the c-Myc oncogene on chromosome 8, are characteristic of Burkitt's lymphoma with or without EBV association. In contrast, early posttransplant lymphoproliferative disorders generally lack cytogenetic abnormalities. Hodgkin's and primary effusion lymphoma show very complex cytogenetics. Carcinomas caused by EBV also have complex cytogenetics.

The association of virus with cells within a patient's tumor varies somewhat with tumor type. In a subset of peripheral T cell lymphomas, evidence of viral infection is found in only a small subset of the tumor cells [39]. In nasopharyngeal carcinoma, there is a subset of cells in some tumors that seem to lack virus [40, 41]. In other tumors, if evidence of virus is found in 1 tumor cell, it tends to be in all.

With the exception of some of the lymphoid proliferations arising in profoundly immunocompromised patients, these tumors are clonal [42]. Clonality in lymphoid malignancies is readily established by studying antigen receptor rearrangements and in tumors in general by examining a variety of polymorphisms in X chromosomes

(where in women, one or the other X will be inactivated). For the EBV-associated malignancies, clonality is also inferred from the study of the viral terminal repeat sequences.

Viral gene expression and its contribution to tumorigenesis and maintenance of the malignant phenotype also varies with tumor type. In posttransplant lymphoproliferative disease, particularly the variety that arises early and is classed as polymorphous (see below), there is broad expression of viral latency antigens including all of those that play a role in lymphocyte immortalization in vitro [1]. These tumors generally do not have chromosomal translocations or other mutations that explain their proliferation. Thus it is presumed that these viral proteins explain the uncontrolled proliferation. In contrast, Burkitt's lymphoma expresses only the EBV nuclear antigen 1 (EBNA1) protein and the EB early region (EBER) RNAs. There is agreement that EBNA1 is required for the maintenance of the viral episome, but whether the viral protein also contributes to tumor maintenance in other ways, is unresolved. Both EBNA1 and EBER RNAs have been implicated in protecting cells from apoptosis, but the evidence is inconclusive. As noted above, Burkitt's lymphomas consistently show a chromosomal translocation that leads to c-*myc* dysregulation. However, studies of Burkitt's cells that are grown as single cell clones show that episomes are regularly lost. It is possible to isolate subclones of some Burkitt's lines such as Akata that have lost all viral episomes [43]. These subclones do not have the growth characteristics of the parent line and are not tumorigenic in immunodeficient mice. Thus it seems likely that viral gene expression even in tumors with highly restricted patterns of expression plays some role in the maintenance of the malignant phenotype.

Hodgkin's disease, nasal lymphoma and nasopharyngeal carcinoma fall between these 2 extremes. These tumors express genes known to be required for lymphocyte immortalization and known to transform in vitro [latent membrane protein 1 (LMP1)], but they also have a multitude of genetic abnormalities. In Hodgkin's lymphoma the virus may contribute in a fashion that is distinct from these other settings. The lymphoma is of B lineage cells but these cells fail to express immunoglobulin. In many instances, particularly with EBV-associated Hodgkin's lymphoma, there are actually stop codons that prevent protein expression. In normal B cell development such cells undergo apoptosis [44]. Membrane immunoglobulin molecules provide a tonic signal required for B cell survival. LMP2A provides this signal as well, thus allowing survival of B lineage cells that lack immunoglobulin.

Specific Tumors

Lymphoma

Burkitt's lymphoma is a common childhood tumor in malarial areas of Africa [45]. This B cell tumor typically arises in the jaw at the time that adult teeth appear. Viral gene expression is very restricted [46]. EBNA1 is expressed but not the other latency

Table 1. Risk factors for transplant-related lymphoma

Risk factor	High	Intermediate	Low
Solid organ Immunosuppression	Bowel OKT3 (a monoclonal antibody targeting CD3 T cells)	Kidney Cyclosporine, tacrolimus, mycophenylate	Corneal Rapamycin
EBV serology Organ rejection Marrow or stem cell	Seronegative		Seropositive
T cell depletion	OKT3 T cell depletion, antithymocyte globulin		Campath, elutriation
Graft versus host disease	OKT3 T cell depletion, antithymocyte globulin		

nuclear antigens or LMP1. The promoters that drive expression of the latency nuclear antigens are densely methylated [47]. The very restricted pattern of viral expression and a lack of surface adhesion molecules, and a failure to process antigens for presentation in MHC class I complex [48] all contribute to evasion of immune surveillance by tumor cells. Malaria is generally recognized as a cofactor but its role remains poorly understood. Malaria may serve as a stimulus to B cell proliferation, as an immunosuppressive factor disrupting T cell or NK cell immune surveillance, or possibly through an entirely different mechanism. Burkitt's lymphoma occurring in North America and Western Europe is much less common, has a very different clinical presentation, generally as abdominal disease or as a lymphocytic leukemia. Although it occurs in children, it is also found in adults. Moreover it appears in patients with HIV [49]. Indeed the observation of a surge in cases in men who have sex with men and had otherwise rare opportunistic infections was one of the phenomena that led to the identification of AIDS. Burkitt's lymphoma is an AIDS-defining illness, i.e. HIV infection that would not otherwise be referred to as AIDS is classified as AIDS if the patient also has Burkitt's lymphoma. Burkitt's lymphoma in North America and Western Europe with or without HIV infection is not caused by EBV in most instances [49]. In all cases of Burkitt's lymphoma, whether EBV-related or not, HIV-associated or not, presenting as a mass or as a leukemia there is a characteristic histologic appearance with small cells with minimal cytoplasm and a very high growth rate (nearly 100% of the cells express the proliferation-associated marker Ki-67). There is also a family of balanced chromosomal translocations.

Following solid organ or bone marrow transplantation, lymphoproliferative diseases arise in approximately 0.5–10% of the recipients [50]. Determinants of risk are listed in table 1. Several of these warrant comment. Allogeneic immune responses,

whether those associated with organ rejection in solid organ transplant recipients or those related to graft versus host disease in bone marrow and stem cell recipients, entail an increased incidence of lymphoproliferative disorder. Similarly, many immunosuppressive therapies used to prevent or reduce these responses are also associated with increased risk such as the use of OKT3 antibodies in either setting. However, not all such therapies involve increased risk. In particular in the bone marrow or hematopoietic stem cell setting the selective removal of T cells from the graft product (such as is accomplished with OKT3 antibody that targets CD3 on T cells) dramatically increases the risk, whereas balanced removal of T and B cells (such as is accomplished with the Campath antibody that targets CD52 on T and B cells) is not associated with any increased risk. Whether B cell depletion protects against the development of EBV tumors because it reduces the pool of virus-infected cells or because it reduces the pool of cells that might potentially be infected by virus, is unknown. Another difference in risk factors between organ and bone marrow or hematopoietic transplantation is that EBV seronegativity in the recipient is only important as a risk factor in organ transplantation. This is presumably because in that setting the organ transplant recipient has no immunologic experience with EBV. In contrast, in the bone marrow/hematopoietic transplant setting, the recipient's immune system is generally replaced by the donor's and thus the recipient's immunologic experience is largely irrelevant.

EBV is usually associated with B cell lymphoproliferative disease arising soon after transplantation. Lesions occurring several years after transplantation are often but not always related to EBV. Some of these tumors express the full spectrum of antigens found in EBV-immortalized lymphoblastoid cell lines, while others show more restricted patterns of viral gene expression. Lack of immune surveillance is thought to play a critical role in the pathogenesis of posttransplant lymphoma [51, 52]. This idea was strengthened when it was demonstrated that adoptive cellular immunotherapy was useful particularly in the bone marrow transplant setting [53, 54]. In some instances tumors regressed with administration of EBV-specific T cells. In other instances, high-risk patients received EBV-specific T cell prophylaxis. Several investigators have advocated monitoring viral load as a guide to immunosuppression or to 'preemptive therapy' [55], building on observations that the viral copy number in peripheral blood mononuclear cells, plasma or whole blood has generally been higher in patients with posttransplant lymphoproliferative disease [55, 56].

An antibody targeting CD20, a B cell surface antigen, has proven very useful in the management of posttransplant lymphoproliferative disease [56, 57]. Approximately half of the patients will respond to this therapy. However, following its administration, there is virtually always a fall in EBV copy number in peripheral blood mononuclear cells that accompanies the depletion of B cells. This B cell depletion and fall in EBV copy number occurs independently of tumor response and thus rituximab therapy may render viral copy number measurement in peripheral blood mononuclear cells irrelevant.

Table 2. Viral gene expression in tissues

Tissue	EBNA1	EBNA2	LMP1/LMP2	EBERs 1 and 2
Acute mononucleosis, B lymphocytes	+	+	+	+
Healthy seropositive, B lymphocytes	+[1]	–	–[1]	+
Oral hairy leukoplakia, epithelial cells	+	–	+	+
Posttransplant lymphoma, early, polymorphous	+	+	+	+
Burkitt's lymphoma (African)	+	–	–	+
Nasal lymphoma, NK	+	–	+	+
Hodgkin's lymphoma	+	–	+	+
Nasopharyngeal carcinoma	+	–	+/–	+
Gastric carcinoma	+	–	–	+
Leiomyosarcoma	+	?	–	+

[1]Reverse transcriptase PCR studies consistently identify LMP2 but not LMP1 transcripts in lymphocytes from peripheral blood. EBNA1 RNA has also been detected by many authors. Because of the rarity of these cells, nothing is known of actual antigen expression.

Approximately half of the AIDS lymphomas are caused by EBV. Primary central nervous system lymphomas in AIDS patients are virtually always related to EBV [58, 59]. Other large B cell lymphomas, particularly those with immunoblastic features, are also usually EBV-associated [30]. Low CD4 T cell counts identify AIDS patients at especially high risk for EBV non-Hodgkin's lymphoma. Patients with brain lymphoma generally have CD4 counts $<20/\mu l$ [60]. Higher but not normal CD4 counts ($>200/\mu l$) that are often seen in patients receiving antiretroviral therapy are associated with an increased risk of Hodgkin's lymphoma [61].

EBV is detected in the tumor cells of approximately one third of Hodgkin's lymphoma cases in North America and Western Europe, with a higher fraction in the rest of the world approaching 100% in areas of Africa and Latin America [62–64]. Most common in young adults (15–35 years), Hodgkin's lymphoma is least frequently caused by EBV in patients in this age range. Infectious mononucleosis entails an increased incidence of EBV-associated Hodgkin's lymphoma but not other Hodgkin's lymphoma [35]. The interval between infection and Hodgkin's lymphoma is from approximately 6 months to 20 years, with most cases arising in the first several years. Tumor cells express EBNA1, LMP1 and LMP2.

Carcinoma

Nasopharyngeal carcinoma is consistently associated with undifferentiated nasopharyngeal carcinoma in all populations [36]. Most common in Cantonese, it also occurs in other Southern Chinese, North African and Eskimo populations. The viral antigens expressed in tumor tissue comprise EBNA1, LMP2 and sometimes LMP1 (table 2). High antibody titers against many EBV antigens including lytic viral antigens are well

recognized [65, 66]. Antibody titers are sometimes used clinically in the evaluation of patients at high risk. Viral DNA present in the serum or plasma of these patients appears to be derived largely from tumor cells undergoing apoptosis and fragmenting the nuclear DNA [67]. The DNA copy numbers in plasma or serum are much higher in patients with active nasopharyngeal carcinoma than in healthy individuals or in patients in remission. The failure to clear viral DNA with therapy is an extremely bad prognostic sign.

In approximately 10–15% of the gastric cancers, EBV is present in tumor cells [68, 69]. The incidence of this malignancy varies widely, being high in Japan and Korea and lower in North America and Western Europe. The percentage of cases involving virus seems to be nearly constant, whether high- or low-incidence populations are being studied. Viral protein expression is limited to EBNA1. EBV-associated gastric cancer has a somewhat better prognosis than other gastric cancers.

Smooth-Muscle Tumors

Leiomyosarcomas are smooth-muscle tumors. When they arise in immunodeficient patients (HIV, organ transplant, congenital immunodeficiency), they are consistently associated with EBV. They are extremely rare. EBER expression is well documented as is the absence of LMP1 expression.

Conclusion

EBV was discovered almost 50 years ago. In the beginning, when the virus was isolated from Burkitt's tumor cell lines, it appeared to be the simple and direct cause of the lymphoma. Investigation has revealed the virus to be ubiquitous and has linked it to a multitude of diseases, benign and malignant. However, large gaps in our knowledge remain. A better understanding of the determinants of symptomatic infection, of the character of the immune response, as well as of the peculiar environmental, genetic and infectious cofactors that lead to malignancy will likely guide the way to new interventions to prevent or treat the disease.

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The Biology and Molecular Biology Underlying Epstein-Barr Virus Oncogenesis

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Abstract

Epstein-Barr virus (EBV) is a ubiquitous γ-herpesvirus. After exposure, individuals remain EBV-infected for life. EBV-infected B cells are the primary site of in vivo persistence but mucosal epithelial cells may contribute to the production of infectious virus and to virus secretion into saliva. The maintenance of B cell infection is dependent upon the expression of EBV latency proteins and noncoding regulatory RNAs and upon the integration into the natural biology of the B cell. Latency gene expression is complex with different subsets of the 9 EBV latency proteins being expressed in different settings. Latency proteins mimic constitutive signaling of cellular pathways and have growth-stimulatory and cell survival properties. While EBV infection is usually asymptomatic, the ability of the EBV latency genes to promote proliferation and the need for tight immunological control of EBV-infected B cells throughout life can lead to disease. The functions of the EBV latency genes are discussed in the context of in vivo EBV latency and EBV-associated malignancies.

Epstein-Barr virus (EBV) was discovered in 1964 [1]. EBV is a γ-herpesvirus virus that infects ~95% of the adult population worldwide. Infection in childhood is usually asymptomatic, whereas in adolescence or early adulthood it is associated in 30–40% of the cases with infectious mononucleosis [2]. A Scandanavian serological study examining samples collected from children aged 9–12 years found no change in seroprevalence to EBV in samples in this age group collected at a 30-year interval (1967 and 1997), indicating stable rates of childhood infection despite social changes over this period [3]. EBV is transmitted predominantly in saliva [4]. Low levels of EBV are detected in genital secretions but this is not considered to be a significant source of virus transmission [5]. As is typical of the herpesvirus family, EBV has a large (172 kb) double-stranded DNA genome that is maintained in latently infected cells as an extrachromosomal episome, and once infected with EBV an individual remains infected for life.

Epstein-Barr Virus Biology

EBV efficiently infects B cells in the lymphoid tissues of the Waldeyer ring [6]. B cells are the site of maintenance of long-term viral persistence. In healthy EBV-seropositive individuals, 1–50 per million circulating B cells carry the EBV genome [7]. EBV infection of B cells is biased towards establishment of latency with limited viral gene expression. The expression of the EBV replicative cycle and production of progeny virus takes place in terminally differentiated plasma B cells [8, 9]. Whether other cell types are obligatory participants in the maintenance of persistent infection, remains unresolved. Although it has proven difficult to document in vivo, there is evidence suggesting that epithelial cells may contribute to the cycle of virus replication and spread that is an important component of both persistent infection of the individual and transmission of virus from one individual to the next [10, 11]. Lytic viral replication has been observed in differentiated epithelial cells in the lesions of AIDS-associated oral hairy leukoplakia [12], indicating the potential for an epithelial contribution to virus production. In vitro, epithelial cell infection and lytic gene expression also occur preferentially in differentiated cells [13, 14]. EBV entry into cultured epithelial cells is inefficient unless the virus is associated with B cells [15] or with antibody [16]. Cultured tonsillar epithelial cells can be EBV infected [17] and this may be a biologically relevant source of orally secreted virus. EBV infected monocytes/Langerhans cells have also been suggested as players in the trafficking of virus between the B cell compartment and mucosal epithelial cells [18, 19].

Studies on the process of EBV entry into B cells and epithelial cells also support a model in which both cell types are involved [11]. The EBV membrane glycoprotein gp350/220 binds to the complement receptor type 2 (CD21) on B cells [20, 21] and virus entry is triggered by an interaction between a tripartite gp42 containing EBV glycoprotein complex and cellular HLA class II molecules [22, 23]. Infection of CD21 and MHC class II negative epithelial cells involves a bipartite EBV glycoprotein complex that lacks gp42. The net result is an alternating tropism in which B-cell-derived virus is slightly more infectious for epithelial cells, while epithelial-cell-derived virus is significantly more infectious for B cells [24]. EBV shed into saliva has a glycoprotein composition consistent with being epithelial-derived [25] and hence would have a predilection for infecting B cells upon transmission to a naïve host.

Epstein-Barr Virus Gene Expression Programs

EBV encodes ~86 proteins [26, 27] of which only a small number are expressed during latent infection of B cells or in EBV-associated malignancies [1]. The expression of latency genes differs in different settings from the full-growth proliferative latency program (type III latency) through the more restricted type II and type I programs to the type 0 or in vivo latency program, where viral protein expression is absent or occurs

only transiently [28]. The type III latency program is expressed in B cell lines established by in vitro EBV infection and comprises the nuclear proteins EBNA1, EBNA2, EBNA3A, -3B, -3C and EBNA-LP, the membrane-spanning proteins LMP1 and LMP2A and -2B plus noncoding viral RNAs. These latter include the small polymerase III transcribed EBER1 and EBER2 RNAs and polyadenylated transcripts from the BamHI-A rightward transcript (BART) locus that gives rise to multiple micro-RNAs [29, 30]. Type III latency expression induces B cell activation and proliferation and produces an immortalized phenotype that allows long-term cell growth in culture. In vivo, type III latency occurs upon EBV infection of naïve tonsillar B cells [31] and in disorders such as posttransplant lymphoproliferative disease and primary central nervous system lymphoma in AIDS that arise in immunocompromised patients [32, 33]. In type II latency, the EBNA2, EBNA3A, -3B, -3C and EBNA-LP proteins are not expressed, while in type I latency protein expression is further limited by lack of expression of the latency membrane proteins, leaving EBNA1 as the only EBV protein produced. In the tissue culture setting, type II latency is seen in EBV-infected epithelial cells. This mimics the expression pattern seen in the EBV-associated epithelial malignancy nasopharyngeal carcinoma and in B cell Hodgkin lymphoma. Type I latency is found in Burkitt lymphoma, both in vivo and in cultured B cell lines derived from tumor specimens. Type 0 occurs in circulating, EBV-genome-positive, memory B cells [34] and represents the long-term site of viral persistence in which the lack of viral gene expression allows EBV to escape T cell surveillance and survive in an immunologically competent host.

The differential EBV gene expression seen in the different types of latency is a consequence of alternative promoter usage. Upon primary infection of a B cell, transcripts for the latency nuclear EBNA2 and EBNA-LP proteins derive from the Wp promoter, which is regulated by B-cell-specific transcription factors. EBNA2 expression results in promoter switching to the EBNA2-responsive Cp, which drives the expression of all of the latency nuclear proteins through alternatively spliced transcripts and is active in type III latently infected cells. In type III latency EBNA2 also drives the expression of LMP1 and LMP2. In type I and type II latency, the only nuclear EBV protein present is EBNA1, which is expressed from a different promoter, Qp. LMP1 expression in type II latency also derives from a different promoter than in type III latency. The EBNA2-responsive ED-L1 promoter is used in type III latency and an alternative L1-TR promoter is utilized in type II latency.

Mimicry of Cell-Signaling Pathways by the Epstein-Barr Virus LMP1 and EBNA2 Proteins

The essential latency III proteins LMP1 and EBNA2 are functional mimics of activated cellular CD40 [35–38] and the intracellular domain of Notch (NotchIC) [39] respectively. The mimicry of CD40 signaling by LMP1 has been substantiated using chimeric CD40-LMP1 receptors [40–42].

LMP1 and CD40 Signaling

Domains in the LMP1 cytoplasmic tail signal through tumor-necrosis-factor-receptor-associated factors and tumor-necrosis-factor-receptor-associated death domain proteins to activate NF-κB, and the JNK, MAPK and p38 kinases [43]. In this way LMP1 provides ligand-independent tumor-necrosis-factor-like signaling. A key component of LMP1 activity is the activation of both canonical inhibitory-κB-kinase-dependent and noncanonical NF-κB-inducing-kinase-dependent NF-κB signaling [44] and treatment of type III latently infected cells with NF-κB inhibitors induces apoptosis [45]. Signaling mediated by AP-1, TGF-α, STAT-3, PI3K/Akt and epidermal growth factor receptor is also activated by LMP1 [46–52] and gene array analyses particularly identified NF-κB, c-Myc and AP-1 genes as being LMP1 regulated [45, 53]. NF-κB upregulation of proteins such as Bmi-1 [54] and the antiapoptotic proteins c-FLIP, survivin, A20, c-IAPs, BFL-1, BCL-XL and Mcl-1 [45, 52, 53, 55–59], and c-Myc upregulation of telomerase activity [60] are likely to contribute to LMP1-mediated oncogenesis, as is LMP1-mediated inhibition of p16 to counter cellular senescence [61]. LMP1-mediated induction of the cytokine IL-6 induces STAT-3 phosphorylation and activation, which in turn induces c-Myc and activates the upstream LMP1 promoter [46].

The expression of LMP1 in Rat1 fibroblasts causes anchorage-independent growth and the cells form tumors in nude mice [62]. In transgenic mouse models, the expression of LMP1 in B cells increases the incidence of lymphoma [63, 64], while the expression in epithelial cells leads to epithelial hyperplasia [65], which can progress to carcinoma [51]. In type III B cells, conditionally expressing LMP1 withdrawal of LMP1 resulted in cells becoming cell-cycle-arrested and quiescent [53].

Cells are very sensitive to the levels of LMP1 and in some settings LMP1 can have paradoxical effects such as growth inhibition and sensitization of cells to chemotherapeutic agents like cisplatin and bleomycin [66–68]. These effects are often also related to NF-κB activation. Although LMP1 can protect against interferon-α through interaction with Tyk2 and blockage of STAT-2 phosphorylation [69], LMP1-mediated NF-κB upregulation also induces interferons-α and -γ [70], which in turn leads to activation of STAT-1 and interferon-regulatory factor 7 (IRF-7) [71]. Downstream interferon-stimulated genes such as the proapoptotic protein CD95 (Fas) are consequently upregulated [53, 72]. These effects may be modulated in the B cell setting by EBNA2. EBNA2 upregulates c-Myc, which in turn downregulates genes of the NF-κB and interferon pathways and reduces interferon induction and induction of interferon-stimulated genes [73]. However, the expression of LMP1 protein itself must be tightly controlled and LMP1 expression is consequently regulated at multiple levels. Transcriptionally, LMP1 is expressed from 2 separate promoters, ED-L1 and L1-TR. ED-L1 is regulated by EBNA2 in type III latency, while the upstream L1-TR promoter is used in type II latency and is regulated by STATs and Sp1/Sp3 [74–76]. Both promoters are subject to positive autoregulatory loops, ED-L1 through LMP1 activation of IRF-7 [77] and L1-TR through IL-6-mediated activation of STAT-3 [46].

Loss of expression through CpG methylation of the LMP1 promoter has been described in nasopharyngeal carcinoma with undetectable LMP1 [78]. However, there is frequently no direct correlation between LMP1 mRNA levels and LMP1 protein levels and modulation of LMP1 expression through targeting of the LMP1 3′ untranslated region by EBV-encoded micro-RNAs is an important component of posttranscriptional regulation [79].

EBNA2 and the Notch Pathway

EBNA2 functions as a transcriptional transactivator and is the first gene expressed after EBV infection of B cells. Immediately after infection EBNA2 is expressed from the Wp promoter, which is regulated by the B-cell-specific protein BSAP/Pax5 [80]. This promoter subsequently becomes CpG methylated and there is a switch to the Cp promoter, which is EBNA2 regulated and gives rise to all of the latency nuclear proteins through alternative splicing. Since EBNA2 also activates the LMP1 and LMP2A promoters, this places the complete latency III program under EBNA2 regulation. EBNA2 does not bind directly to DNA and a breakthrough in the understanding of EBNA2 function came with the discovery that EBNA2 is targeted to DNA through interaction with Cp-binding factor 1 (CBF1) [81–84]. CBF1 is the human homolog of Drosophila Suppressor of Hairless (SuH), murine recombination-binding protein Jκ and *Caenorhabditis elegans* Lag-1. These proteins are often referred to as CSL for CBF1/SuH/Lag-1. CSL is the nuclear effector of the Notch signaling pathway which is highly conserved from worms to mammals and influences cell fate decisions and proliferation versus differentiation [85]. There are 4 Notch receptors in mammals, Notch 1–4 and 5 Jagged and δ-like ligands. Both ligands and receptors are single-pass transmembrane proteins. Following ligand-receptor interaction in cell-to-cell signaling, a series of proteolytic cleavage events occur which culminate in cleavage by a γ-secretase complex that releases NotchIC [86]. In the absence of signaling the CSL protein is associated with a corepressor complex that includes Mint/Sharp, SMRT, CIR, SKIP, mSin3A and HDACs [87–90]. Upon signal induction, NotchIC enters the nucleus and binds CSL in association with a coactivator complex that includes p300 and MamL1 [91]. This results in transcriptional activation of the targeted genes. Although EBNA2 lacks amino acid homology with NotchIC, it nonetheless targets CSL in a mechanistically similar manner and likewise recruits coactivators to trigger promoters containing CSL-binding sites (GTGGGAA) [92, 93]. There is evidence that the EBNA2/NotchIC-bound CSL complex has a greater affinity for DNA than the CSL/corepressor complex and this may contribute to the switch from repression to activation [94]. Interestingly, the mammalian CSL protein lacks a classical nuclear localization signal and nuclear transfer requires interaction with SMRT of the corepressor complex or NotchIC/EBNA2 [95]. This raises the possibility that the corepressor/coactivator complexes are assembled in the cytoplasm prior to nuclear entry. A major difference between Notch and EBNA2 signaling is that Notch signaling is transient, whereas EBNA2 is constitutively active.

Interference with the EBNA2-CSL interaction either by mutagenesis [96] or by introducing a cell-permeable competitive peptide prevents EBV-induced growth stimulation of primary B cells and the peptide also abolishes the growth of type III B cell lines [97]. Notch signaling is sensitive to cell context and to signal intensity. Notch can induce either proliferation or differentiation in different settings [91]. EBNA2 signaling is also subject to modulation through interactions with the latency EBNA3 and EBNA-LP proteins. Binding of EBNA3 proteins is competitive with EBNA2 and also inhibits CSL binding to DNA, thus downregulating EBNA2 activation. Functional mapping of EBNA3A found that the regions of EBNA3A needed for interaction with CSL were essential for type III B cell proliferation [98]. Further, conditional expression of EBNA2 in a type I B cell line, and therefore in the absence of the EBNA3 proteins, leads to growth arrest [99], as does disturbing the balance by overexpression of EBNA3A. The differences in differentiation versus proliferation responses seen with Notch signaling in different cell types are also observed with EBNA2. EBNA2 is not expressed upon EBV infection of epithelial cells but forced expression of EBNA2 in this setting is associated with cessation of cell growth and induction of p21/WAF1 [100].

Several RNA array and proteomic analyses have been performed to identify EBNA2-regulated genes using conditional loss of EBNA2 expression in the context of type III latency or conditional expression in B cells in the absence of other EBV proteins [101–104], and EBNA2-interacting proteins have also been identified by yeast 2-hybrid as part of systematic interactome mapping of EBV proteins [105]. A key finding from these analyses was the validation of c-Myc and c-Myc-regulated genes as downstream EBNA2 effectors. This finding is consistent with the observation that c-Myc is also a target of NotchIC [106, 107]. Outside of the c-Myc axis and some of the basic-helix-loop-helix (Hes/Hey) genes, there was relatively little overlap between EBNA2-regulated genes and known Notch targets. This lack of a broad correlation between EBNA2 and Notch likely reflects the contributions of tissue background and dosage sensitivity to the signaling readout. NotchIC and EBNA2 also function by CSL-independent mechanisms. One example is the ability of both NotchIC and EBNA2 to interact with Nur77 [108, 109]. Nur77, a member of the nuclear hormone receptor superfamily, is a transcription factor that has a second function as a mediator of apoptosis. In response to an apoptotic stimulus, nuclear Nur77 translocates to the cytoplasm, where it targets mitochondria to induce cytochrome c release [110]. NotchIC/EBNA2 protect cells from Nur77-mediated apoptosis by preventing Nur77 nuclear export. The conserved region 4 of EBNA2 binds Nur77 and conserved region 4 deleted EBNA2, when expressed in the background of an EBNA2 deleted, complementing EREB2–5 type III cell line or, within a recombinant EBV virus, results in decreased cell viability and loss of protection against Nur77-mediated apoptotic stimuli [111, 112].

A conundrum raised by the finding of EBNA2 as a NotchIC mimic is that while dysregulation of Notch 1 signaling through translocation or mutation occurs in ~55% of all human T cell acute lymphoblastic leukemias [106], Notch signaling has

not been consistently linked to B cell malignancies. Conditional deletion of Notch 1 in the mouse impaired T cell development but did not affect the development of mature B cells [113]. Conditional deletion of CSL or Notch 2 specifically prevented the development of marginal zone B cells [114, 115]. In the hematopoietic stem cell compartment, the role of Notch appears to be to inhibit differentiation and favor stem cell self-renewal [116, 117] rather than to contribute directly to proliferation, and EBNA2 may similarly provide an antidifferentiation signal. The recent description of synergy between Notch signaling and B cell receptor (BCR) activation and CD40 signaling to optimize activation of follicular B cells may be particularly relevant [118]. The EBV LMP1 and LMP2A proteins mimic constitutive CD40 and BCR signaling respectively, and EBNA2 may thus be providing a Notch-like signal to the EBV-infected B cell to maximize an activated, proliferative response.

Growth-Stimulatory and Cell Survival Properties of Other Epstein-Barr Virus Latency Proteins and RNAs

The EBNA1, EBNA2, EBNA3A, EBNA3C and LMP1 proteins are required for EBV-induced growth immortalization of primary B cells in vitro. The EBNA-LP and LMP2A, -2B proteins and the noncoding EBERs and BART RNAs are nonessential in vitro but are likely to have a role in efficient EBV persistence in vivo.

EBNA1

EBNA1 binds to sites in the EBV latency origin of replication, ori-P, and is necessary for replication of the episomal EBV genomes and efficient segregation of the EBV genomes upon cell division [119, 120]. EBNA1 acts as a transcriptional enhancer of EBV latency gene expression when bound to ori-P and this property is also necessary for B cell immortalization. A genetic approach revealed that the latency Cp promoter was quiescent in the absence of EBNA1 enhancer activity, thus preventing Cp-driven expression of the nuclear EBNA proteins [121]. EBNA1 binding to USP7/HAUSP, a ubiquitin-specific protease, protects cells from apoptotic challenge by lowering p53 levels [122, 123]. EBNA1 expression has been found to alter cell gene expression in array analyses [124]. This property is insufficient to directly cause tumorigenesis in that EBNA1 transgenic mice either did not develop malignancies [125] or showed limited lymphomagenesis after a long latency [126]. EBNA1 contains a central glycine-glycine-alanine repeat region that slows translation and reduces the presentation of CD8+ T cell epitopes [127, 128], thus allowing EBNA1 to be expressed without eliciting a strong CD8+ immune response.

EBNA3A, B, C

The EBNA3 proteins provide the dominant CD8+ epitopes during EBV latency [129]. EBNA3B is not required for in vitro immortalization of B cells. EBNA3A and -3C are

believed to contribute to immortalization through several mechanisms. EBNA3 downmodulation of EBNA2 transcriptional activity through competition for the EBNA2 DNA-targeting partner CSL (CBF1/recombination-binding protein Jκ) is one key activity [98, 130]. EBNA3C can abrogate cell cycle checkpoints through inhibition of p27/KIP1 and interaction with the checkpoint regulator Chk2 [131, 132]. In an inducible system, loss of EBNA3C led to accumulation of p16/INK and a decrease in hyperphosphorylated pRb [133]. Both EBNA3A and -3C provide a cell survival advantage by downregulating the expression of the cell death mediator Bim [134].

EBNA-LP

EBNA-LP acts as a coactivator for EBNA2-mediated transcriptional activity. EBNA-LP coactivation is mediated by Sp100. EBNA-LP interacts with Sp100 and displaces it from promyelocytic leukemia nuclear bodies [135, 136].

LMP2A, 2B

The cytoplasmic N terminus of LMP2A contains immunoreceptor tyrosine activation motifs and LMP2A modulates BCR signaling by associating with the cellular tyrosine kinases Lyn and Syk. LMP2A mimicry of BCR signaling provides a survival signal for B cells in vivo that allows B cells lacking a functional BCR to avoid their predestined apoptotic fate and survive [137, 138]. In vitro, LMP2A recruitment of Lyn and Syk has the opposite effect and blocks BCR-induced signaling. Since BCR crosslinking causes EBV lytic cycle reactivation, the expression of LMP2A may favor the maintenance of EBV latency [139]. LMP2A provides cell survival signals through activation of PI3K and Akt in both B cells and epithelial cells [140, 141] and activation of β-catenin in epithelial cells [142]. LMP2B negatively regulates the activity of LMP2A [143]. Although LMP2A is not essential for in vitro B cell immortalization by EBV, LMP2A is believed to provide important survival signals during the maintenance of in vivo EBV persistence.

EBERs

Because of their abundant expression in all EBV-infected cells ($\sim 5 \times 10^6$ copies per cell), the small noncoding EBER RNAs form the targets for in situ hybridization assays to identify EBV-infected cells in clinical samples [144]. The polymerase III transcribed EBER RNAs provide protection against interferon-induced cell death by binding to the interferon-inducible protein kinase, PKR, and blocking its kinase activity [145, 146]. EBER RNAs also protect against the interferon response induced through RIG-I, which responds to viral double-stranded RNA [147]. The mechanistic basis for the blockage of innate immune responses is unclear in the context of the finding that the EBERs are located solely in the nucleus [148]. Infection of primary B cells with recombinant EBV deleted for either EBER1 or EBER2 revealed that EBER2 significantly impacted on B cell outgrowth following virus infection, whereas loss of EBER1 had little effect in this assay [149].

BamHI-A Rightward Transcripts

The BARTs are highly spliced, polyadenylated EBV transcripts that are detected in all EBV-infected cells and tumors and are particularly abundant in EBV-infected epithelial tissues [150–152]. The function of the BARTs had been enigmatic until the discovery that they give rise to multiple micro-RNAs [29, 30]. A cluster of the BART micro-RNAs target the 3′ untranslated region of the LMP1 transcript [79]. These micro-RNAs negatively regulate LMP1 protein levels and modulate LMP1-induced NF-κB signaling. Since elevation of LMP1 levels by as little as 2-fold is growth inhibitory and proapoptotic [153, 154], the BART micro-RNAs may be important in modulating LMP1 levels to ensure a proproliferative outcome. Interestingly, in B cells LMP1 upregulates IRF-4 [155], which blocks IRF-5 activation through competition for MyD88. IRF-5 is expressed in type III latently infected cells and downregulates the BART promoter [156]. Thus a regulatory feedback loop exists in which a reduction in LMP1 would reduce IRF-4 expression, increase the levels of activated IRF-5 and reduce the expression of the BART micro-RNAs. A reduction in the negative regulatory micro-RNAs would in turn increase the LMP1 protein levels (table 1).

In vivo Epstein-Barr Virus Persistence

A model has developed in which EBV establishes and maintains life-long persistence by utilizing natural B cell biology while at the same time enhancing the survival of the EBV-carrying B cell population [28, 157]. EBV primarily infects B cells of Waldeyer's ring (tonsils and adenoids). Using B cell markers to sort cells, naïve (IgD+) tonsillar B cells were found to be the population expressing type III EBV growth proliferative genes. These EBV-infected cells also expressed CD80, a marker of activated, proliferative lymphoblasts [31]. Naïve B cells are normally activated by a combination of antigen presentation, which leads to BCR signaling, and T cell help which initiates CD40 signaling. In the case of EBV infection these 2 signals are provided constitutively by LMP2A and LMP1 respectively. Recently, Toll-like receptor (TLR) signaling has been recognized as a requisite third signal for the activation of naïve B cells. BCR stimulation and T cell help were found to induce only limited proliferation and signaling by any of the TLRs was necessary to produce a full proliferative response [158]. EBV interactions with plasma-membrane-associated TLR2 and with intracellular TLR7 have been described. EBV binding to the B cell in the absence of gene expression upregulates NF-κB through TLR2 [159] and upregulates the expression of a number of interferon-stimulated genes including TLR7. Furthermore, treatment of naïve B cells with a TLR7-inhibitory oligonucleotide impairs EBV-induced proliferation of these cells [155]. Thus EBV interaction with naïve B cells also induces TLR signaling to provide the third signaling stimulus. Activated TLR2+ B cells from tonsils are induced to differentiate and secrete IgM by TLR2 ligands [160]. The anti-differentiation function of EBNA2 may provide protection from the differentiated cell fate. EBV also

Table 1. EBV latency products

	Key functions
Nuclear proteins	
EBNA1	Binds sites in the latency origin of replication, ori-P, and the Qp promoter; essential for replication and maintenance of the EBV episomal genome in latency; provides enhancer function for latency Cp promoter; regulates p53 stability
EBNA2	Transcription factor; mimics Notch signaling through interaction with CSL; upregulates c-Myc; alters cell gene expression; antiapoptotic function
EBNA3A, 3B, 3C	3A and 3C are required for in vitro B cell immortalization; modulate the EBNA2-CSL interaction; overcome cell cycle checkpoints; downregulate proapoptotic Bim
EBNA-LP	Coactivator for EBNA2; interacts with Sp100 and displaces it from promyelocytic leukemia nuclear bodies; nonessential for in vitro immortalization of B cells
Membrane proteins	
LMP1	Mimics activated CD40 signaling; activates NF-κB, JNK, MAPK and p38 pathways; induces epithelial hyperplasia and B cell lymphoma in transgenic mice; alters cell gene expression
LMP2A	Manipulates BCR signaling; provides cell survival signals; blocks lytic cycle reactivation; nonessential for in vitro immortalization of B cells
Noncoding RNAs	
EBER1, EBER2	Abundant ~172 nt polymerase III transcribed RNAs; inhibit apoptosis; nonessential for in vitro immortalization of B cells
BARTs	Large alternatively spliced, polyadenylated transcripts; source of multiple miRNAs; cluster 1 miRNAs regulate LMP1 protein level; nonessential for in vitro immortalization of B cells

encodes 2 Bcl-2 genes, BHRF1 and BALF1, that are expressed as part of the viral lytic program but are additionally expressed immediately after infection to protect B cells from apoptosis [161] (fig. 1).

Activated B cells expand into germinal centers, where survival depends on the B cell expressing a high-affinity BCR that can receive survival signals from cognate antigen presented by dendritic cells and on T helper cell activation of CD40 signaling. Cells lacking BCR expression or expressing BCRs with crippling mutations are eliminated by apoptosis. In vitro infection of germinal center B cells with EBV generates B cell lines lacking functional BCRs, indicating that EBV infection can rescue such cells [162, 163]. This correlates with in vivo studies documenting destructive BCR

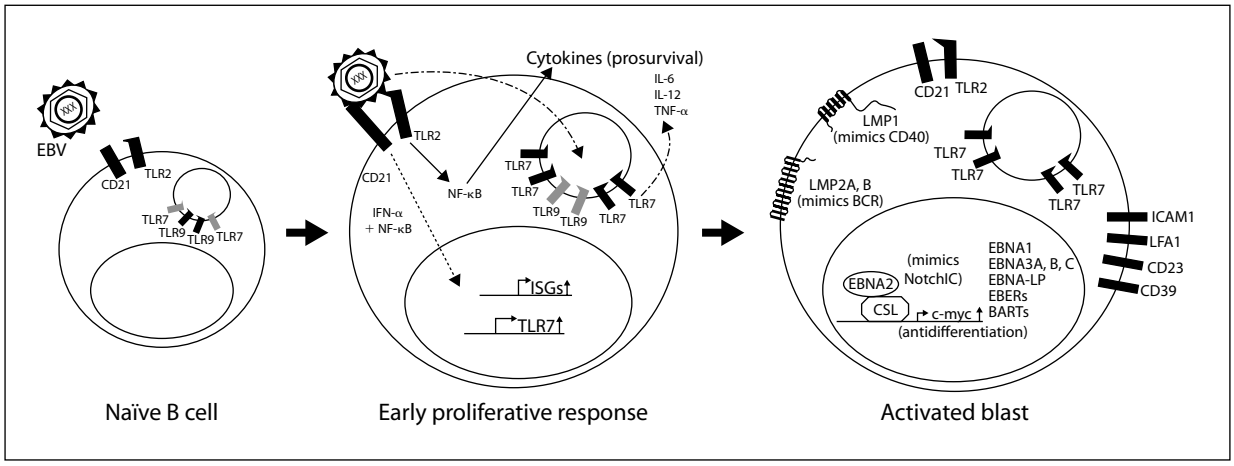

Fig. 1. EBV infection of B cells. The naïve B cell is the predominant target of EBV infection during maintenance of viral persistence in vivo. Contact between the virion and its receptor, CD21, activates NF-κB and interferon-α pathways and NF-κB activation is further induced by interaction with TLR2. This initial signaling combines to upregulate interferon-stimulated genes (ISGs) and TLR7 (dark shading) and to downregulate TLR9 (light shading). NF-κB and TLR7 signaling induce expression of proproliferative and prosurvival cytokines. With progression of the infection, EBV latency proteins are produced that mimic constitutively activated cell receptors and drive proliferation. LMP1 mimics activated CD40, LMP2A mimics BCR activation and EBNA2 mimics activated Notch. Expression of the complete latency III program, including EBNA1, EBNA-LP, EBNA3A/B/C and the noncoding EBER and BART RNAs, converts B cells into activated blasts and drives long-term proliferation. LFA1 and ICAM1 are cell adhesion proteins. CD23 and CD39 are activation antigens.

mutations in EBV-associated Hodgkin's lymphoma [164]. RT-PCR studies on tonsillar germinal center B cells found a type II latency pattern with expression of the EBV EBNA1, LMP1 and LMP2A genes but no expression of EBNA2 or the EBNA3s [165]. Since LMP1 and LMP2A provide the equivalent of CD40 and BCR signaling, the expression of these EBV genes presumably allows the EBV-infected B cells to survive germinal center passage independent of the normal exogenous survival signals. Downregulation of EBNA2 at this point may be important, since EBNA2 represses the expression of the BCL-6 transcription factor [166] that is essential for the formation of germinal centers in mice [167]. B cells exit from the germinal center as memory B cells. The EBV-infected memory B cell transiently expresses EBNA1 but otherwise does not express viral proteins [34]. This allows the infected cell to circulate in the periphery in an immunologically competent individual without being eliminated. In long-term latency, 1–50 per million circulating memory B cells carry the EBV genome. In immunocompromised patients who are not suffering from overt disease this number can increase up to 30-fold, but the carrier cell remains a resting

memory B cell [168]. Cells exiting the germinal center can also differentiate into plasma cells and the plasma cell is the site of lytic viral reactivation [9]. Immunocompromised individuals have an increase in the number of cells expressing the lytic viral program. It should be noted that in infectious mononucleosis, most EBV-positive B cells are detected, not in germinal centers but in interfollicular areas, and may represent direct infection of memory B cells or germinal center cells [169]. The situation during active primary infection may therefore differ from that existing after establishment of a persistent infection.

The Relationship between in vivo Latency and Epstein-Barr-Virus-Associated Cancers

Just as the number of EBV latency genes that are expressed differs in different B cell compartments, so does the extent of latency gene expression that occurs in different EBV-associated malignancies. In posttransplant lymphoproliferative disease and in primary central nervous system lymphoma in AIDS, full latency III gene expression occurs. These cells have the characteristics of activated B cells and are polyclonal or oligoclonal proliferations. The loss of host immune surveillance may allow increased direct infection of memory or germinal center B cells in a manner reminiscent of primary infection during infectious mononucleosis, or cells that do not participate in the germinal center reaction and would normally be eliminated by T cell surveillance may survive and expand.

EBV-associated Hodgkin's disease and Burkitt's lymphoma cells show evidence of having gone through a germinal center reaction. The EBV-positive malignant Hodgkin/Reed-Sternberg cells express the type II latency profile of EBNA1, LMP1 and LMP2, EBERs and BARTs. This is the profile seen in tonsil germinal center cells and suggests that these malignant cells exit the germinal center without having completed the memory B cell differentiation step and while still retaining their proliferative capacity. Burkitt's lymphoma expresses the type I program of EBNA1, EBERs and BARTs. All Burkitt lymphoma cells carry a translocation of the c-Myc gene that places its regulation under that of the immunoglobulin locus. Endemic Burkitt's lymphoma occurs in the malarial belt of Africa. It has recently been reported that a membrane protein of the malarial parasite stimulates EBV-positive peripheral blood memory B cells to reactivate the lytic cycle [170]. Thus increased EBV viral load along with malarial perturbation of the immune system may enhance the frequency of erroneous recombination events such that an EBV-infected cell may exit the germinal center as a memory B cell but with constitutive upregulation of c-Myc providing a proliferative signal.

In the epithelial malignancy nasopharyngeal carcinoma, EBV also expresses the type II latency program. EBV infection of epithelial cells in vitro is biased towards the lytic program and epithelial infection in vivo is believed to provide a mechanism for

amplification of virus replication and spread. In type II latency the promoters for the EBNA1 (Qp) and LMP1 (L1-TR) genes are regulated by STAT transcription factors [46, 74]. STATs are cytoplasmic proteins that require phosphorylation for nuclear import and transcriptional activity. Activated STATs are not present in normal epithelium, but nuclear STAT-3 and STAT-5 are detected in nasopharyngeal carcinoma cells [74]. Entry of EBV into a cell that has already undergone disregulation of STAT signaling could be a predisposing event for the development of nasopharyngeal carcinoma by inducing the expression of EBNA1 and LMP1 and favoring the establishment of a latent EBV infection.

Concluding Remarks

Infection with EBV is prevalent worldwide and yet malignancies associated with the virus are largely restricted to particular geographic areas and/or to populations who are immunocompromised. The immune defect may be genetic such as occurs in severe combined immunodeficiency disorder [171], be driven by another infectious agent like human immunodeficiency virus or the malaria parasite or be imposed as part of a clinical treatment regimen as occurs in organ transplantation. The relatively limited frequency of EBV-associated malignant disease indicates that life-long infection is usually well controlled by the host immune system. However, as part of the biology required to maintain life-long persistence, EBV expresses regulatory RNAs plus multiple latency genes whose protein products induce cell proliferation and enhance cell survival. These properties allow survival of cells that would otherwise be destined for elimination. The resulting B cell population has the potential to accumulate the kind of cellular genomic mutations or rearrangements that can ultimately drive the development of EBV-associated B cell malignancies.

Information on EBV gene expression in different B cell populations in vivo has enhanced the understanding of the integration of the EBV-infected B cell into pathways of natural B cell trafficking and differentiation and has produced insight into the derivation of the different types of EBV-associated malignancy. There remain discrepancies between the models for persistent EBV B cell infection and the observations made in primary EBV infection in the context of infectious mononucleosis. Additional studies are needed to evaluate the extent to which EBV behavior in infectious mononucleosis is representative of the more common asymptomatic primary EBV infection.

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Kaposi-Sarcoma-Associated Herpesvirus

Clinical Diseases and Viral Pathogenesis

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Abstract

Kaposi-sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 , is a member of the γ-herpesvirus family. KSHV is associated with different malignancies including, Kaposi sarcoma, primary effusion lymphoma and multicentric Castleman disease. In this review we will discuss the pathogenesis of Kaposi sarcoma and KSHV-induced lymphoma, including primary effusion lymphoma and multicentric Castleman disease. We will also provide a brief introduction to the virus itself. We particularly focus on the viral gene expression pattern together with the current animal models that allow us to study the above-mentioned diseases and overall pathogenesis by KSHV. We conclude with the mention of some currently prevalent therapeutics and some novel strategies with increased potential.

Kaposi-sarcoma-associated herpesvirus (KSHV) causes Kaposi sarcoma (KS), an endothelial cell tumor. The link between the virus and the cancer fulfills Koch's postulates: every KS patient carries KSHV and every KS tumor cell expresses ≥1 viral proteins [1, 2]. As with other cancers, and unlike infectious diseases, there exists a time gap between primary infection and disease; an estimated 7 years pass between seroconversion and disease manifestation [3, 4]. An increase in viral load in the peripheral blood and KSHV seropositivity predicts subsequent disease state. Full-length KSHV has been cloned from a KS lesion (Genbank entry: AF148805) and KSHV-carrying primary effusion lymphoma (PEL), which are a type of B cell lymphoma. PEL can be induced to release infectious virus particles [5] and this virus is capable of infecting cells in culture [6, 7] and in mouse models [8]. KS is a tumor of

endothelial cells and upon infection with KSHV immortalized endothelial cells take on a KS-like morphology [9–11] and some attain the additional ability to form tumors in mice [12, 13]. Transcriptional profiling of these mouse tumors, suggest that they closely resemble patient KS lesions.

Kaposi Sarcoma

KS is a tumor of lymphatic endothelial cell origin [14–16] and was identified as an AIDS-defining malignancy. It is tightly correlated with loss of CD4+ T-cells and the ensuing loss of immune surveillance resulting in viral reactivation from a latent reservoir, which we believe is a subset of B cells [17, 18]. Other forms of immune suppression, such as that resulting from organ transplantation, can also lead to viral reactivation and KS even in the absence of HIV infection. In organ transplant patients, the KS incidence rates correlate with regional KSHV prevalence. Then there is classic KS, initially described by Moritz Kaposi [19, 20] in 1872, requiring no known cofactors, other than age. Lastly, there is endemic KS, always widespread in parts of Sub-Saharan Africa and now shown to be the most abundant cancer in children, many of whom are HIV-positive at birth. All 4 forms of KS, HIV-associated, iatrogenic, classic/sporadic and endemic, are related to KSHV. At present there is no evidence to suggest that they are caused by different virus strains or that the key molecular mechanism is different. However, there is variation in their overt presentations leading to the clinical classifications of patch, plaque and nodular KS. There are differences in the tumor locations too, as KS foci can materialize on the skin or involve internal organs.

A rise in viral load predicts imminent clinical lesions independent of HIV or immune status [21] [Dittmer and Martin, unpubl.]. The KSHV viral load rises in peripheral blood mononuclear cells 1–6 months before lesion formation [22] and KSHV is also found in the circulating B cells, macrophages and endothelial cells [2, 17, 23]. The presence of anti-KSHV antibodies documents prior exposure but does not predict KS development, since in HIV-positive individuals the median time from seroconversion to disease is $\geq$7 years [3, 24].

Lymphoma Related to Kaposi-Sarcoma-Associated Herpesvirus

Coincidentally, lymphoproliferative diseases, such as PEL or multicentric Castleman disease (MCD) [25, 26], often accompany KS in AIDS patients. The lymphatic effusions harbor KSHV and maintain the virus upon continuous growth in culture or in tumor xenograft models [5, 11, 27, 87]. PEL, also referred to as body-cavity-based lymphoma, represents a specific subset of non-Hodgkin's B cell lymphomas that involves peritoneal, pleural or pericardial cavities, thus representing a distinct clinicopathologic

group [28]. All PEL are KSHV-positive, and many are often coinfected with Epstein-Barr virus. They are typically large-cell immunoblastic or anaplastic lymphomas that express CD45, clonal immunoglobulin gene rearrangements, lack c-*myc*, bcl-2, ras and p53 gene alterations [28, 29]. PELs have characteristics analogous to a preterminal stage of B cell differentiation. Since PELs have mutations in their immunoglobulin genes, they are thought to arise from postgerminal center B cells. Most PELs express CD138/syndecan-1 antigen, which is normally found in a subset of plasma cells, but unlike plasmacytomas, PELs do not express immunoglobulins. Recently, other rare B cell lineage lymphoproliferative diseases like germinotropic lymphoproliferative disease have been linked to KSHV [30]. Germinotropic lymphoproliferative disease involve plasmablasts but unlike plasmablastic lymphomas contain polyclonal immunoglobulin receptors. There have also been other case reports showing KSHV present in solid HIV-associated immunoblastic/plasmablastic non-Hodgkin's lymphomas [31, 32]. This suggests a model where KSHV infects early germinal center B cells that can still differentiate into multiple phenotypes depending on the secondary mutations in the cellular genome.

Kaposi-Sarcoma-Associated Herpesvirus

KSHV is the eighth member of the human herpesvirus (HHV) family and is also named HHV-8. The herpesviruses comprise a group of double-stranded DNA viruses with varied biology and disease induction properties. The pathogenesis caused by these viruses is usually dependent upon host immune suppression. All herpesviruses share a common evolutionary origin, as highlighted by the homology between a substantial number of the viral genes [33]. Based on their biological characteristics and genomic organization, herpesviruses are classified into 3 subfamilies: α, β and γ. The γ-herpesviruses are lymphotropic, i.e. they infect and establish long-term latency in the lymphoid compartment. They are further subdivided into: lymphocryptoviruses (γ-1) and rhadinoviruses (γ-2). Whereas Epstein-Barr virus or HHV-4 is a lymphocryptovirus, KSHV (HHV-8) is a rhadinovirus. In addition to establishing latent infection, KSHV is capable of undergoing lytic reactivation in epithelial, endothelial or fibroblast cells.

Every KS tumor cell transcribes high levels of the canonical KSHV latency genes latency-associated nuclear antigen (LANA), viral FADD-like interferon-converting enzyme or caspase 8 inhibitory protein (vFLIP), viral cyclin (vCYC) and kaposin [1, 2, 41] together with all the viral micro-RNAs [Dittmer, unpubl.]. LANA, vFLIP, vCYC and the viral micro-RNAs [34–36] are all under the control of the same LANA promoter (LANAp). Despite being located several kilobases downstream of these 3 genes, kaposin is also transcribed from the LANAp by alternative splicing during latency. It is also noteworthy that kaposin, mirK12–10 and mirK12–11, which are embedded in the Kaposin message [36–38], can be transcribed from an inducible proximal promoter. Kaposin was further shown to stabilize cellular cytokine mRNAs

[39]. Hence, these 4 genes: LANA, vFLIP, vCYC and kaposin, are all believed to be essential for KS tumorigenesis. However, using viral profiling studies we were able to discern primary tumors into distinct subsets based upon the extent of expression of lytic viral genes, including KSHV K1 [40], the viral interferon regulatory factor (vIRF-1) and the viral G-protein-coupled receptor (vGPCR) [41], suggesting that a subset of KS phenotypes is attributable to these genes [42–44]. Interestingly, vIRF-3, a duplicated KSHV IRF homolog, is constitutively (latently) transcribed in KSHV-infected PEL [45] but not in KS. We speculate that in order for KSHV to interfere with the host innate interferon response, the virus has placed copies of the vIRFs in infected cells which can interfere with normal IRF signaling, under different control elements: one specific to B cells (vIRF-3) and another to endothelial cells (vIRF-1).

Animal Models of Kaposi Sarcoma and Kaposi-Sarcoma-Associated Herpesvirus

Animal models are essential for understanding the biology of human disease. KS and KSHV-associated lymphomas are no exception. Additionally, KSHV is particularly difficult to grow in cell culture and does not effectively infect any species other than humans. The current animal models include rhesus macaque monkeys infected with rhesus rhadinovirus (RRV), the primate homolog of KSHV. Human KSHV does not infect primates, even though most primates carry their own rhadinoviruses, such as RRV. Additionally, human KSHV does not infect mice. However, humanized immune-deficient mice are capable of being infected by KSHV and serve as a small animal model for drug efficacy [8] and pathogenesis [46]. Humanized mouse models are not only used for KSHV, but many other human viruses such as HIV-1, Epstein-Barr virus, human T lymphotropic virus 1, HHV-6, varicella-zoster virus human cytomegalovirus (HCMV) and others. In newer mouse models, human cells of all hematopoietic lineages (including monocytes and B cells) and stromal endothelial cells survive for prolonged periods in the animal host. Depending on the biology of the particular virus and the design of the human graft, the resulting infection may be noncytopathic (e.g. HCMV) or induce severe target cell depletion (HHV-7, HIV-1). Experiments by our group and others have revealed a biphasic infection, an early phase of lytic replication followed by long-term latency. Infection is dependent upon intact virions as shown by inhibition of lytic replication upon ganciclovir treatment. Viral DNA persistence and gene expression is most abundant in CD19-positive B lymphocytes, suggesting that these models faithfully mimic the hitherto known tropism of KSHV [17, 18, 47].

Work on the primate and murine homologs has contributed significantly to our understanding of KSHV. Murine herpesvirus 68 (MHV-68) in particular has been invaluable, since many of the viral proteins show relatively high sequence and functional homology to KSHV. For instance, MHV-68 vCYC induces tumors in transgenic mice [48] analogous to KSHV. Studies have shown that MHV-68 required LANA [49]

and a pool of proliferating B cells to establish long-term splenic latency [50]. Because MHV-68 replicates to high titers as well as establishes latency in mice, detailed pathogenesis studies are possible. However, there are also differences between the human and murine viruses, such as the absence of homologs of kaposin, the KSHV micro-RNAs and vFLIP [51].

The primate homolog of KSHV, RRV, is a natural infectious agent of macaques. Most macaques in captivity are seropositive for RRV, but for experimental purposes, newborn macaques can be raised free of RRV (and other herpesviruses) by hand-rearing [52]. This allows us to study de novo infection of immunologically naïve animals. Two complete RRV strains have been sequenced at the Oregon Regional Primate Research Center and the New England Primate Research Center. Analysis of the RRV26–95 and RRV17577 genomes indicates that these are independent isolates of the same virus species and are closely related in structural organization and overall sequence to KSHV [53, 54]. All KSHV genes have at least 1 clearly discernable homolog in RRV except KSHV K3, K5, K7 (nut-1), and K12 (kaposin). RRV contains 1 macrophage inflammatory gene (MIP-1/vCCL) and 8 IRF (vIRF) homologs compared to 3 MIP-1/vCCLs and 4 vIRF genes in KSHV. In addition to sequence identity, there is also similarity with respect to gene expression and splicing. For example, the RRV Orf50, R8 and R8.1 polycistronic transcript and the LANA, vCYC and vFLIP transcripts are all spliced similarly to the transcripts encoding the corresponding genes in KSHV, [55, 56]. Although there is no sequence homology between the RRV and KSHV micro-RNAs, the RRV micro-RNAs are located at the same relative position downstream of vFLIP [57]. The transcription program of RRV resembles that of KSHV [55] and RRV capsid structure and virion assembly are analogous to KSHV [58, 59].

As mentioned above, the lack of a traditional permissive culture system for KSHV limits the ability to study the lytic aspects of the virus lifecycle. Although 2 KSHV bacterial artificial chromosomes (BAC) have been constructed [60, 61] for genetic manipulation of the virus, the methods to analyze properties of recombinant KSHVs are limited due to low-efficiency tissue culture models and humanized SCID mice. In contrast, RRV can be grown to high titers (1×10^6 pfu/ml) in rhesus fibroblasts and plaque assays are routinely used to measure RRV replication [62]. RRV recombinants can be generated by standard gene replacement techniques, an RRV-BAC or transfection of overlapping cosmids [62–64]. RRV-negative rhesus macaques inoculated with RRV demonstrate persistent viral infection [52] in 100% of the animals, providing a robust and biologically significant animal model.

RRV infection of macaques results in lymphoproliferative diseases reminiscent of KSHV-associated MCD [65]. In contrast to control animals inoculated with SIVmac239, the HIV homolog in macaques, or RRV alone, 2 of 2 animals coinfected with SIVmac239 and RRV17577 developed hyperplastic lymphoproliferative disease resembling MCD, characterized by persistent angiofollicular lymphadenopathy, hepatomegaly, splenomegaly and hypergammaglobulinemia. Similar coinfection studies were also performed by Mansfield et al. [52] at the New England Primate

Research Centrer using RRV (strain 26–95) and SIVmac239. Experimental infection of macaques was associated with lymphadenopathy that subsequently was replaced by marked follicular hyperplasia. In the severest cases, this follicular hyperplasia destroyed the medullary sinuses and completely effaced the normal lymph node architecture, similar to KSHV-infected, HIV-negative patients with histologic features of angioimmunoblastic lymphadenopathy and reactive lymphadenopathy. B cell proliferation is a common occurrence between KSHV-associated MCD and angioimmunoblastic lymphadenopathy [52]. However, these pathologies were transient; 12 weeks after RRV infection they appeared to be resolved and the macaque immune system was able to prevent progression to lymphoma. In addition, Mansfield et al. [52] observed that 3 of the 4 monkeys coinfected with RRV and simian immunodeficiency virus developed an arteriopathy. This arteriopathy was similar to the vascular endothelial lesion seen in patients with KS and to the large-vessel arthritis in MHV-68-infected mice. This suggests that acute infections with KSHV, RRV or MHV-68 in the host induce similar pathologies.

To study established KSHV-associated cancers, xenograft models have proven invaluable. PEL cells are readily transplantable onto immune-deficient SCID and nude mice [27, 87, 91]. Intraperitoneal injection typically results in ascites tumors, whereas intravenous or subcutaneous injection leads to tumor formation. For some cell lines, ascites as well as subcutaneous tumors were observed [67]. KS tumors have not been propagated in mice or tissue culture at this point. However, artificial KSHV infection of endothelial cells of both human [68] or murine [12] origin form cell lines that are fully tumorigenic in nude mice and maintain the KSHV genome in the absence of selection. In these examples, KSHV contributed directly to the transformed phenotype and represent the best KS tumor model to date. However, this is an infrequent event, since most KSHV-infected endothelial cells do not form tumors in mice.

An alternative approach to infection studies uses transgenic mice, where individual KSHV proteins are expressed in the hopes of recapitulating selected aspects of KSHV pathogenesis. For instance, we found that the KSHV latent promoter (LANAp) showed B cell lineage specificity in transgenic mice [69]. KSHV LANA expression in transgenic mice resulted in B cell hyperplasia, though fully neoplasic lymphomas were rare in the Bl6 strain of mice [70]. In addition to LANA single transgenic mice, vCYC single transgenic mice and vFLIP single transgenic mice have been studied. By comparison endothelial cells were more resistant to the action of KSHV genes than developing B cells (summarized in table 1). These transgenic models reported a tumor incidence of ~10% compared to ~2% in littermate control mice, which is significant but low and similar to our observations for LANA single transgenic mice. These data suggest that individual viral genes can promote growth but to a very limited extent.

The importance of animal models is underscored by the rather paradoxical behavior of the KSHV cyclin. Ectopic expression of KSHV vCYC alone in cultured cells was not associated with transformation but rather with apoptosis [71–73]. However, loss of p53 uncovered the transforming potential of vCYC in vivo; while KSHV vCYC

Table 1. Latent oncogene cooperation is required for KSHV tumorigenesis

Viral gene	Incidence, %	Mean time to onset, days	Reference
PEL model (transgenic mice)			
Lck-MHV-68 CYC1	40	240	Van Dyk et al. [48]
Eμ-vCYC	17	300	Verschueren et al. [75]
Eμ-vCYC and $p53^{del}$	100	80	Verschueren et al. [74]
H2Kb-vFLIP	11	600	Chugh et al. [106]
LANAp-vCYC	0	300	Dittmer [unpubl.]
LANAp-LANA	10	300	Fakhari et al. [70]
KS model (transgenic mice)			
vCYC	0	≥180	Montaner et al. [44]
vFLIP	0	≥180	Montaner et al. [44]
Kaposin	0	≥180	Montaner et al. [44]
vCYC and vFLIP	0	≥180	Montaner et al. [44]
VEGFR-LANA	80	200	Sugaya et al. [107] (lung failure)

single transgenic mice did not develop tumors, lymphomas developed rapidly when crossed in a p53null background [74, 75]. Presumably, loss of p53 counteracted the proapoptotic signals that were associated with forced KSHV vCYC expression.

Two other KSHV genes, vGPCR [76, 77] and K1 [78], have also been ectopically expressed in transgenic mice. In these models vGPCR and K1 activated the same signaling pathways, as predicted from human tissue culture studies. These transgenic mice are characterized by dysplastic, highly angiogenic lesions, which underscore a role for these genes in the pathogenesis of KS. Additionally the K1 mice also developed B cell lymphomas [78].

Therapeutic Approaches to Kaposi Sarcoma: Highly Active Antiretroviral Therapy

In general, AIDS- and transplant-associated KS declines upon immune reconstitution, but not classic and endemic KS, although the immune status of these patients is typically not assessed. Controlling KS disease in HIV patients by restoring CD4+ T cell levels through highly active antiretroviral therapy (HAART) is very effective. HAART serves multiple purposes: it controls the HIV-positive viral load, which in the context of AIDS KS may reactivate KSHV directly [79, 80] or otherwise exacerbate the KS phenotype through changes in the local cytokine milieu [81, 82], it enables a functional immune response against KSHV, thereby limiting systemic spread [83], and lastly some HAART drugs themselves may also be efficacious against KS tumor growth.

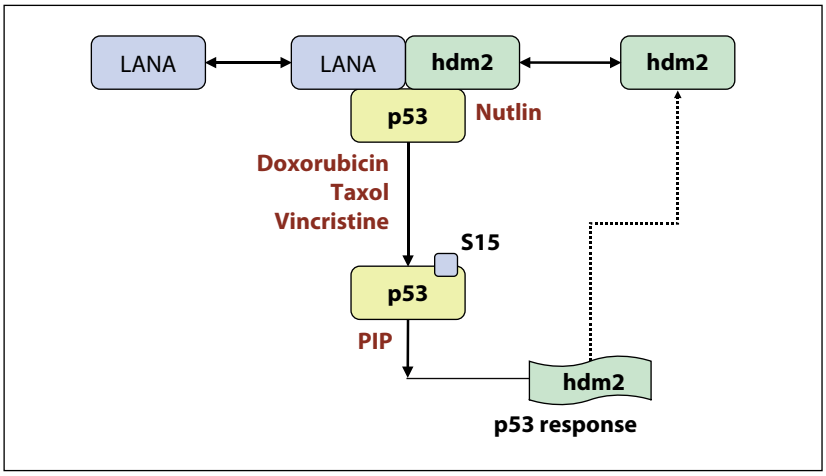

Fig. 1. Model of p53-LANA interaction in PEL. Shown are the interactions between the LANA, hdm2 and p53 proteins (indicated by boxes) based on published observations. These are the LANA:p53:hdm2 trimolecular complex. This complex can be disrupted by nutlin to release free p53, free LANA and free hdm2. The complex can also be disrupted by DNA-damaging agents to yield activated p53 that is phosphorylated at S15. At present, it is not clear whether the activated p53 stems from the complex or whether there is free, i.e. non-LANA-bound p53 in PEL as well. Activated p53 can induce the transcription of canonical p53 responsive genes such as hdm2 (indicated by the wavy box) despite the presence of LANA. PIP = Pifithrin.

In the post-HAART era AIDS KS is seen more and more in patients with reasonable CD4 counts (>400) and undetectable HIV viral load. These patients as well as all those with internal organ involvement are in requirement of novel anticancer therapy.

Therapeutic Approaches to Kaposi Sarcoma: Chemotherapy

The standard of care for systemic KS consists of chemotherapy, most often taxol or liposomal doxorubicin. Current treatments for MCD, PEL and other AIDS lymphomas include combination chemotherapy such as CHOP, which contains 4 drugs: prednisone, vincristine, cyclophosphamide and doxorubicin, or EPOCH that contains etoposide in addition to CHOP. The relative success of chemotherapy against KS and KSHV-associated lymphoma may be linked to the unusual but almost universal wild-type status of p53 in KS. Since p53 is a necessary mediator of DNA and microtubule damage responses, tumors with wild-type p53 function tend to be susceptible to DNA-damaging drugs such as doxorubicin and vincristine. In the case of PEL, susceptibility to DNA-damaging agents correlated perfectly with p53 functionality [84, 85] (fig. 1).

While effective, the side effects of chemotherapeutic drug regimens limit their efficacy, especially in immunocompromised KS patients. Anthracycline drugs in particular

(e.g. doxorubicin, epirubicin, idarubicin, daunorubicin) have known cardiotoxic side effects. To limit these adverse reactions, many of these agents are now formulated as liposomes, the leading commercial utilization of nanotechnology. The liposomal structure is hypothesized to stabilize encapsulated drugs in vivo and thus, liposomes flowing in the blood stream are unable to extravasate intact blood vessels, thereby accumulating in areas of discontinuous capillaries, such as tumor tissue. Since KS is the most highly vascularized tumor characterized by capillary leakage, this property of liposomal drug formulations has increased therapeutic efficacy. Liposomal formulations of daunorubicine and taxol are currently used against KS, even though animal models of KS did not become available until recently and these drugs were never tested preclinically.

There is a methodological problem with the comparative review of anti-KS drugs. KS patients in the USA today are universally on HAART and often show acceptable ($\geq$400 cells/μl) CD4+ T cell counts and thus exhibit KS development in the absence of detectable HIV loads. By contrast, many of the initial clinical trials were conducted in the pre-HAART era, where patients were experiencing HIV- as well as chemotherapy-mediated immune suppression. This may have led to a biased enrollment into trials or part of the chemotherapy effect may have been a result of unintended depletion of HIV target cells.

Therapeutic Approaches to Kaposi Sarcoma: Antiviral Agents

It is tempting to employ antiherpesvirus drugs to fight KSHV-associated cancers, since they would be highly selective against the virally infected tumor cells. A single study showed that systemic ganciclovir reduced the incidence of KS [86], yet it had no effect on established PEL tumors in the mouse model [87]. Ganciclovir requires activation by the KSHV thymidine kinase or phosphotransferase [88], thus latent cells that do not express these viral proteins are resistant to the drug. However, some KS lesions that do express these viral lytic genes in a great proportion of the cancer cells would be susceptible to the drug. More recently, the combination of azidothymidine and ganciclovir together with induction of viral replication followed by an antiviral drug showed promising results in animal studies [89–91]. Ganciclovir, cidofovir and other antiherpesviral drugs most certainly limit KSHV replication and peripheral viremia [92]. Furthermore, antiherpesviral drugs restrict HCMV replication, which frequently reactivates in AIDS patients and in turn was also shown to induce KSHV [93]. This may either be the direct result of HCMV present in coinfected monocytes or through the upregulation of inflammatory cytokines that also reactivate KSHV [94–97].

IFN-α can induce KS tumor regression in a subset of patients with AIDS-associated KS. Its mechanisms of action are diverse but include inhibition of angiogenesis. Most patients treated in the 1980s and early 1990s received IFN-α without concomitant antiretroviral therapy or in combination with single-nucleoside reverse transcriptase

inhibitors and occasionally showed dramatic tumor regression. Recently, Krown et al. [98] reported on a trial where increasing doses of recombinant IFN-α2b were administered daily subcutaneously to successive cohorts of patients with AIDS-associated KS who were also concomitantly undergoing protease-inhibitor-based HAART therapy. This trial established the maximum tolerated dose of IFN-α2b in combination with protease-inhibitor-based HAART therapy. Although KS regression was observed, the study only included 14 patients and the limited analysis failed to show clearance of KSHV from plasma or peripheral blood mononuclear cells, even among patients whose KS regressed. In culture models of PEL, IFN-α was the only cytokine that could inhibit KSHV reactivation [94], suggesting that both virus-specific and generalized antitumor activity may mediate its clinical efficacy.

Therapeutic Approaches to Kaposi Sarcoma: Emerging Molecular Targets

The defining characteristics of KS include extreme angiogenesis, vascular leakage and its endothelial cell origin. In fact, other than hemangioma, which is the hyperproliferation of blood vessels in the skin or the liver, KS is the only cancer of endothelial cell lineage. KS tumor cells are highly growth factor (particularly VEGF-1) dependent. Clinical trials involving daily doses of imatinib mesylate (Gleevec), which targets c-*kit* and platelet-derived growth factor receptor signaling [99, 100], or a matrix metalloproteinase inhibitor [101] both showed clinical and histologic regression of cutaneous KS. A clinical trial using an angiogenic inhibitor, IM862, targeting the angiogenic nature of KS proved ineffective in obliterating KS [102], yet the clinical interest in using antivascular/anti-VEGF agents remains high. Rational candidates include Bevacizumab/ Avastatin™, a monoclonal antibody against VEGF and Medi522/ Vitaxin™, a humanized antibody that targets αVβ3 integrin. Moreover, there is emerging, but as of yet controversial, evidence that protease inhibitors such as indinavir, which also inhibit matrix metalloproteinase, may have direct anti-KS activity in addition to HAART-associated reconstitution of the immune system [103].

The Akt/mTOR signaling pathway has emerged as a promising new target in KS. Akt is one of the most frequently activated kinases in human cancer. It is a kinase that triggers mTOR, either directly or indirectly via TSC-1/TSC-2 and it is negatively regulated by the tumor suppressor, PTEN. Stallone et al. [104] showed that KS tumor biopsies from renal allograft recipients expressed high levels of VEGF, the VEGF receptor, Flk-1/kdr, and phosphorylated Akt and p70S6 kinase, all molecular players in the signaling pathway targeted by rapamycin. Sodhi et al. [105] reported that cell lines expressing HHV-8 vGPCR and vascular tumors in vGPCR transgenic mice had all upregulated Akt/mTOR signaling and were susceptible to inhibition by rapamycin. Wang et al. [40] have also shown that the KSHV K1 viral protein can activate PI3K, Akt and mTOR in endothelial cells and in B cells [106]. Recently our group demonstrated

that KSHV-associated PEL cells were also uniquely susceptible to inhibition by rapamycin [107]. Inhibition of mTOR in KS or PEL resulted in reduced protein synthesis of IL-6, IL-10 and VEGF [unpublished observation].

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Molecular Biology of Human Herpesvirus 8 Neoplasia

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Abstract

Human herpesvirus 8 (HHV-8, Kaposi's-sarcoma-associated herpesvirus) was discovered in 1994, accompanied by tremendous interest in the field of human viral oncology and rapid development of research projects on molecular biological and pathogenesis-related aspects of this novel human γ-herpesvirus. The virus was found, in short order, to be associated with 2 rare B cell lymphomas, primary effusion lymphoma and multicentric Castleman's disease, in addition to Kaposi's sarcoma, in which HHV-8 genomic sequences were first identified. This review summarizes our current understanding not only of the likely mechanisms contributing to HHV-8 oncogenic pathogenesis but also of the functions of key γ-herpesvirus-conserved and HHV-8-specific genes in virus biology. The underlying message is that HHV-8-induced oncogenesis is a complex process that is likely to involve both paracrine-mediated promotion of cell proliferation and survival by viral lytic gene products and classical cellular transformation induced by the activity of latency proteins. Furthermore, the 3 malignancies associated with HHV-8 infection are likely to be the products of different pathogenic mechanisms, including differential involvement of individual HHV-8 latency and lytic genes.

Infection with human herpesvirus 8 (HHV-8), a lymphotropic γ2-herpesvirus, has been linked etiologically to 3 distinct malignancies: Kaposi's sarcoma (KS), primary effusion lymphoma (PEL) and a subset of multicentric Castleman's disease (MCD) [1]. Although presence of HHV-8 genomes is a feature common to all these malignancies, their distinctive clinical, histopathological and molecular characteristics point to distinct underlying pathogenic mechanisms. KS, the most common cancer among patients with AIDS, is a histologically complex highly vascular tumor that is characterized by the presence of distinctive proliferative 'spindle' cells, a perfusion of irregular slit-like endothelial lined spaces (vascular slit-neo-angiogenesis), extravasated erythrocytes and infiltrating inflammatory cells [2]. Although the spindle cells, which are believed to originate from HHV-8-infected vascular or lymphatic endothelial cells, are thought to represent the tumor cells in the KS lesions, they lack many features of classical neoplastic cells, such as clonality, tumorigenicity and aneuploidy [2–4]. Instead, the growth of KS spindle cells, at least in the early stages of the lesions, is believed to be highly dependent on autocrine

and paracrine growth signals provided by the inflammatory and angiogenic cytokines abundantly present in the KS microenvironment [2]. In contrast to KS, PEL and MCD are relatively rare lymphoproliferative disorders that arise from HHV-8 infection of B cells, a fact in keeping with the classification of HHV-8 as a γ-herpesvirus. PEL is a malignant neoplasm of postgerminal center B cells which resembles multiple myeloma in its gene expression profile and typically presents as bloody effusions in body cavities without a contiguous tumor mass [1]. The malignant cells in PEL are clonal, fully immortalized, tumorigenic in nude mice and frequently coinfected with the Epstein-Barr virus (EBV). Finally, HHV-8 infection is frequently, but not invariantly, associated with the 'plasma cell variant' of MCD, an aggressive systemic illness characterized by polyclonal expansion of lymphoid cells resembling plasma cells, which is accompanied by fever, weight loss, splenomegaly and diffuse lymphadenopathy [1]. The presence of systemic symptoms and polyclonal B cell expansion suggest that MCD is primarily a cytokine-mediated process triggered by the systemic release of viral and/or host-derived cytokines which then act in a paracrine fashion to drive B cell proliferation.

Our understanding of the pathogenesis of HHV-8-associated malignancies is further complicated by the debate over the relative contribution of the latency versus lytic genes to the process. In the case of other γ-herpesviruses, such as EBV and herpesvirus saimiri (HVS), latency genes are believed to be key players in cellular immortalization and oncogenicity. In contrast, there is limited or no evidence that latent HHV-8 infection can immortalize cells on its own and, on the contrary, accumulating evidence suggests that lytic replication and expression of lytic genes make important contributions to the pathogenesis of HHV-8-associated malignancies. This notion is supported by the oncogenic potential of a number of HHV-8 lytic genes [5], by the increased incidence and severity of KS in patients with active HHV-8 lytic replication [6, 7] and by the reduced risk of KS development in patients treated with antiviral drugs that target the HHV-8 lytic life cycle [8]. However, since lytic replication eventually culminates in cell death, this raises the important question as to how the expression of lytic genes in cells destined to die can cause cancer. Further complicating the issue of the contribution of lytic genes to HHV-8 oncogenesis is the observation that the 3 HHV-8-associated malignancies not only show considerable difference in the proportion of HHV-8-infected cells undergoing lytic replication (or expressing lytic genes) but also in their spectrum of lytic gene expression [1]. This review outlines the present state of knowledge with regard to the properties, mechanisms of action, and functions of HHV-8 latency and lytic proteins that may contribute to virus-associated neoplasia and attempts to address some of the above issues.

Signaling Receptors

Viral G-Protein-Coupled Receptor

The constitutively active (ligand-independent) chemokine receptor specified by open reading frame 74 (ORF74) of HHV-8 has been implicated in Kaposi's sarcomagenesis

largely because of its ability to induce KS-like tumors in transgenic mice [9–12]. This process involves paracrine mechanisms, believed to be mediated by viral G-protein-coupled receptor (vGPCR)-induced cytokines, and therefore potentially provides a means by which the lytically expressed viral protein could play a role in neoplasia. However, initial studies of vGPCR noted its ability to transform immortalized cell lines to grow in soft agar and to form tumors in nude mice [13], and it was speculated that such activity was relevant to HHV-8-induced malignancies. Indeed, vGPCR can immortalize primary endothelial cells to grow continuously in culture, of potential relevance to KS, by a mechanism that appears to involve induction of hTERT activity and vascular endothelial growth factor (VEGF)/VEGF receptor 2 autocrine signaling [14]. Proliferation and survival of primary endothelial cells was also found to be induced by vGPCR transduction, by a process possibly involving the induction of heme oxygenase-1 activity by vGPCR-induced VEGF [15]. However, it is far from clear that these experimental systems of vGPCR-mediated 'autocrine' cell immortalization and transformation reflect the true role of vGPCR in HHV-8 malignancies because all available evidence indicates that vGPCR is expressed only during lytic replication, in cells destined to die [16–18].

It is in the context of paracrine signaling, then, that the role of vGPCR in virus-induced malignancies is most appropriately considered, in light of our current understanding of vGPCR expression during lytic cycle replication. It is not insignificant that in the murine transgenic model of vGPCR-induced sarcomagenesis only a minority of cells within KS-like lesions actually express the viral receptor and that there are elevated levels of angiogenic cytokines produced in these tissues [9–11]. It is important to note, however, that degradation of most host mRNAs is promoted by the HHV-8 ORF37-encoded exonuclease (SOX), and this presumably would restrict the spectrum of gene products induced by vGPCR [19]. On the other hand, it is also known that some cytokines, such as interleukin-6 (IL-6), and other gene products escape the host shutoff phenomenon, perhaps in part explained by the stabilization of AU-rich-element-containing mRNAs by kaposin B (see below). Notwithstanding these considerations, an important clue to understanding the mechanisms underlying the observed vGPCR-induced pathogenesis was the finding by Holst et al. [10] that chemokine responsiveness of vGPCR was necessary for efficient induction of sarcomagenesis. HHV-8 vGPCR can be activated and inhibited by cellular chemokines, such as CXCL1/GROα (agonist) and IP-10/CXCL10 (inverse agonist), and it is now known that although vGPCR couples functionally to q, i, and 12/13 classes of Gα protein in the absence of chemokine, agonist-induced vGPCR couples specifically to $G\alpha_q$, thereby restricting its signal transduction and downstream targets [20]. As $G\alpha_q$ activates mitogen-activated protein kinase (p38, Erk, Jnk) signaling in endothelial cells and p38/Erk are implicated in induction of VEGF via hypoxia-inducible factor 1α activation [21, 22], this may explain, at least in part, the requirement for agonist responsiveness of vGPCR for sarcomagenesis. Other relevant cytokines, such as IL-6, IL-8/CXCL8, basic fibroblast growth factor, VEGF-C and IL-1β, are induced by

vGPCR, possibly also via $G\alpha_q$-initiated signaling in endothelial cells [21, 23, 24]. Another potential mechanism by which vGPCR may contribute to KS includes its activation of Akt, important for murine sarcomagenesis induced by the viral receptor [25]. Akt-activated mammalian target of rapamycin (mTOR), via targeting and inactivation of mTOR negative regulator TSC2 by Akt, is also key to vGPCR-induced endothelial cell growth in culture and to allograft-induced tumorigenesis [26]. Sarcomagenesis in this allograft model was demonstrated to be mediated via paracrine mechanisms.

Fitting in with the idea of paracrine-induced Kaposi's sarcomagenesis, naturally and in the vGPCR-transduced mouse model, is the demonstration in mice conditionally expressing vGPCR that continued expression of the HHV-8 receptor is necessary for sustained sarcomagenesis [27]. Furthermore, the secreted HIV Tat protein enhances KS-like lesion development in mice, pointing again to paracrine mechanisms of disease development and providing a means by which HIV might contribute to KS [28]. Finally, it has been observed that vGPCR-positive endothelial progenitors are more abundant in early-stage murine sarcomas than in later-stage lesions [29], clearly inconsistent with a classical 'autocrine transformation' model and indicating that vGPCR contributes to disease development primarily indirectly via angiogenic cytokine induction.

K1/Variable ITAM-Containing Protein

The K1 gene of HHV-8 specifies a single-span membrane protein that is a constitutively active Ig-related signal transducer with an immunoreceptor tyrosine-based activation motif (ITAM) similar to the Ig-α and Ig-β chains of the B cell receptor [30, 31]. Due to extensive heterogeneity in the extracellular loops of the receptor, the K1 protein is referred to as the variable ITAM-containing protein (VIP). The K1 ORF is located at the extreme left end of the HHV-8 genome, collinear with genes encoding functionally similar (although structurally distinct) transforming proteins in other γ-herpesviruses, such as EBV latency membrane protein-1 (LMP-1) and HVS transformation-associated protein (STP) [32]. This led to early speculation about a possible role of VIP in the development of HHV-8-assoctiated malignancies, and indeed there are several lines of experimental evidence that are supportive of such a hypothesis, as discussed below. It is important to note, however, that the expression of K1 appears to be primarily during the lytic cycle rather than latency [17, 18, 33], and this needs to be borne in mind when considering its potential role as a classical viral transforming protein.

The fact that HVS STP is required for virus-mediated T cell transformation, both in culture and in in vivo systems, provided a means to test the functional equivalency in this respect of HHV-8 K1. Recombinant virus expressing HHV-8 ORF K1 in place of HVS ORF1 was found to retain its ability to transform cells; thus, VIP was able to substitute functionally for STP [34]. Furthermore, VIP was found to induce plasmablastic lymphomas and sarcomatoid tumors in K1 transgenic mice, and salivary

gland carcinomas developed in a significant proportion (25%) of mice infected with ORF K1-containing recombinant murine γ-herpesvirus-68 [35, 36]. These types of experiment indicate that VIP mediates cellular transformation via dysregulation of cell signaling to promote the growth and survival of the cells in which it is expressed. Perhaps consistent with this model is the finding that VIP is expressed in at least some cells in KS and MCD lesions [37, 38], although whether this represents latent expression, is not clear. VIP signal transduction, in endothelial cells and B cells, has been demonstrated to promote cell survival, in part via Akt-mediated inactivation of proapoptotic GSK-3, Bad and forkhead transcription factors and activation of mTOR [38, 39]. Indeed, K1-mediated immortalization of primary endothelial cells [38] indicates a potential role of VIP in KS development, but this would be dependent on latent expression of the viral receptor.

It is possible, also, that VIP contributes to viral neoplasia via the induction of proinflammatory and angiogenic factors. VIP has been demonstrated to signal via SH2 domain Src family kinases, PI3K-p85 and PLC-γ to activate various signaling cascades [30, 31, 39–41]. With respect to cytokine induction, the PI3K/Akt/NF-κB pathway is of principal importance, and VIP can induce expression of proinflammatory cytokines, such as IL-1β, IL-6, IL-12 and granulocyte-macrophage colony-stimulating factor that are responsive to such signaling [40, 41]. The angiogenic factors VEGF, matrix metalloproteinase 9 and CXCL8/IL-8 also are induced by VIP, via ITAM-SH2 signaling protein interactions but unresolved pathways [38, 40]. These data suggest that regardless of the stage of VIP expression, lytic or latent, the receptor could play a role in promoting HHV-8-induced neoplasia via paracrine mechanisms involving inflammatory and angiogenic cytokines, of particular importance in KS [2] but also of relevance in MCD and PEL [42, 43].

K15/Latency-Associated Membrane Protein

The HHV-8 latency-associated membrane protein (LAMP), a 12-transmembrane-spanning constitutive signaling receptor, is specified by an 8-exon gene at the extreme right end of the viral genome, collinear with EBV LMP-2, although with no sequence or structural similarities [44–46]. There are 2 alleles of K15, a predominant and a minor form that are significantly diverged with respect to amino acid sequence but conserved in overall structure and in functional signaling motifs in the cytoplasmic C tail [32, 46, 47]. These motifs comprise 2 SH2- and 1 SH3-binding sequences and a site interacting with TNF-receptor-associated factors [44, 45, 48]. Interactions between LAMP and TNF-receptor-associated factors 1, 2 and 3 have been demonstrated and lead to activation of mitogen-activated protein kinases ERK and JNK and transcription factors NF-κB and AP-1 [32, 45]. Interactions with members of Src-family kinases occur via Y_{481} in the SH2-binding motif YEEV, which is the predominant site of tyrosine phosphorylation of LAMP in B cells [44, 48]. This motif together with the SH3-binding sequence (PPLP) is necessary for BCR signaling suppression mediated by LAMP, a function paralleling EBV LMP-2 activity [32, 44]. The other

SH2-binding motif in LAMP, Y_{432}SIL, is not significantly phosphorylated and its function has not been determined. The suppression of BCR signal transduction could potentially serve the virus by blocking signals that promote B cell activation and virus latent-to-lytic switching, as proposed for EBV LMP-2. However, this is speculation, and it may be that such BCR inhibition serves to promote virus productive replication via blocking of apoptotic functions of BCR signaling in the infected cell.

Antiapoptotic functions of LAMP have been proposed based on the observed interactions of the receptor with antiapoptotic HS-1 associated protein X1, both in the endoplasmic reticulum (ER) and on mitochondria [49]. Furthermore, LAMP recently has been demonstrated to induce transcriptional expression of several other antiapopotic proteins, including A20, Birc2, Birc3 and Bcl-2A1 [50]. Additionally, LAMP stimulates expression of several angiogenic and inflammatory factors, such as IL-6, CXCL8/IL-8, CXCL3/GRO-γ and Cox-2, which may be relevant in KS development, and also induces dscr1, a target of VEGF signaling [50]. Thus, prosurvival and proangiogenic functions of LAMP could, as proposed above for K1, contribute to neoplasia induced by HHV-8.

As with consideration of the role of VIP in virus biology and pathogenesis, determining the roles of LAMP in these respects is complicated by uncertainty about when it is expressed. A further issue is that different splice or proteolytic isoforms of LAMP may be present and expressed differently during latent and lytic infection [48, 49]. A 23-kDa form of LAMP incorporating the signaling motif-containing C tail has been detected in latently infected PEL cells [49], but K15 transcripts, while expressed at low levels in latently infected PEL cultures, are highly induced with TPA treatment and onset of lytic replication [44–46]. The full-length K15 product has been detected in HEK293 cells harboring HHV-8 bacmid genomes, providing the only evidence to date for 45-kDa LAMP expression during latency, in transfected rather than infected cells [50]. It would appear that LAMP is, in fact, essentially a lytically expressed protein and therefore that any role of the protein in virus-associated pathogenesis would be via paracrine rather than autocrine mechanisms. However, at present the possibility cannot be excluded that there are low levels of latently expressed LAMP and/or higher levels of latent expression in particular in vivo settings that could allow for direct contributions of LAMP to virally-induced neoplasia.

Cytokines

Viral Interleukin-6

The HHV-8-specified homologue of interleukin-6 (vIL-6) has been speculated since its discovery to play roles in the development and/or progression of KS, MCD and PEL because its cellular counterpart, human IL-6 (hIL-6), had previously been associated with KS and MCD and was a known B cell growth factor [51–54]. IL-6 was found at elevated levels in KS lesions and in serum of MCD patients, with a positive correlation between IL-6 concentration and MCD disease severity. Thus, vIL-6 was predicted

when identified to be involved in promoting HHV-8-associated malignancies, and indeed subsequently derived data relating to its activities and mechanisms of action support this view.

While hIL-6 and vIL-6 share only 25% amino acid sequence identity, the 3-dimensional structures of the 2 cytokines are very similar, comprising closely superimposable 4-helix bundle structures with analogous, although not identical, interfaces for interactions with the signal transducer, gp130, and the nonsignaling α-subunit of the IL-6 receptor, gp80 [55]. The major functional difference between vIL-6 and hIL-6 is that the viral cytokine does not require gp80 for formation of stable signaling complexes with gp130 [56]. Evidence from mutational analysis of vIL-6 indicates that gp80 independence of the viral cytokine is based on the precise conformation of the molecule [57]. Notwithstanding, vIL-6 signaling complexes that include gp80 can form and experimental evidence suggests that gp80 can modulate quantitatively and qualitatively signal transduction induced by the viral cytokine [55, 58, 59]. Furthermore, vIL-6 and hIL-6 signal transduction via hexameric ($IL\text{-}6_2$:$gp130_2$:$gp80_2$) complexes can be distinguished at the level of STAT1 and STAT3 activation profiles and duration of STAT signaling [59]. Thus, the precise structures of vIL-6 versus hIL-6 signaling complexes may determine the biological activities and functions of these homologous cytokines. Another major difference between HHV-8 and cellular IL-6 proteins is that the viral cytokine is inefficiently secreted and largely retained intracellularly. Experimentally induced localization of vIL-6 within the ER, via tagging with an ER retention motif, has demonstrated that signal transduction by vIL-6 is possible within this compartment [60]. Indeed, our own data from subcellular fractionation experiments have confirmed induction of gp130 signaling initiation by (untagged) vIL-6 in the ER, the major site of intracellular vIL-6 localization as determined by membrane fractionation and confocal immunofluorescence microscopy [Chen and Nicholas, unpubl.]. Thus, unlike hIL-6, vIL-6 is able to signal intracellularly, and this could be of consequence with regard to virus biology and pathogenesis (discussed below).

Viral IL-6 is predominantly a lytic gene, being massively induced in lytically induced PEL cultures [17, 18]. However, vIL-6 protein has been detected in uninduced PEL cultures, and in PEL, MCD and KS tissues in the absence of other lytic antigen expression, indicating restricted expression also during latency, at least under some circumstances [16, 61–63]. It is noteworthy that vIL-6 is one of a few lytic genes that are induced by Notch signaling, independently of the immediate-early lytic activator RTA, raising the possibility of lytic gene expression programs that are distinct from the full replicative cycle [64]. Thus, vIL-6 has the potential to impact virus-induced neoplasia both by direct, autocrine mechanisms promoting cell survival and proliferation in latently infected cells and via paracrine mechanisms during lytic replication. In the former situation, cytokine concentration via ER sequestration coupled with intracellular signaling from this compartment may enable the appropriate functioning and biological activities of vIL-6 under conditions of restricted expression. These activities, which presumably would include positive effects on cell survival and

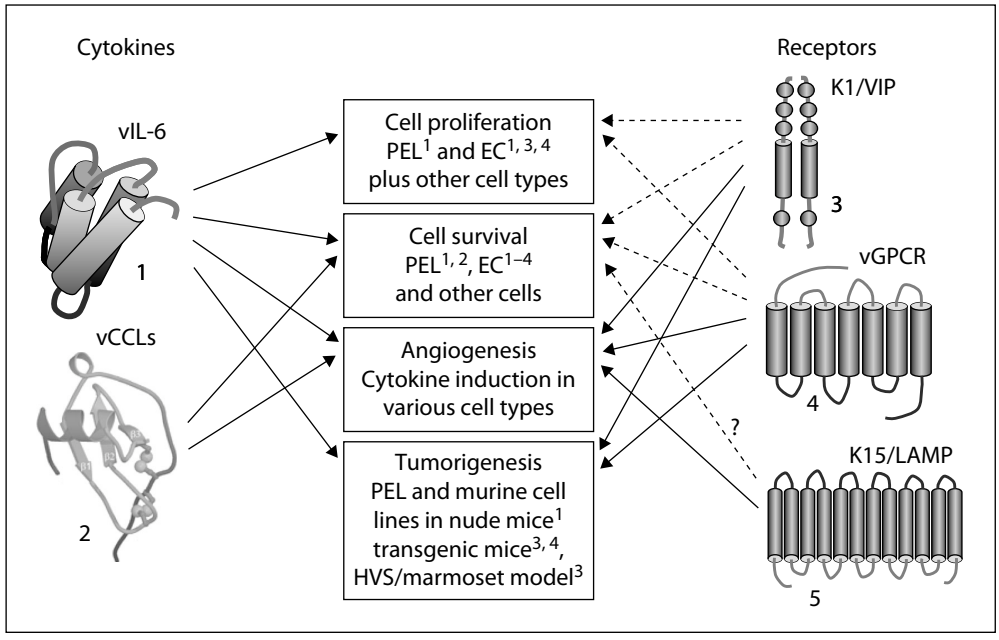

Fig. 1. Pathogenic functions of HHV-8 cytokines and receptors. The activities of potential relevance to HHV-8 malignant pathogenesis are indicated. All of the viral proteins are expressed during lytic replication and, apart from vIL-6, have not been detected in latently infected cells. Therefore, at present there is no evidence for the operation in neoplasia of the direct autocrine activities of the receptors demonstrated in experimental systems (dotted lines), although it is conceivable that they may occur under particular circumstances in vivo. However, the HHV-8 receptors could potentially contribute to pathogenesis also via induction of cellular secreted factors (e.g. VEGF, IL-6, IL-8, basic fibroblast growth factor, matrix metalloproteinases) and therefore act in a paracrine manner, like the HHV-8 cytokines.

proliferation, known to be mediated by vIL-6 in PEL cells [62, 65], may serve to promote the maintenance of viral latent load within the host but could also contribute to HHV-8-induced hyperproliferative and malignant disease. On the other hand, it is entirely possible that paracrine signal transduction by lytically expressed vIL-6 is the major means by which vIL-6 contributes to neoplasia (fig. 1). Proproliferative and prosurvival effects could be mediated directly on neighboring uninfected and latently infected cells but could also result from the influence of vIL-6-induced cytokines, such as VEGF and cellular IL-6 [66–68]. Unlike the situation for vGPCR, the range of cytokines induced by paracrine-acting vIL-6 would not be subject to restriction by exonuclease/SOX-mediated host shutoff.

vCCL Chemokines

The viral chemokines vCCL-1, vCCL-2 and vCCL-3, specified by ORFs K6, K4 and K4.1, respectively, are expressed early during the lytic cycle [17, 18, 67]. vCCLs 1 and 2 are most closely related to both CCL3/vMIP-1α and CCL4/vMP-1β with respect to

primary structure, whereas vCCL-3 has no obvious cellular counterpart but is similar to a number of CC chemokines. Functionally, however, the viral chemokines have properties that are unique and distinct from their structural homologues; vCCL-1 is an agonist for CCR8, vCCL-2 for CCR3, CCR8 and possibly CCR5, and vCCL-3 has been reported to signal via CCR4 and XCL1 [69–73]. The agonist properties of the viral chemokines suggest that they play roles in Th2 polarization of the immune response, and vCCL-2 may further contribute to blocking antiviral immunity by targeting a number of receptors as a neutral ligand to prevent normal chemokine function (reviewed in [74]). Indeed, in vivo experiments have demonstrated such Th2 polarization and evasion of immune-induced cytotoxicity and pathogenesis by vCCL-2 [75, 76]. The demonstrated recruitment of monocytic (THP-1) cells by vCCL-1 and vCCL-2 [73] suggests another role for these v-chemokines, namely the recruitment of HHV-8-permissive cells into sites of ongoing lytic replication to allow the dissemination of virus within the host.

What might the roles of the HHV-8 chemokines be in neoplasia? Like vIL-6, the viral chemokines have the potential to act in a paracrine fashion on neighboring cells and to induce cellular secreted factors independently of the effects of shutoff of host gene expression induced by SOX in lytically infected cells in which the vCCLs are produced [19]. In this regard, perhaps the most notable property of the viral chemokines is their proangiogenic activity, likely to be mediated in part via induction of VEGF [67, 69, 72]. Promotion of angiogenesis by the v-chemokines would be predicted to contribute to KS [77], and also to PEL, in which VEGF has been shown to play an important role in cell growth and dissemination in mice [43]. However, in addition to this indirect role in pathogenesis via induction of cellular cytokines, vCCL-1 and vCCL-2, at least, have the potential to contribute directly to neoplasia via CCR8-medaited prosurvival signaling. Thus, we have determined that vCCL-1 and vCCL-2 can block apoptosis induced in endothelial cells by stress (serum starvation, lytic replication) in a CCR8-dependent manner, and that v-chemokine antiapoptotic efficacy is equivalent to that mediated by VEGF, a known survival factor for endothelial cells [Choi and Nicholas, unpubl.]. Antiapoptotic activities of the v-chemokines have been reported previously by us and others in PEL and murine BW5147 murine lymphoma cells [67, 78]. Unlike other antiapoptotic effectors specified by HHV-8 [79], the v-chemokines can escape the confines of lytically infected cells to act in a paracrine manner on neighboring cells and thereby potentially contribute, as lytic proteins, to virus-induced neoplasia, likely in cooperation with latency functions.

Latent Proteins

Latency-Associated Nuclear Antigen

LANA, encoded by ORF73, is a large multifunctional protein that is consistently expressed in all HHV-8-associated malignancies and is considered a universal marker

of HHV-8 latency [80, 81]. LANA binds to the sequences in the terminal repeats of the HHV-8 genome via its C terminus and to the nucleosomes through its N terminus, thereby tethering the viral episomes to the host chromosome and ensuring their efficient partitioning during cellular division [82, 83]. Additional activities of LANA, including its ability to support replication of HHV-8 DNA in dividing cells [84, 85] and inhibit the transcriptional activity of HHV-8 lytic regulator RTA/ORF50 [86], probably contribute to its role in the establishment and maintenance of latency.

LANA is also known to interact with a number of cellular proteins and affect diverse signaling pathways, which may play a role in HHV-8 tumorigenesis (fig. 2). Thus, LANA was shown to interact with p53, and this interaction was found to block p53 transcriptional activity and p53-induced cell death [87]. The rarity of p53 mutations in PEL cell lines lends further support to the hypothesis of its functional inactivation by constitutively expressed LANA [88, 89]. However, both LANA and p53 are relatively 'sticky proteins' and have been shown to bind to many partners in vitro [90, 91], which make it difficult to establish the specificity and functional significance of their observed interaction, a notion supported by the presence of a functional p53 signaling pathway in PEL cell lines [89].

LANA was also reported to interact with the retinoblastoma (Rb) protein, resulting in the transcriptional activation of E2F-responsive genes and, in combination with H-Ras, to the transformation of rat embryo fibroblast cells [92]. Again, the functional significance of LANA-Rb interaction has been called into question by the finding that Rb function is intact in PEL cell lines [93].

An interaction of LANA that has received recent attention is with GSK-3β, a kinase involved in phosphorylation of β-catenin and its subsequent degradation by ubiquitin-mediated proteolysis. Interaction of LANA with GSK-3β induces a cell-cycle-dependent nuclear accumulation of the latter, thereby stabilizing cytosolic β-catenin, which can then bind to the transcription factor lymphoid enhancer-binding factor and activate transcription of its target genes [94]. Additionally, interaction of LANA with GSK-3β leads to inactivation of nuclear GSK-3β, with functional consequences for the activity of proteins that are GSK-3β substrates [95]. One such GSK-3β substrate is c-Myc, which is phosphorylated by GSK-3β at Thr58 and subsequently ubiquitinated and degraded, a process that is blocked in LANA-expressing cells [96, 97]. Interestingly, LANA also stimulates phosphorylation of c-Myc at Ser62 by activating ERK, which stimulates the transcriptional activity of c-Myc [97]. Finally, in addition to regulating gene expression via the p53, Rb and GSK-3β pathways, LANA is also known to affect host gene expression directly [98, 99], which could contribute to dysregulation of gene expression observed in HHV-8-associated malignancies.

Transgenic mice expressing LANA under its own promoter were recently described [100]. These animals demonstrated B-cell-specific expression of the transgene, which was accompanied by splenic follicular hyperplasia and increased germinal center formation [100]. Approximately, 11% of the LANA transgenic mice developed lymphomas, which was twice the expected rate [100]. The low frequency

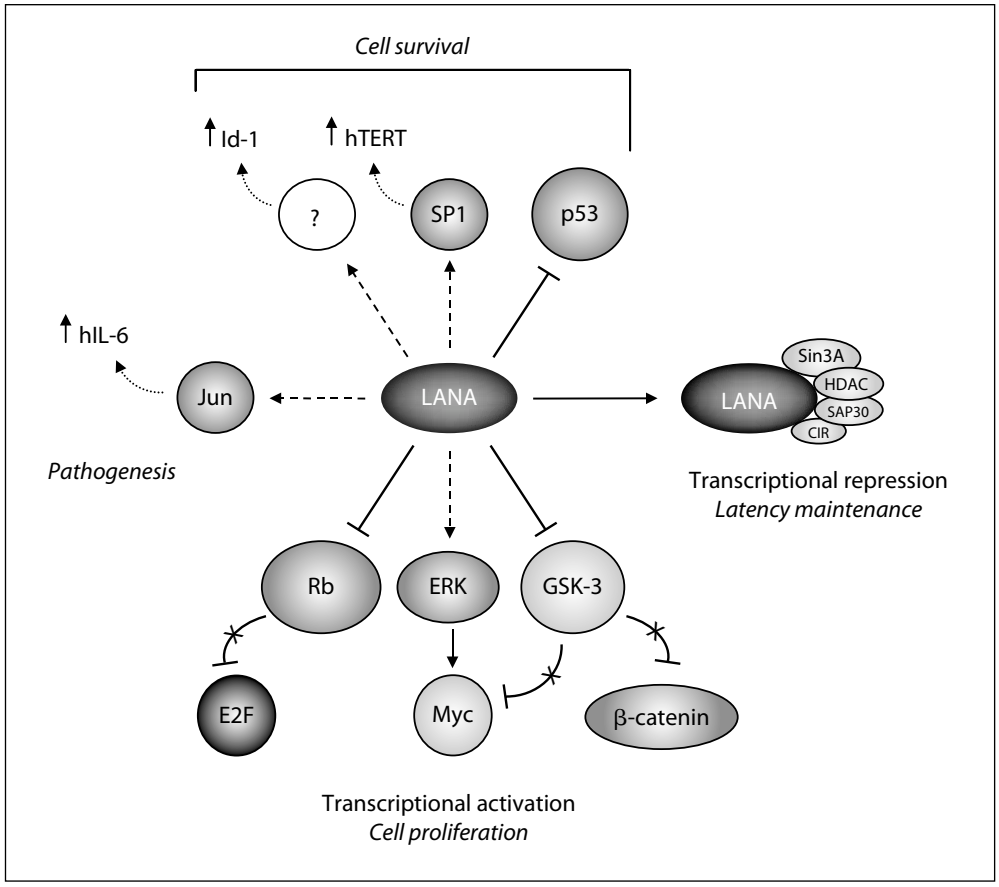

Fig. 2. Overview of LANA activities. Various activities of LANA have been identified, including transcriptional activation either by direct promoter targeting (e.g. via binding to SP1 or Jun) or by binding-mediated inhibition of Rb, p53 or GSK-3, transcriptional repression via recruitment of corepressor complexes to LANA-targeted promoters, and induction of cellular proteins such as IL-6, hTERT and Id-1 via activation of transcription factors such as Jun and SP1. Combined, these activities are likely to promote cell proliferation and survival in the context of HHV-8 latent infection and also to inhibit lytic reactivation. They probably also are important contributors to HHV-8-induced cellular transformation and malignant disease.

and long latency to lymphoma development in LANA mice suggest the contribution of additional viral- and/or host-derived factors in LANA-dependent lymphomagenesis [100].

v-Cyclin

HHV-8 v-cyclin is encoded by the ORF72 and is cotranscribed with LANA and viral FLICE inhibitory protein (vFLIP) from the major latency promoter. v-Cyclin is a structural and functional homologue of cyclin D2 and like the latter binds to and activates

Cdk6, directing its kinase activity towards Rb [101]. However, in contrast to the cellular D-type cyclins, v-cyclin is much less active towards Cdk4 [102]. Another unique feature of the v-cyclin/Cdk6 complex is its relative resistance to inhibition by Cdk inhibitors, such as p27, p21 and p16 and, accordingly, ectopic expression of v-cyclin in quiescent fibroblast cells has been shown to stimulate cell cycle progression and to overcome G_1 arrest imposed by these inhibitors [103]. In the case of p27, this resistance is further enhanced by the fact that the v-cyclin/Cdk6 complex is known to phosphorylate p27, targeting it for degradation [104, 105]. As compared to the cyclin D/Cdk6 complex, the v-cyclin/Cdk6 complex also possesses enhanced kinase activity towards its substrates [106, 107] and targets an extended array of substrates for phosphorylation [104, 105]. For example, substrates of v-cyclin/Cdk6 include proteins targeted by the cyclin E/Cdk2 complex, such as p27, histone H1, Id-2 and Cdc25a [102, 104, 105, 107], as well as those that are substrates of cyclin A/Cdk2, such as Cdc6 and Orc1 [108]. Thus, the v-cyclin/Cdk6 complex possesses the functional activities of both G_1 and S phase cyclin/Cdk complexes and therefore has the potential of dysregulating G_1/S phase progression. Cells expressing v-cyclin have also been reported to undergo continued DNA synthesis and nuclear division, without cytokinesis, thus potentially dysregulating mitotic progression, activating p53, and inducing apoptosis and growth arrest [109]. Accordingly, targeted expression of v-cyclin in the B cell compartment of transgenic mice resulted in the development of lymphomas, but only in p53-deficient animals [110]. Finally, another consequence of v-cyclin-induced mitotic dysregulation is genomic instability, which may contribute to HHV-8 tumorigenesis [111].

It must be noted, however, that a number of discrepancies emerge when the activities attributed to v-cyclin based on in vitro biochemical studies or overexpression studies in heterologous cell culture systems are checked against PEL cells with endogenous v-cyclin expression. For example, despite the presence of v-cyclin (and LANA), the Rb pathway is functional in PEL cells [93]. Similarly, although v-cyclin is supposed to promote p27 degradation, PEL cells generally express abundant amounts of this protein [112]. The difficulty of delineating the true contribution of v-cyclin to HHV-8 oncogenesis is further complicated by the lack of suitable animal models of HHV-8-induced cancers and the difficulty of generating cell lines with stable expression of this protein.

K13-Viral FLICE Inhibitory Protein

K13, the third protein encoded by the major latency locus, contains 2 tandem death effector domains which are also found in the prodomain of caspase 8/FLICE. Proteins with a similar domain structure exist in other viruses and include MC159L and MC160L from the molluscum contagiosum virus and E8 from equine herpesvirus 2 [113–115]. At the time of their discovery, these proteins were believed to protect virally infected cells from death-receptor-induced apoptosis by blocking the homophilic interactions between the death effector domains of caspase 8/FLICE and FADD and, as such, were collectively referred to as vFLIPs [113–115]. Accordingly, vFLIPs E8 and MC159L were shown to protect cells from apoptosis induced by tumor

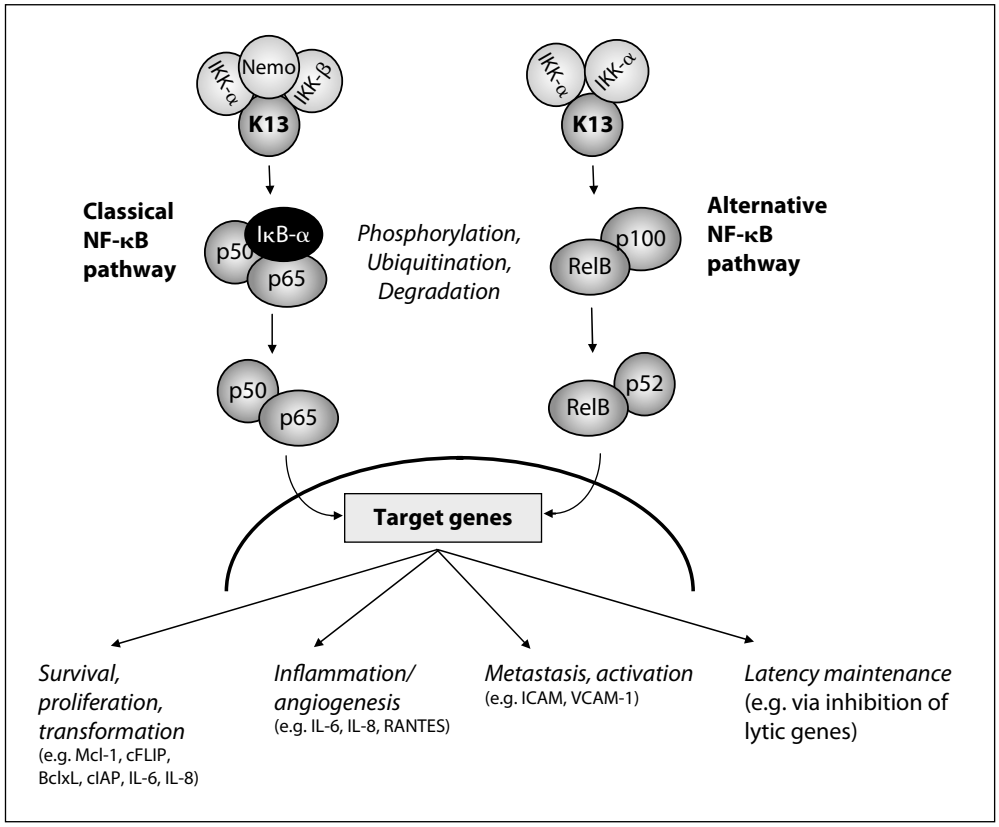

Fig. 3. Contribution of K13 to HHV-8 oncogenesis. Interaction of K13 with the IKK signalsome complex, consisting of IKK-α, IKK-β and Nemo/IKK-γ, results in the activation of the classical NF-κB pathway, whereas its interaction with IKK-α activates the alternative NF-κB pathway. In turn, the 2 NF-κB pathways transcriptionally activate a number of NF-κB responsive genes with diverse biological activities important for HHV-8 oncogenesis. K13-induced NF-κB activation may also promote HHV-8 latency by inhibiting the expression and activity of lytic genes.

necrosis factor receptor 1 and Fas/CD95 [113–115]. In initial studies, stable expression of K13 was also shown to protect A20 (a mouse lymphoma cell line) [116] from Fas-induced apoptosis and PC-12 (a rat pheochromocytoma cell line) against TNF-α-induced apoptosis [117]. However, these results have not been independently confirmed and subsequent studies have revealed that K13 is unique among the vFLIPs in possessing the ability to activate the classical and alternative NF-κB pathways [118, 119] and does not act as an inhibitor of caspase 8 [120]. K13 is believed to trigger the NF-κB pathway by directly interacting with and activating an IκB kinase (IKK) signalsome complex, consisting of IKK-α, IKK-β and IKK-γ/Nemo, thereby bypassing the upstream components of TNF-α-induced NF-κB signaling pathways, such as receptor-interacting protein and TNF-receptor-associated factor 2 [121–123] (fig. 3). This allows for selective activation of the prosurvival NF-κB pathway without

concomitant JNK activation [122]. Since the NF-κB pathway is known to upregulate the expression of a number of antiapoptotic genes, it is likely that the protective effect of K13 against death-receptor-induced apoptosis observed in the earlier studies reflected an indirect outcome of this activity rather than a direct consequence of caspase 8/FLICE inhibition. This hypothesis is supported by the lack of any reported interaction between K13 and caspase 8/FLICE [123], and by the phenotype of K13 transgenic mice, which do not show any features seen in mice deficient in caspase 8 or FADD, and are not resistant to Fas-induced apoptosis [120].

The biological effects of K13-induced NF-κB, however, are not limited to protection against apoptosis, and this activity has been implicated in the ability of this protein to promote cellular proliferation and transformation, cytokine secretion (e.g. IL-6, IL-8, RANTES, etc.) and protection against growth-factor-withdrawal-induced apoptosis [120, 124–128] (fig. 3). Thus, ectopic expression of K13 in Rat-1 fibroblast cells leads to classical signs of transformation, such as focus formation, growth in soft agar and tumor formation in nude mice, which are accompanied by NF-κB activation and can be reversed by genetic and pharmacological inhibitors of this pathway [124]. Similarly, constitutive NF-κB activation in K13 transgenic mice is associated with enhanced proliferation of lymphocytes to mitogenic stimuli and an increased incidence of lymphoma [120].

The biological relevance of K13-induced NF-κB activity has been confirmed by study of PEL-derived cell lines. Both the classical and alternative NF-κB pathways are constitutively active in PEL cell lines [119, 121, 129], and gene silencing studies have also confirmed that K13 is primarily responsible for these activities [119, 126, 130, 131]. Remarkably, silencing of K13 expression in PEL cells not only blocked their constitutive NF-κB activity but also resulted in induction of apoptosis, suggesting that K13-induced NF-κB activity is essential for the survival of PEL cells [130, 131]. However, a recent study reported that K13 also blocks HHV-8 lytic replication and the expression of lytic genes and, conversely, its silencing induces lytic gene expression. This leaves open the possibility that induction of lytic replication might also have contributed to the death of PEL cells observed following K13 silencing, a notion supported by the relatively delayed occurrence of cell death upon K13 silencing found in the prior studies [130, 131]. Interestingly, the suppressive effect of K13 on the expression of lytic genes is not uniform and it fails to block lytic replication-induced vIL-6 production, which may provide a possible explanation for the dysregulated expression of vIL-6 observed in cells latently infected with HHV-8 [132].

Ectopic expression of K13 in vascular endothelial cells, in the absence of other latent genes, is sufficient to transform them into spindle cells resembling the distinctive spindle cells observed in KS lesions [127, 128]. This phenotype of K13-expressing cells is due to NF-κB activation and is accompanied by exuberant production of proinflammatory cytokines and upregulated expression of cell adhesion molecules [127, 128]. Similarly, ectopic expression of K13 in lymphatic endothelial cells was shown to upregulate ICAM-1 and MHC-1 expression [133]. Taken collectively, these

results suggest that K13 may also contribute to the pathogenesis of KS lesions, a notion also supported by the fact that there is a dramatic upregulation of K13 expression in late-stage KS lesions [134].

Kaposins

A family of 3 proteins specified by the K12 locus, the kaposins are the products of alternative reading frames, 1 (that encoding kaposin A) initiated by a conventional AUG codon and those for kaposins B and C by CUG initiators upstream of ORF K12 [135]. Kaposin B comprises sequences derived from 23-nucleotide direct repeat elements DR1 and DR2 and is translated from the most 5′ CUG codon (in frame 2) in the major kaposin transcript. Kaposin C is initiated from a downstream CUG codon and comprises DR1 and DR2 translations (frame 1) fused to K12. While K12-locus-containing transcripts are found in high abundance in latently infected KS and PEL cells [17, 18, 136–138], as they are during lytic cycle also, these are not K12-specific products but rather include the DR1 and DR2 sequences [135]. A larger, spliced transcript initiates 5 kbp upstream of K12, 3′ of ORF73 (encoding LANA) [139]. Potential non-AUG translation start codons are present in the first exon and exon 2 (5′ of DR2) and these potentially could be utilized to derive additional translation products with unique N termini. The spliced transcript was initially identified in primary PEL tissue but since identified also in established PEL cell lines. The relative expression of kaposins A, B and C in different cells varies; for example, while only kaposins A and C were detectable in primary PEL [139], kaposin B was the predominant protein in BCBL-1 cultured PEL cells [135].

The expression of K12 locus mRNAs and proteins during latency, as determined in some cell lines and primary tissues, makes it appropriate to consider kaposins as potential direct contributors to HHV-8 pathogenesis. In this regard, it is particularly noteworthy that kaposin A (6-kDa ORF-K12 translation product) functions as an oncogene in Rat-3 transformation assays [140]. The mechanism of action appears to be by membrane recruitment of cytohesin-1, an ADP-ribosylation factor guanine nucleotide exchange factor, transforming activity of kaposin A, being dependent on cytohesin-1 [141]. Kaposin A is predicted to be a type II membrane protein, with 2 hydrophobic domains, and confocal microscopy and subcellular fractionation studies indicate that it localizes to perinuclear and plasma membrane regions [141, 142]. Kaposin A expression in transfected cells leads to activation of serine/threonine kinases, such as PKC, ERK, CAM kinase II, Cdc2 and cGMP-dependent protein kinase [141–143], but the underlying mechanisms have not been elucidated. Kaposin B, on the other hand, has been shown to target through its DR2-specified sequences MK2 kinase via its so-called C lobe, a region targeted for activating phosphorylation by p38 kinase and which is bound intramolecularly by the C terminus of MK2 to bring about autoinactivation [144]. Thus, binding of kaposin B to MK2 mediates kinase activation in part via alleviation of negative regulation. MK2 activity stabilizes mRNAs containing AU-rich elements; these messages include those for cytokines,

such as IL-6, that are likely to promote HHV-8-associated malignancies. As p38-phosphorylating and -activating kinase MKK6 is specified by an AU-rich-element-containing mRNA, the activity of MK2 would be predicted to be enhanced by kaposin B via increased phosphorylation of MK2 by p38 in addition to direct prevention of autoinactivation via C lobe binding. While a specific serine residue in the DR1-encoded C-terminal region of kaposin B is targeted by p38 [145], the relevance of p38 activation to kaposin B activity and virus biology and pathogenesis remains to be determined. Notwithstanding, kaposin B clearly has the potential to influence virus pathogenesis via control of inflammatory and angiogenic cytokines in addition to possible direct autocrine effects on latently infected cells.

Viral Interferon Regulatory Factor 3

There are 4 viral interferon regulatory factor (IRF) homologues specified by the ORF K9 to ORF K11 region of the HHV-8 genome [146]. One of these, vIRF3 (also referred to as LANA2), has been detected in latently infected PEL cell lines and therefore could play a direct role in HHV-8 neoplasia [17, 18, 146, 147]. However, while vIRF3 expression has been detected in MCD lymphocytes, it is apparently absent in latently infected KS cells. This contrasts vIRF3 with the other latent proteins listed above and means that its role during latency and any contribution to virus-associated malignancies are probably restricted to B lymphocytes.

In common with the other vIRFs, vIRF3 can block the activities of cellular IRFs and thereby suppress interferon responses to viral infection [148]. However, it has also been demonstrated to block apoptotic signaling downstream of the IFN-activated protein kinase, to interact directly with 14-3-3 proteins and proapoptotic transcription factor FOXO3a and to inhibit p53 activity, thereby protecting cells against apoptotic signals [147, 149, 150]. The consequence of these activities coupled with latent expression could conceivably contribute to viral malignant pathogenesis (PEL, MCD) in addition to viral persistence.

Discussion and Perspectives

Research on HHV-8 has had a significant impact on our understanding of γ-herpesvirus genetic diversity and on the range of mechanisms that can potentially contribute to viral oncogenesis. As highlighted in this review, viral latency and lytic functions are believed to act in concert to allow the onset and progression of neoplasia. This perhaps follows naturally from the discovery that KS is essentially a cytokine-driven angioproliferative disease and the identification of angiogenic and proinflammatory cytokines and cellular cytokine-inducing viral lytic genes encoded by HHV-8. Similar lytic functions can contribute to PEL and MCD also, for example via angiogenic, proproliferative and prosurvival activities of the viral cytokines vIL-6 and vCCLs. However, also evident is that in neoplasia these lytic gene products must act in a

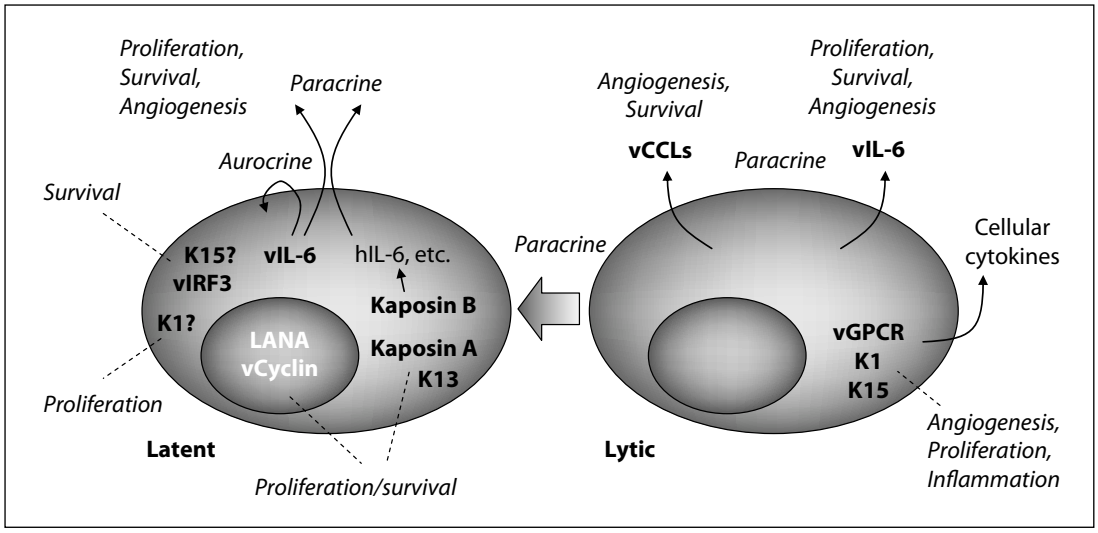

Fig. 4. Contributions of HHV-8 functions to malignant pathogenesis: envisioned functions of viral latent and lytic proteins in virus-induced neoplasia. Latent proteins LANA, v-cyclin, K13, vIRF3, and kaposins A and B can be expressed in normal latently infected cells, and also in transformed premalignant and malignant cells derived from them. As such, they have the potential to contribute directly to HHV-8 pathogenesis. K1/VIP and K15/LAMP have been speculated to play such a role, but to date conclusive evidence is lacking that the proteins are expressed during latency. However, these receptors, like the viral cytokines, can contribute to disease via paracrine signaling, affecting both latently infected and uninfected cells in the locality. Angiogenesis, cell growth and cell survival all are predicted to be impacted by the viral cytokines and the HHV-8 receptor-induced cellular cytokines alike.

paracrine manner, since the cells in which they are expressed will not survive the viral productive replication cycle. Direct, autocrine contributions to viral oncogenesis are mediated by the latent proteins, and in this respect LANA and K13 appear to be pivotal to promoting cell proliferation and cell survival, via direct targeting of proteins such as GSK-3β, Rb and p53 (LANA) or indirectly via prosurvival effects mediated by NF-κB activation (K13). These functions satisfy the natural biological requirements of the virus by maintaining the viability and proliferative capacity of latently infected cells, but clearly these same activities can lead to cell transformation by allowing the cell to escape the normal checks on cell division and loss of genetic integrity. Prosurvival and proliferative functions of the viral cytokines (vIL-6, vCCLs) and cellular secreted proteins induced by other lytic proteins (vGPCR, K1, K15) would be expected to combine synergistically for both viral maintenance and pathogenesis with the actions of latent proteins in cell populations in which there is some ongoing lytic replication (fig. 4).

What is the evidence for the contribution of the various experimentally determined activities of HHV-8 proteins to HHV-8 malignancies? So far, in the absence

of data from suitable animal model systems (e.g. macaques infected with appropriately mutated rhesus rhadinoviruses) the evidence is far from conclusive and one must rely on informed speculation. Perhaps the most dramatic and convincing demonstration of the contribution of a viral lytic protein to HHV-8-associated disease comes from the generation of KS-like lesions in vGPCR transgenic mice [9, 11, 12]. These studies were key in providing evidence for the central contribution of induced cellular cytokines to KS. However, there is an important caveat: most cellular gene expression is shut off in the context of virus productive replication, due to the actions of SOX, and so the repertoire of vGPCR-induced cellular cytokines that can potentially contribute to KS is presumably highly restricted in the natural setting of HHV-8 infection [19]. Another notable experimental observation is that the K1 gene of HHV-8 can substitute functionally for the genetically collinear STP gene of HVS in in vivo transformation assays utilizing infected marmosets [34]. Two caveats apply here: the first is that K1 expression has not been shown conclusively to occur during latency; the second is that experimentally infected marmosets, which are not the natural host, develop rapidly progressive polyclonal T cell tumors that do not represent the types of lymphoma (PEL, MCD) associated with HHV-8. HVS itself is nontransforming in the squirrel monkey, the natural host. All other evidence for the involvement of HHV-8 proteins in neoplasia comes from speculative projections from activities identified in vitro or from inoculations of viral gene-transduced cells into immunocompromised mice. Thus, vGPCR, vIL-6, K1, K13 and kaposin A each have the capacity to transform primary or immortalized cells to support growth in culture or lesion development in mice. These types of experiment are informative with respect to identifying potential transforming proteins in the context of virus infection, but it must be recognized that such systems, in which isolated viral genes are expressed constitutively and often at unnaturally high levels, do not in themselves indicate that these activities operate in and are relevant to virus-induced neoplasia. Clearly, this is especially true of lytically expressed proteins which in experimental assays are dependent on autocrine mechanisms of transformation; such a situation is unlikely to occur naturally because the cells in which they are expressed are presumably undergoing full productive replication and will be lysed.

Based on these considerations and the detailed accounts in this review of the properties and activities of the various putative contributors to HHV-8-induced cancers, the reader will appreciate that there is still some way to go towards elucidating the actual mechanisms of viral malignant pathogenesis. However, it is impressive how much progress has been made in identifying novel properties of various HHV-8 proteins since the discovery of the virus only 13 years ago. Importantly, studies of HHV-8, as outlined in this review, have led to an appreciation of the real potential for cooperation between latent and lytic functions, acting via autocrine and paracrine mechanisms, in the establishment and progression of HHV-8-associated cancers.

Acknowledgments

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Human T Cell Leukemia Virus Type 1 and 2: Mechanisms of Pathogenesis

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Abstract

Human T cell leukemia virus type 1 (HTLV-1) and type 2 (HTLV-2) are complex retroviruses that have been studied intensely for nearly 30 years because of their association with neoplasia, neuropathology and/or their primary T lymphocyte transforming capacity. HTLV-1 and HTLV-2 are highly homologous at the nucleotide sequence level, but the clinical manifestations differ significantly. HTLV-1 is the causative agent of adult T cell leukemia, an aggressive CD4+ T cell malignancy, and immune-mediated disorders including HTLV-associated myelopathy/tropical spastic paraparesis. In contrast, HTLV-2 is much less pathogenic with only a few cases of reported disease association. Both HTLV-1 and HTLV-2 efficiently transform T lymphocytes in cell culture and persist in infected individuals or experimental animals. The study of HTLV, particularly the properties of the viral Tax oncoprotein, has allowed investigators to dissect many cellular processes, several of which are likely key contributors to the pathobiology of the virus. Furthermore, studies utilizing genetically manipulated infectious molecular clones of HTLV-1 and HTLV-2 in cell culture and a rabbit model of infection have revealed a critical supporting role of other viral gene products in virus biology and provided fundamental insights into the mechanisms of pathogenic outcomes associated with the infection of HTLV-1 or HTLV-2.

The human T cell leukemia virus (HTLV) types 1–4 are classified as complex retroviruses and members of the genus *Deltaretrovirus* [1]. HTLV-1 and HTLV-2 are the most prevalent worldwide with approximately 10–20 million people infected, whereas HTLV-3 and HTLV-4 were discovered recently in a very limited number of individuals in Africa. HTLV is a highly cell-associated virus and infection is spread horizontally via sexual transmission and exposure to contaminated blood products, or vertically via breast milk. Once in the host following the initial burst of replication, HTLV primarily increases its copy number by proliferation of the infected cells. Infection with HTLV-1 has clearly been linked to the development of adult T cell

leukemia (ATL)/lymphoma, an aggressive CD4+ T lymphocyte malignancy, and various lymphocyte-mediated inflammatory diseases including HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), uveitis, infectious dermatitis and arthropathy [2–4]. HTLV-1 infection is endemic in Japan, Africa, South America and the Caribbean basin with associated diseases occurring in approximately 2–5% of the persistently infected individuals. HTLV-2 has been linked to only a few cases of a rare variant of hairy cell leukemia and sporadic myelopathy resembling HAM/TSP [5]. Geographically, HTLV-2 infections are less defined; although high concentrations of infected people can be found in Central and West Africa, native Amerindian populations in the Americas, and in small populations of intravenous drug users in the USA and Europe. This chapter will discuss important aspects of HTLV-1 biology and, where appropriate, will highlight comparative studies between HTLV-1 and HTLV-2 disease association, molecular pathogenesis and cellular transformation. We will primarily emphasize studies utilizing genetically manipulated infectious molecular clones of HTLV in cell culture and a rabbit model of infection that have provided fundamental insights into HTLV mechanisms of replication and leukemogenesis.

Human T Cell Leukemia Virus Genome and Replication

HTLV-1 and HTLV-2, as well as the related simian and bovine T cell leukemia viruses, are complex retroviruses that have similar genome structures (fig. 1). These leukemia viruses differ from animal transforming retroviruses such as Rous sarcoma virus or Abelson murine leukemia virus in that they do not encode viral homologues of cellular proto-oncogenes. HTLV-1 and HTLV-2 share approximately 70% nucleotide sequence homology and contain the essential genes *gag*, *pol* and *env* typical of all replication-competent retroviruses. In addition, HTLV uses alternative splicing and internal initiation codons to produce several regulatory and accessory proteins encoded by open reading frames (ORFs) in the pX region, which is located in the 3′ portion of the viral genome (fig. 1). ORF IV and III encode the positive Tax and Rex regulatory proteins, respectively, and are essential for efficient viral replication and, ultimately, cellular transformation. Tax increases the rate of transcription from the viral promoter in the long terminal repeat (LTR) and modulates the transcription or activity of numerous cellular genes involved in cell growth/survival and differentiation, cell cycle control and DNA repair [6]. Rex is a nucleolar localizing and shuttling phosphoprotein that acts posttranscriptionally by preferentially binding, stabilizing and selectively exporting the unspliced and incompletely spliced viral mRNAs from the nucleus to the cytoplasm, thus primarily controlling the expression of the structural and enzymatic proteins [7]. HTLV-1 ORFs I and II encode the accessory proteins p12 and p30/p13, respectively [8]. HTLV-1 also encodes an mRNA and protein from the minus strand of the genome,

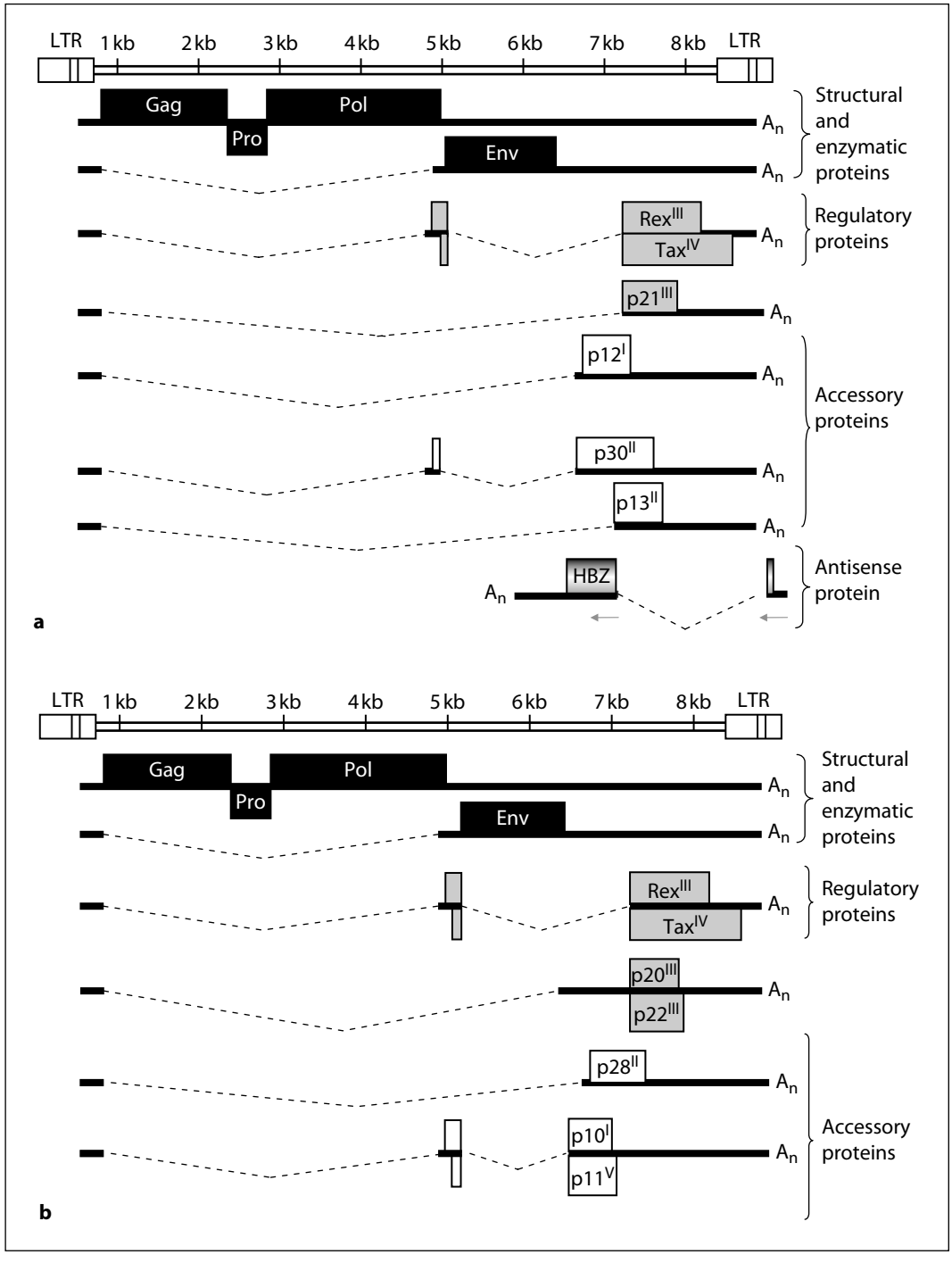

Fig. 1. Genome organization of HTLV-1 and HTLV-2. HTLV-1 (**a**) and HTLV-2 (**b**) proviral genome in kilobases containing the long terminal repeats (LTR), mRNAs and ORFs are shown. ORFs are indicated by boxes: structural and enzymatic proteins (black), regulatory protein ORFs (grey), accessory protein ORFs (white), HTLV-1 anitisense ORF (shaded). A_n = PolyA.

termed HTLV-1 basic leucine zipper factor (HBZ) [9]. HTLV-2 ORFs I, II and V encode the p10, p28 and p11 accessory gene products, respectively [10]. The functional roles of the accessory proteins in HTLV biology are not clearly understood but are beginning to emerge. Although the accessory proteins are dispensable for infection and transformation of primary human T lymphocytes in culture, they are clearly important for the ability of the virus to infect, spread and persist in inoculated experimental animals.

Human T Cell Leukemia Virus Experimental Systems

Cell Culture

Since the discovery of HTLV almost 30 years ago, experimental assay systems for the study of the virus have been complicated by its poor replication in culture, lack of consistent animal models, and the low frequency of infection and protracted time course of the disease as compared to the avian and murine retroviruses, as well as HIV-1. In cell culture, HTLV has the capacity to infect a number of cell types including B cells, T cells, endothelial cells, glial cells and monocytes of both human and nonhuman origin. However, only primary T lymphocytes are susceptible to immortalization/transformation, which historically represented an accepted experimental system for exploring the early events associated with malignancy. HTLV is a highly cell-associated virus: cell-free infection is very inefficient; efficient infection of cells requires co cultivation of target cells, primary peripheral blood mononuclear cells (PBMCs), with irradiated HTLV producer cells. Immortalization is defined as continuous growth of T lymphocytes in the presence of exogenous IL-2 and typically evident in culture microscopically as refractile cell clusters within 7–10 weeks of cocultivation. Transformation is defined as continuous growth in the absence of exogenous IL-2; the establishment of hearty IL-2-independent transformed T cell lines typically requires months in culture.

Initial HTLV studies were restricted to examination of infected patient material, overexpression of individual viral genes using reporter assays in cell lines, or characterization of infected cell lines with viral isolates obtained directly from patients. Although these types of study have been very informative, the understanding of HTLV biology and pathogenesis has benefited further from the isolation and manipulation of proviral clones capable of generating infectious virus, and the development and refinement of methodologies for characterization of these clones in primary human T lymphocytes and relevant animal models.

HTLV Animal Models

The use of a variety of animal models of HTLV infection and transformation has provided important insight into the viral and host determinants of the malignant process. As with most all animal models of infection and disease, the HTLV animal

models each have unique advantages and disadvantages [11]. HTLV consistently infects only rabbits, some nonhuman primates and to a lesser extent rats. HTLV does not efficiently infect murine cells, thus limiting the mouse as an infection model. However, tumor transplant models in genetically engineered severe combined immunodeficiency (SCID) mice have yielded important information on the proliferative and tumorigenic potential of ATL cells as well as allowed assessment of potential therapeutic drugs on tumor outgrowth. In addition, transgenic mouse models have revealed the role of the viral Tax and Tax-mediated dysregulation of cellular processes leading to lymphocyte transformation and leukemogenesis. The squirrel monkey has been infected successfully with HTLV-1 and offers an attractive nonhuman primate model of HTLV-1 for vaccine testing. Rats are a useful model for the neurologic disease associated with the viral infection and have been employed to test the role of cell-mediated immunity to the infection.

Among the HTLV infection models, the rabbit has been used the most extensively because of the ease and consistency of HTLV transmission. However, in the majority of studies, the rabbit infection has only paralleled the asymptomatic infection in humans. Early studies utilizing the rabbit model of HTLV infection provided important information regarding transmission of the virus, bodily fluids likely to contain the virus (blood, semen, breast milk) and effective methods to prevent transmission [11]. The rabbit model also has been used for the evaluation of immune responses against infection and in attempts to generate a vaccine. Early studies applied the model to define the sequential development of antibodies to HTLV-1 in infected rabbits and to detect proviral DNA in infected tissue. More recently, the rabbit model has been used successfully to evaluate infectious molecular clones of HTLV-1 and HTLV-2 [12, 13]. Essentially, molecular cloned proviral DNA is transfected into human PBMCs or established cell lines to generate virus producer cells. Lethally irradiated producer cell lines are inoculated into rabbits, where viral replication, immune response and persistence are monitored over time. The conclusions and implications of these experiments will be discussed in specific sections below.

Human T Cell Leukemia Virus Cellular Tropism

HTLV-1 and HTLV-2 exhibit differences in in vivo T cell tropism and this has been hypothesized to be important for their distinct leukemogenic capacity [5, 14]. Investigation of HTLV cell tropism in asymptomatic patients and those with neurological disease indicated that HTLV-1 has a preferential tropism for CD4+ T cells with CD8+ T cells being an additional viral reservoir in HAM/TSP patients. In contrast, HTLV-2 in vivo tropism is less clear but seems to favor CD8+ T lymphocytes. One study revealed that proviral sequences were detected predominantly in CD8+ T lymphocytes from HTLV-2-infected individuals, whereas others detected HTLV-2 in both CD4+ and CD8+ T cell subsets, with a greater proviral burden in CD8+

T cells. The distinct in vivo T cell tropism of HTLV-1 and HTLV-2 has been recapitulated in vitro using immortalization/transformation assays where irradiated HTLV producer cells were cocultured with freshly isolated human PBMCs. The results from these studies showed that the majority of cells transformed by HTLV-1 in vitro were CD4+ T lymphocytes [5], whereas HTLV-2 preferentially transformed CD8+ T cells [15]. Studies using HTLV recombinant infectious clones indicated that Tax and overlapping Rex did not confer the distinct HTLV-1 and HTLV-2 transformation tropism in vitro [16]. This suggested that other viral genes or sequences were responsible for the differential ability to transform CD4+ or CD8+ T cells. Follow-up recombinant studies revealed that the *env* gene was the major viral determinant of the distinct in vitro cellular transformation tropism of HTLV-1 and HTLV-2 [17]. This differential tropism was hypothesized to be a postentry phenomenon, since at the time HTLV-1 and HTLV-2 were considered to utilize the same cellular receptor.

Using various assay systems, several cell surface molecules have been shown to be important for HTLV entry into cells including the glucose transporter 1, heparan sulfate proteoglycans and neuropilin 1, suggesting that the HTLV receptor may be multicomponent [14, 18, 19]. Recently, careful examination of the cell surface of activated primary T cells revealed that CD4+ T cells expressed significantly higher levels of heparan sulfate proteoglycans than CD8+ T cells, whereas CD8+ T cells expressed glucose transporter 1 at dramatically higher levels than CD4+ T cells. Jones et al. [18] showed that HTLV-2 Env binding and viral entry were significantly higher on CD8+ T cells, while HTLV-1 Env binding and viral entry were higher for CD4+ T cells. Moreover, the authors reported that overexpression of glucose transporter 1 in CD4+ T cells enhanced HTLV-2 entry, while expression of heparan sulfate proteoglycans on CD8+ T cells increased HTLV-1 entry. These studies demonstrated that HTLV-1 and HTLV-2 differ in their T cell entry requirements and together with the viral recombinant studies suggest that the distinct differences in the in vitro cellular transformation tropism and in vivo pathobiology of these viruses result from different interactions between their related Env proteins and molecules on CD4+ and CD8+ T cells involved in entry.

Cellular Transformation and Pathogenesis

HTLV-1 and HTLV-2 display distinct clinical manifestations, but a hallmark feature of both of these viruses is their ability to infect and transform primary human T lymphocytes in cell culture. Although the molecular basis for cellular transformation is not completely understood, data generated from multiple experimental systems clearly identified the viral transactivator Tax as the critical determinant. Initial experiments revealed that Tax alone can morphologically transform rodent fibroblasts, induce tumors in transgenic mice and immortalize primary human T cells [6]. Studies using infectious molecular clones showed directly that Tax is essential for HTLV-1- and HTLV-2-mediated cellular transformation of primary human T cells

[15, 20]. A key advantage of a molecular clone approach, compared to overexpression studies, is that the transforming capacity is evaluated in the context of all viral genes using natural target cells, in this case, primary human T lymphocytes. The precise mechanism by which Tax initiates the malignant process is unclear but is proposed to involve several points of cellular dysregulation culminating in the accumulation of genetic mutations and uncontrolled lymphocyte growth. Although there are many similarities between HTLV-1 and HTLV-2 Tax (Tax-1 and Tax-2, respectively), a number of distinct phenotypic differences have been documented in certain cell culture model systems [5] (fig. 2). These differences have been hypothesized to hold the key as to why HTLV-1 and not HTLV-2 is associated with disease. Although Tax clearly is a critical component of the transforming capacity of the virus, data are emerging that other viral genes have important roles in the biology of the virus and ultimately its oncogenic potential. We discuss in more detail below specific Tax activities implicated in the transformation process and the supporting contribution of other viral gene products. Our discussion will emphasize studies utilizing infectious molecular clones both in in vitro culture systems and in vivo.

Human T Cell Leukemia Virus Regulatory Genes and Pathogenesis

HTLV encodes 2 positive regulatory proteins, Tax and Rex, from the same completely spliced mRNA in separate but overlapping reading frames. At the molecular level, the basic role of Rex is to regulate cytoplasmic levels of viral genomic unspliced mRNA (*gag/pol*) and singly spliced *(env)* mRNA, thus controlling the expression of the structural and enzymatic gene products that are essential for production of viral progeny. Therefore, it is proposed that Rex is critical for the transition from the early, latent phase to the late, productive phase of HTLV infection. Ye et al. [21] utilized an infectious molecular clone to investigate the contribution of Rex in HTLV-1 immortalization of primary T cells in vitro and viral survival in an infectious rabbit animal model. It was reported that the ability of Rex to modulate viral gene expression and virion production is not required for in vitro immortalization of primary human T lymphocytes by HTLV-1. However, this Rex-deficient virus was significantly hampered in its ability to spread and persist in inoculated rabbits.

Since the *tax* and *rex* genes are in partially overlapping reading frames, mutation in one gene frequently disrupts the other, confounding the interpretation of mutational analyses in the context of the virus. Younis et al. [22] generated and characterized a unique proviral clone HTLV-1 IRES Tax in which the *tax* and *rex* genes were separated by expressing Tax from an internal ribosome entry site. HTLV-1 IRES Tax was competent to infect and immortalize primary human T cells similarly to wild-type HTLV-1. In contrast, HTLV-1 IRES Tax failed to efficiently replicate and persist in inoculated rabbits. This study emphasizes the importance of temporal and quantitative regulation of specific viral mRNA for virus survival in vivo.

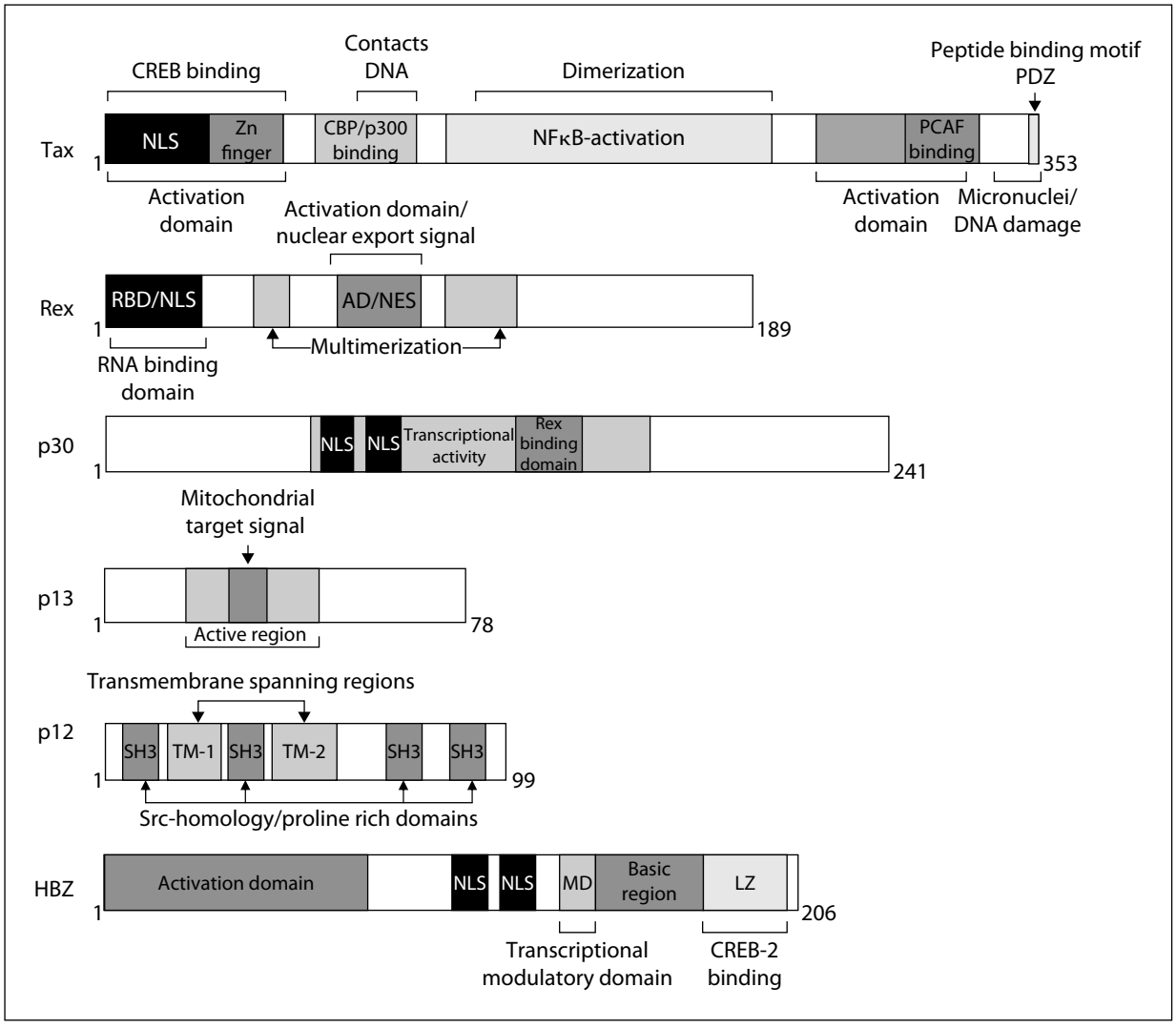

Fig. 2. Structural and functional domains of HTLV-1 regulatory and accessory proteins. Highlighted within each protein are the identified domains required for protein function, host cofactor interactions and cellular localization. CREB = Cyclic-adenosine-monophosphate-responsive element binding protein; PDZ = postsynaptic density, Drosophila discs large, Zonula occludens-1; NLS = nuclear localization signal; RBD = receptor binding domain; AD/NES = activation domain/nuclear export domain; MD = modulatory domain; LZ = leucine zipper.

Tax and Viral Transcription

Tax is one of the first proteins expressed early after viral infection and is a transactivator of viral gene expression. Tax transcriptionally activates the HTLV promoter through three 21-bp repeat sequences termed the Tax response element (TRE). The TRE

contains DNA sequences identical to part of the cyclic-adenosine-monophosphate-responsive element (CRE). The CRE, which is contained in many cellular gene promoters, is responsive to cyclic adenosine monophosphate and binds members of the CRE-binding protein/activating transcription factor (CREB/ATF-1) family of transcription factors in a Tax-dependent manner. In vitro, Tax contacts GC-rich DNA that flanks the TRE-1 sequence and recruits the cellular coactivator (CREB) to the transcription complex. The Tax/CREB heterodimer interacts with the CRE-like sequence of the viral promoter to activate viral transcription. Tax directly interacts with CREB-binding protein (CBP) and p300 to form a Tax/CREB/p300/CBP complex. Recruitment of another host cell factor, PCAF, which directly interacts with Tax, is essential for transcription initiation. Tax also modulates the activity of other cellular transcription factors including serum response factor and AP-1, which activate a plethora of early response genes that regulate proliferation. Tax-1 and Tax-2 mutants have been identified that fail to activate the CREB/ATF pathway and are defective for transactivation of the viral promoter [23, 24]. Overexpression of these and other Tax mutants in various assay systems has been invaluable for dissecting cell signaling pathways and for determining the interplay between Tax and cellular transformation. However, Tax functional analysis in the context of an infectious virus presented a unique challenge, since a knockout of Tax, or more specifically, the inability of Tax to activate the CREB/ATF pathway, disrupts overall viral gene expression and replication, thus resulting in essentially a dead virus. Ross et al. [23] circumvented this problem by generating a unique HTLV-2 provirus, which replicates by a Tax-independent mechanism due to replacement of the TRE with the cytomegalovirus immediate-early promoter enhancer. The advantage of this novel approach is that viral gene expression and replication are not disrupted significantly by mutations in Tax. Initial Tax knockout studies revealed that Tax was required for T lymphocyte transformation, providing the first direct evidence in the context of a virus that Tax was the critical viral transforming protein [23]. Subsequent studies revealed that CREB/ATF activation by Tax was required to promote sustained cell growth and IL-2-independent cellular transformation [25].

Tax and NF-κB

In addition to transactivating the viral promoter, Tax modulates the transcription or activity of numerous cellular genes involved in cell growth and survival, cell cycle control and DNA damage/repair [6]. One of the major cell growth and survival pathways that Tax targets involves the activation of NF-κB. Tax directly interacts with inhibitory κB kinase (IKK)-γ, which ultimately induces the phosphorylation and degradation of IκB-α, resulting in the nuclear expression of NF-κB, which leads to the expression of many gene promoters including IL-2, IL-2 receptor-α, IL-3 and granulocyte-macrophage colony-stimulating factor. Mutational analysis of Tax-1 and Tax-2 has revealed specific mutants and domains important for the activation of NF-κB signaling [23, 26]. Coculture studies utilizing HTLV-1 and HTLV-2 infectious molecular

clones indicated that immortalization of T lymphocytes in cell culture is dependent on Tax activation of NF-κB [20, 25]. The critical role for NF-κB activation by Tax in the HTLV-1 malignant process also is supported by in vivo observations. In addition, NF-κB and NF-κB target genes are found to be activated in ATL, ATL-transplanted NOD-SCID INF-γ knockout mice and tumors arising in Tax transgenic mice [11]. Approaches to block NF-κB using drugs or peptide inhibitors have resulted in tumor cell regression in various animal models [11]. However, there remains a disconnect between Tax activation of NF-κB and ATL, since many leukemic cells no longer express Tax but show constitutive NF-κB activation. Thus, it is clear that Tax activation of NF-κB provides a critical proliferative or survival signal early in the cellular transformation process but not the maintenance of the leukemic state.

Tax and Cell Cycle Control

Perturbation of the cell cycle is a common feature in the transformation of cells by viral oncoproteins. Tax has been shown to modify the cell cycle by directly binding cyclin-dependent kinases 4 and 6 and repressing their inhibitors such as the INK4A-D and KIP1 [6, 27]. Thus, Tax effects G_1/S transition overriding cell cycle control regulated primarily by retinoblastoma and E2F1, thereby releasing cells from growth arrest. Recently, a PDZ-binding motif (PBM) has been identified in the C-terminal fragment of Tax-1; this motif attracted additional interest because of its absence in Tax-2. The PDZ domain was named after the first identified PDZ-containing proteins, postsynaptic density protein 95, *Drosophila* disc large protein and epithelial tight junction protein (zonula occludens 1). It is one of the protein-protein interaction modules commonly used in eukaryotic cells to recruit and organize proteins to sites of cellular signaling. Tax-1, via its PBM, has been shown to interact with the human homolog of *Drosophila melanogaster* disc large tumor suppressor protein, hDLG1 [28]. Tax-1 competes with the binding domain of hDLG and anaphase-promoting complex tumor suppressor protein and rescues cells from cell cycle arrest induced by hDLG. A chimeric Tax-2 encoding the last 53 amino acids of Tax-1 (Tax221), which contains the PBM, demonstrated an increased transforming potential in rat fibroblast cells [29]. It was further found that deletion of the PBM from Tax-1 abrogates hDLG binding and results in reduced transformation activity in rat fibroblasts and an IL-2-dependent mouse cell line [30, 31]. The contribution of the Tax-1 PBM to HTLV-induced proliferation and immortalization of primary T cells in vitro and viral survival in an infectious rabbit animal model was recently investigated [32]. Using both virus gene knockout and knockin approaches, the Tax-1 PBM was found to significantly increase both HTLV-1- and HTLV-2-induced primary T cell proliferation. Viral infection and persistence were severely attenuated in rabbits inoculated with an HTLV-1 provirus containing a deletion in the 4 amino acid PBM motif. Together, these studies support the conclusion that the PBM of Tax-1 and its interacting partners, the cellular PDZ domain containing proteins (e.g. hDLG1), are important in cellular transformation. Thus, the absence of the PDZ domain in Tax-2

may be a major determinant of the differences in pathogenicity between HTLV-1 and HTLV-2.

Human T Cell Leukemia Virus Accessory Genes and Pathogenesis

Nearly a decade after the discovery of HTLV and the Tax- and Rex-positive transregulatory proteins, additional alternatively spliced viral mRNAs containing novel ORFs were identified and characterized [8]. Based on protein sizes expressed from cDNA expression plasmids, HTLV-1 accessory proteins encoded by ORF I and II were named p12 and p30/p13, respectively (fig. 2 and table 1). Although the mRNAs encoding these proteins have been identified in HTLV-infected individuals, to date, detection of these proteins in infected cells has remained elusive. However, cytotoxic T lymphocytes and serum from HTLV-1-infected individuals or experimentally infected rabbits have been demonstrated to recognize peptides against these proteins, providing indirect evidence of expression in vivo [8].

HTVL-1 ORF I p12

p12 appears to be a modulator of T lymphocyte proliferation and immune function [8]. Although it localizes to endomembranes, particularly the endoplasmic reticulum and Golgi, it has been shown to interact with IL-2 receptor-β and -γc chains and MHC class I heavy chains disrupting their surface expression (fig. 3 and table 1). p12 interacts with the 16-kDa subunit of the vacuolar ATPase, a complex important for the function of lysosomes and endosomes and implicated in transformation pathways. In addition, p12 interacts with calnexin and calreticulin, important endoplasmic reticulum regulators of calcium release, NFAT transcriptional activation and the regulation of T cell proliferation. Initial studies utilizing an infectious molecular clone indicated that abrogation of p12 message or protein had no effect on viral replication and immortalization of primary T lymphocytes [33]. Subsequently, studies revealed the essential role of p12 in the establishment of persistent in vivo viral infection using the rabbit model of infection [34]. Studies by Albrecht et al. [35] demonstrated that p12 is required for optimal viral infectivity in quiescent but not activated primary cells, which suggests a role for p12 in T cell activation. More recently, a study using a p12-deficient virus indicated that p12 promoted cell-to-cell spread by inducing LFA-1 clustering on T cells via calcium-dependent signaling [36]. Together these findings suggest that p12 is a multifunctional protein that facilitates viral infection, host cell proliferation and survival, and helps infected cells escape from host immune surveillance.

HTVL-1 ORF II p30/13

p30 is expressed from a doubly spliced mRNA, localizes to the nucleus/nucleolus and physically interacts with CBP/p300, TIP60 and Rex [8, 37] (fig. 3 and table 1). In vitro

Table 1. HTLV-1 regulatory and accessory protein interaction and functions

HTLV-1	Protein	mRNA	Cell localization	Cellular interactions	Function
ORF-I	p12	Singly spliced	Endoplasmic reticulum and Golgi body	Vacuolar ATPases, IL-2Rβ, IL-2Rγc, calreticulin, calnexin, MHC 1-Hc, LAT	STAT and NFAT transcriptional activation, calcium release, immune evasion, down regulates TCR
ORF-II	p13	Singly spliced	Mitochondria and nucleus	Farnesyl-pyrophosphate synthase, actin-binding protein 280	Disrupts inner mitochondrial membrane potential
	p30	Doubly spliced	Nucleus and nucleolus	p300/CBP, TIP60	Retains *tax/rex* mRNA in nucleus for viral latency, modulates CRE and TRE-mediated gene expression
ORF-III	Rex	Doubly spliced	Nucleus and nucleolus	Nuclear pore/ CRM-1	Transports genomic and incompletely spliced viral mRNA to the cytoplasm
	$p21^{rex}$	Singly spliced	Cytoplasm	–	–
ORF-IV	Tax	Doubly spliced	Nucleus and cytoplasm	APC(Cdc20 and Cdc27), CDK-4, CDK-6, CHK1, CHK2, CREB-2 p300/CBP, DLG1, INK4A, INK4B, I KKγ, MAD1, PCAF, P13K, RB, TAX1BP2	Viral transcriptional activation and many other cellular effects
Antisense ORF	HBZ	Singly spliced	Nucleus and nucleolus	CREB-2, CREB, JunB, c-Jun, JunD	Modulates CREB-2 and Jun-mediated transcription

APC (Cdc20 and Cdc27) = Anaphase promoting complex; ATPase = adenosine triphosphatase; c-Jun, JunB, JunD = cellular DNA binding transcription factors; CHK1 = checkpoint kinase 1, 2; CDK-4, 6 = cyclin-dependent kinase 4, 6; INK4A, B = cyclin-dependent kinase inhibitors; CREM-1 = cellular export receptor 1; DLG1 = discs large homologue 1; IL-2Rβ, γ = Interleukin-2 receptor chains β, γ; LAT = linker for activation of T cells; MAD1 = mitotic arrest deficiency protein 1; MHC I-Hc = major histocompatibility complex heavy chain; mRNA = messenger RNA; NFAT = nuclear factor of activated T cells; PCAF = p300/CBP-associated factor; PI3K = Phosphoinositide-3 kinase; RB = retinoblastoma tumor suppressor; STAT = signal transducers and activators of transcription; TAX1BP2 = Tax1 binding protein 2, a novel centrosomal protein; TCR = T-cell receptor; TIP60 = Tat-interactive protein 60; TRE = Tax-1 response element 1.

studies have demonstrated that at low concentrations, p30 differentially regulates cellular and viral promoters through an interaction with CBP/p300. At high concentrations, p30 functions as a repressor of viral gene transcription by competing with Tax for CBP/p300. Similarly, p30 may also repress cellular gene transcription from CREB-responsive promoters by sequestering the limited amount of cellular CBP/p300. p30 via its interaction with TIP60 binds Myc-containing transcription complexes and

enhances Myc-dependent cellular transformation of human fibroblasts. In addition, p30 has been shown to repress viral replication at the posttranscriptional level by binding to and retaining *tax/rex* mRNA in the nucleus [38, 39]. By suppressing Tax protein expression, p30 attenuates HTLV-1 transcription. More recently, it was reported that p30 and the positive posttranscriptional regulator, Rex-1, form ribonucleoprotein complexes specifically on *tax/rex* mRNA [37]. Together, this suggests that p30 may govern the switch between viral latency and replication. Similar posttranscriptional regulation has been reported for HTLV-2, mediated by the p28 ORF II protein [39]. Interestingly, p28 does not appear to share the transcriptional properties of p30. A recent report showed that HTLV-1 p30 expression results in activation of the G_2-M cell cycle checkpoint in Jurkat T cells, which suggests p30 is involved in events that would promote early viral spread and Tcell survival [40]. Although p30 is dispensable for HTLV-1-mediated cellular transformation in culture, inoculation of rabbits with a p30-deficient virus revealed that p30 expression is required early on in infection to sustain high viral loads in rabbits and promote persistence [41]. Thus, it is becoming clear that p30 is a multifunctional protein that may assist the virus at many levels contributing to virus survival and pathogenesis.

p13 is expressed from a singly spliced mRNA. Its ORF corresponds to the 87 carboxyterminal amino acids of p30 [8]. Unlike p30, p13 localizes to the mitochondria, alters its morphology by disrupting the inner membrane potential and ion flux and binds farnesyl pyrophosphate synthetase, an enzyme involved in posttranslational farnesylation of Ras (fig. 3 and table 1). These properties suggest that p13 is involved in cell signaling and apoptosis. p13 was found to negatively influence cell proliferation in high-density cell culture and to interfere with tumor growth in a nude mouse transplant model [42]. In the context of an infectious molecular clone, p13 is dispensable for HTLV-1 infection and immortalization of PBMCs in culture [33], whereas rabbits inoculated with a p13-deficient virus failed to induce a significant immune response and establish a persistent infection [43].

Antisense Gene *HBZ*

The *HBZ* gene is found in HTLV-1 but not HTLV-2 and is encoded on the minus strand of the proviral genome; the mRNA is synthesized from a promoter located in the 3′ LTR [9]. Recent research in the field suggests that the *HBZ* gene may function in 2 molecular forms: mRNA and protein. Exogenously overexpressed HBZ protein interacts with CREB-2 and downregulates Tax-mediated HTLV-1 transcription and interacts with and disrupts the DNA-binding activity of JunB and c-Jun (AP-1 components; fig. 3 and table 1). HBZ also interacts with JunD to activate the transcription of JunD-dependent promoters. Therefore, it has been hypothesized that HBZ may play an important role in HTLV-1 biology by counteracting the effects of Tax-mediated transcription and/or attenuating or activating cellular gene expression. Studies by Arnold et al. [44] utilizing

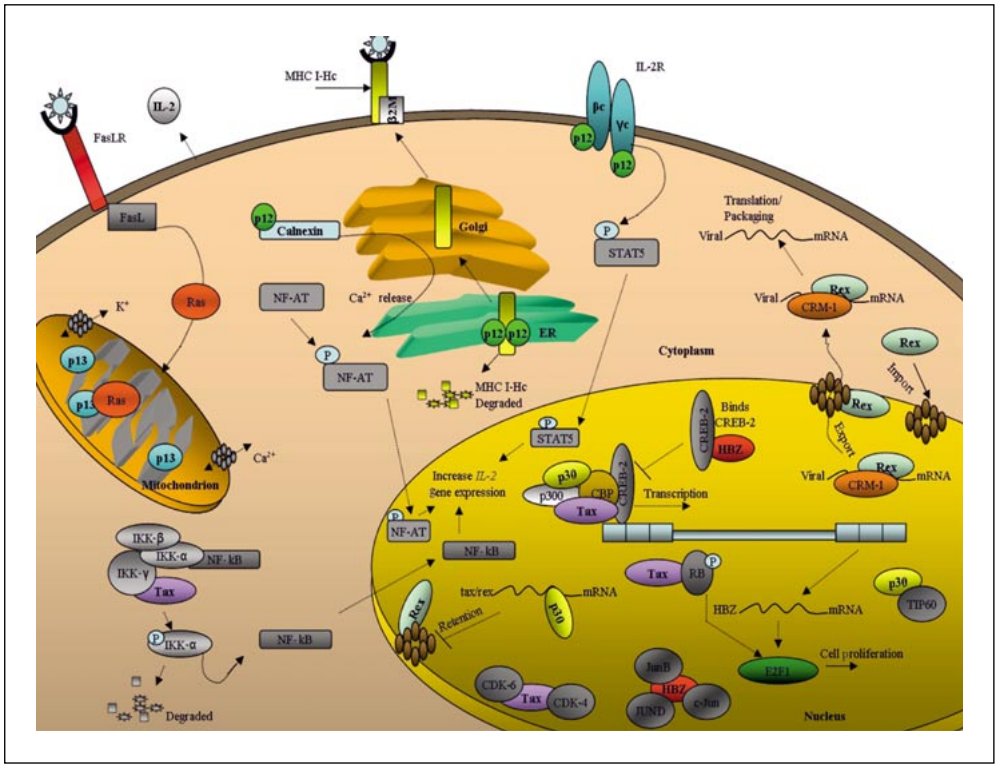

Fig. 3. Key HTLV-1 virus/host interactions in a newly infected T cell. Following proviral integration transcription of viral genes initiates utilizing host cell machinery. The newly synthesized viral proteins hijack the cell by interacting with many host cellular factors at distinct locations and organelles. These interactions and their consequences allow the virus to survive in the infected cell by evading immune surveillance, disrupting cellular homeostasis as well as undergoing replication to produce viral progeny. Tax interacts with CBP/p300/CREB-2 at the viral promoter to initiate viral transcription. Tax activates cyclin-dependent kinases CDK-4 and -6 through direct protein binding leading to the hyperphosphorylation and degradation of retinoblastoma (RB), which frees the E2F1 transcription factor, accelerating cell cycle transition from G_1 to S. Cytoplasmic Tax binds IKK-γ, triggering the phosphorylation of IKK-α and IKK-β, which form a complex (IKK-α/IKK-β/IKK-γ) that phosphorylates IκB-α, leading to its proteasome-mediated degradation. This frees IκB-α-sequestered NF-κB to migrate into the nucleus, where it activates NF-κB-responsive genes. Rex exports unspliced and incompletely spliced viral mRNA to the cytoplasm via interaction with the CRM-1 export pathway through the nuclear pore. Once in the cytoplasm, viral mRNA will be translated, processed and/or packaged into new virions. HBZ downregulates Tax-induced HTLV-1 transcription by interaction with CREB-2 and interacts with and disrupts the DNA-binding activity of JunB and c-Jun, components of activator protein-1 (AP-1). HBZ plays an important role in HTLV-1 biology by counteracting the effects of Tax at the transcriptional level and attenuating the activation of AP-1. Additionally, HBZ interacts with JunD to activate the transcription of JunD-responsive cellular genes that are important in growth, proliferation and apoptosis. *HBZ* mRNA has been shown to increase the expression of *E2F1*, which leads to increased cellular proliferation. p30 acts as a repressor of viral gene expression by retaining *tax/rex* mRNA in the nucleus. p30 also regulates cellular/viral promoters through an interaction with CBP/p300. p13 interacts with the inner mitochondrial membrane and induces membrane permeability and dysregulation of the endoplasmic reticulum (ER) ion flow (K^+, Ca^{2+}). p13 interacts with Ras in the mitochondria, which leads to mitochondrial membrane swelling. p12 increases the DNA-binding and transcriptional activity of STAT5. p12 interacts with calnexin and calreticulin, 2 ER-resident proteins that regulate calcium storage and increase calcium release. p12 modulates

an infectious molecular clone indicated that the HBZ protein is dispensable for immortalization/transformation of primary T lymphocytes in cell culture. Furthermore, rabbits infected with this HBZ protein knockout virus became persistently infected. However, these rabbits displayed a decreased antibody response to viral gene products and reduced proviral load in PBMCs as compared to wild-type HTLV-1-infected animals. The data provide important evidence that HBZ is required for the establishment of chronic viral infections in vivo. Interestingly, in leukemic cells isolated from ATL patients, the 5′ LTR of the provirus is often deleted or hypermethylated, resulting in loss or suppression of the viral genes encoded on the plus strand of HTLV-1. However, the 3′ LTR, which contains the *HBZ* promoter, is hypomethylated and conserved in ATL cells. *HBZ* mRNA is expressed in all ATL cells and suppression of *HBZ* gene transcription by short interfering RNA inhibits proliferation of these cells [45]. Furthermore, *HBZ* mRNA rather than HBZ protein promoted proliferation of a human T cell line [45]. Mutational analysis suggested that the structure of the *HBZ* mRNA is important for its antiproliferative function. It is becoming clear that *HBZ* gene expression is important for viral infection, but further studies are needed to dissect the precise mechanisms that the *HBZ* protein and mRNA play in HTLV-1 pathogenesis.

Conclusions

HTLV-1 and HTLV-2 have the capacity to efficiently immortalize and transform T lymphocytes in vitro and persist in infected individuals or experimental animals. HTLV-1 infection leads to ATL lymphoma and HAM/TSP, whereas HTLV-2 infection is not associated with leukemogenesis. This chapter focused on important aspects of HTLV-1 pathobiology and where appropriate highlighted insightful comparative studies between HTLV-1 and HTLV-2. Multiple-assay systems have provided evidence that the viral Tax protein is the key player in HTLV-mediated oncogenesis. It has also become clear from studies utilizing infectious molecular clones in primary human T cells in vitro and relevant animal model systems that other viral proteins play a supporting role. Such studies will be instrumental to dissect the virus/host interactions associated with HTLV infection and survival, proliferation of infected cells and the development of disease. Ultimately, these model systems can be used to understand the mechanism of viral pathogenesis and to develop potential therapeutic intervention strategies against HTLV-1.

calcium-mediated cellular gene expression by decreasing the threshold of T cell activation through NFAT transcriptional activation and IL-2 production. These interactions increase the responsiveness to IL-2 by STAT5 activation, amplifying physiological stimulation and proliferation of infected cells. p12 binds newly synthesized MHC I heavy chain, preventing its association with β2-microglobulin and increasing its degradation. A reduction in surface expression of MHC I can protect infected cells from cytotoxic T lymphocyte recognition. This function of p12 may play a key role in the establishment and maintenance of infection by HTLV-1, particularly when Tax elicits a strong cytotoxic T lymphocyte response.

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Chromosomal Instability and Human T Cell Leukemia Virus 1 Transformation

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Abstract

Chromosomal instability (CIN) is a hallmark of many cancer cells. Several cellular mechanisms have been proposed to lead to CIN, including abnormal centrosome replication, defects in spindle assembly checkpoint, failed DNA repair and telomere dysfunction. Human T cell leukemia virus 1 (HTLV-1) is the etiological agent for adult T cell leukemia (ATL). The key clinical features of ATL include leukemic cells with multilobulated nuclei called 'flower cells'; this nuclear morphology is suggestive of grossly aberrant chromosomal composition. Notably, after a prolonged asymptomatic period of 20–40 years, 1–5% of the HTLV-1-infected individuals will develop ATL. Therefore, it can be inferred that multiple cumulative genetic and epigenetic alterations in the host genome may be required for ATL leukemogenesis. This chapter will discuss the involvement of CIN and HTLV-1 in ATL. We will explore how the HTLV-1 oncoprotein Tax initiates cellular transformation by inducing CIN.

Chromosomal instability (CIN) is a phenotype exhibited by cells that have an elevated rate of unstable chromosome content. CIN has been described to be associated with tumor development and progression. Some features of CIN include DNA translocation, losses/gains of whole chromosomes or portions of chromosomes, the presentation of chimeric gene fusions and changes in gene copy numbers. Some contributory mechanisms for CIN are reported to encompass loss of spindle checkpoint, abnormal amplification of centrosomes, defects in DNA repair machinery and telomere malfunction, amongst others. Empirically, a correlation between CIN and cancer is supported by findings of high genetic heterogeneity within the same tumor mass [1].

In principle, mutations and chromosomal alterations can be driving forces for beneficial evolution. At the cellular level, nature may favor a cell with a higher replication rate over one with a slower rate. A CIN phenotype may in rare instances allow cells to acquire the right combination of mutations/changes that confer a higher proliferative

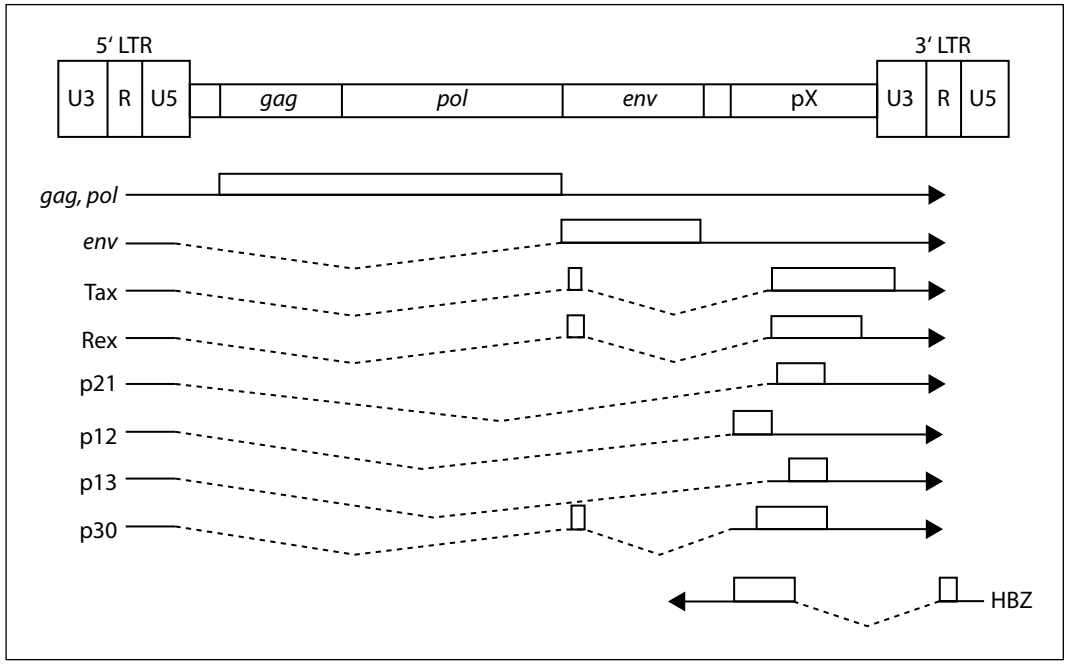

Fig. 1. Genome organization of HTLV-1. HTLV-1 proviral genome, the viral RNA species and the encoded proteins are shown. The genomic unspliced mRNA encodes the structural genes *gag*, *pol* and *env*. A spliced mRNA, which corresponds to the pX region of the provirus, encodes the Tax, Rex, p21, p12 and p30 proteins. In addition, the HTLV-1 encodes a basic leucine zipper factor (HBZ), which is expressed in an antisense fashion. LTR = Long terminal repeat.

capacity. On the other hand, in most settings CIN creates mutations detrimental to normal ambient cellular metabolism; hence, to guard against structural and numerical chromosomal changes, cells have evolved a number of censoring checkpoints [2]. Not surprisingly, cancer cells with CIN are frequently disabled in such checkpoints.

Spontaneous human cancers are difficult to study. By the time a cancer is detected clinically, the original single-cell clonal events have long transpired. Moreover, specific inciting etiologies for the development of many human cancers are difficult to pinpoint. Hence, the first exact transforming event is usually unclear. Given these obstacles, human cancers caused by viral infection present an attractive model for investigating defined stepwise mechanisms for tumorigenesis. Here the viral etiology for the cancer is well defined, and one can usually follow a viral infection prospectively over time monitoring for premalignant changes which may evolve eventually into frank malignancy.

Human T cell leukemia virus 1 (HTLV-1) is a retrovirus which was first isolated in 1980 [3] (fig. 1). This virus has been shown to be the etiological agent for adult T cell leukemia (ATL), an aggressive and frequently fatal leukemia. An estimated 10–20

million people are infected with HTLV-1 worldwide. However, approximately 1–5% of the infected individuals will develop ATL after a long asymptomatic period, spanning 20–40 years [4]. The leukemogenic mechanism of HTLV-1, which leads to ATL, is not fully understood; nonetheless, it is widely accepted that HTLV-1 encodes a viral oncoprotein, Tax, which is key to initiating the transformation of virus-infected cells. Tax is a transcriptional activator that acts to increase viral transcription from the promoter resident in the U3 region of the 5′ HTLV-1 long terminal repeat. In model systems, Tax expression has been shown to be sufficient for transforming cultured human T lymphocytes [5] and for inducing tumorigenesis in transgenic mice [6]. HTLV-1/Tax/ATL represents a good model system for studying how the expression of a viral oncoprotein triggers leukemogenesis. In this regard, it has been reported that Tax-expressing cells have a CIN phenotype, and over time this CIN phenotype leads to the development and selected outgrowth of a rare subpopulation of transformed leukemic cells. Below we review the evidence for Tax-engendered CIN in cells and our current understanding of the mechanisms leading to CIN.

Mechanisms Leading to Chromosomal Instability

Tumorigenesis is a multistep process that cumulates in cellular genetic damage. What is known about the factors that are needed to guard against the manifestation of damaged genetics in human cells? In eukaryotes, a group of CIN-related genes was initially identified in yeast *Saccharomyces cerevisiae* [7, 8]. Based on comparisons with yeast counterparts, many human CIN genes were subsequently identified. Systematic investigation of CIN genes has led to a partial understanding of how CIN can occur mechanistically. These steps are summarized in figure 2 and include (1) an abnormal centrosome cycle; (2) an aberrant spindle assembly checkpoint (SAC); (3) failed DNA repair machinery, and (4) telomere dysfunction.

Aberrant Centrosome Cycle

Centrosomes are cytoplasmic organelles that organize the interphase microtubule cytoskeleton which later morphs into the mitotic spindle poles. A centrosome is composed of 2 centrioles which are surrounded by pericentriolar material. A centrosome duplicates precisely once during the S phase, concurrent with DNA replication. In mitosis, the duplicated centrosomes serve as the microtubule-organizing centers that form the bipolar mitotic spindle poles which anchor the apparatus for the correct segregation of replicated chromosomes.

Dysregulated amplification of centrosomes occurs frequently in many cancers. There are 2 explanations for this finding. First, in cells destined for transformation, a centrosome may duplicate more than once during a single cell cycle. Second, some cells may fail to undergo proper cytokinesis, resulting in supernumerary centrosomes. Cells with >2 centrosomes form tri- or multipolar spindle poles. These cells

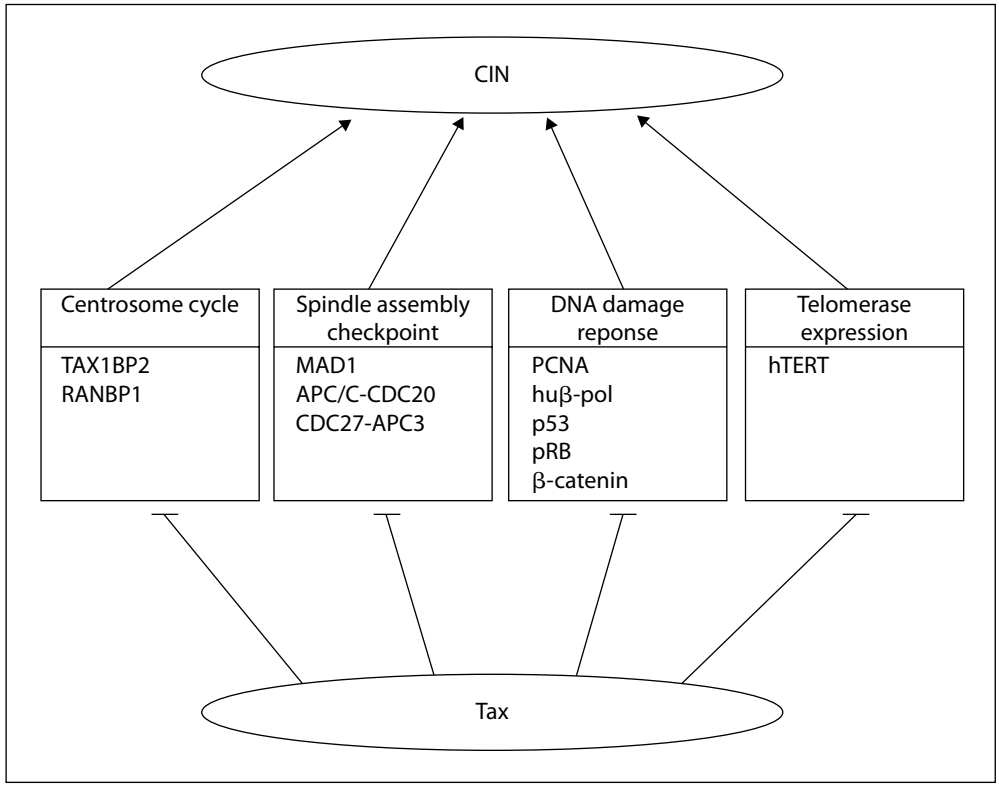

Fig. 2. Cellular factors that may be affected by Tax and contribute to CIN in HTLV-1 infected cells. Tax may disturb many cellular mechanisms that maintain genomic integrity, including the centrosome cycle, the spindle assembly checkpoint, the DNA repair machinery and telomerase activity. RANBP1 = Ran-binding protein 1; MAD = mitotic arrest deficent; APC/C = anaphase-promoting complex/cyclosome; PCNA = proliferating cell nuclear antigen; huβ-pol = human β-polymerase; pRB = retinoblastoma protein; hTERT = human telomerase reverse transcriptase.

may fail to undergo cytokinesis and become either bi- or mononucleated with >2N chromosomal content. Cells with >2 centrosomes, in the absence of the G_1/S p53 checkpoint protein, may continue to progress through the cell cycle to become increasingly aneuploid. Aneuploidy, in turn, may promote the eventual development of a fully transformed phenotype [9, 10].

There is evidence for a centrosome duplication checkpoint. Candidate regulators of this checkpoint include the checkpoint kinases, CHK1, CHK2 and its upstream regulators, ATM and ATR. In addition, a growing number of centrosome localizing proteins have been implicated in centrosomal regulation in the DNA damage response. These factors include the tumor suppressor gene p53, BRCA1/2, the mitotic regulators Aurora A, NEK2, and the polo-like kinases PLK1 and PLK3. Aberrancies in any of these centrosomal proteins may dysregulate the centrosome cycle and promote CIN.

Defective SAC

In mitosis, duplicated chromosomes condense, separate and are partitioned equally into 2 daughter cells. During chromosomal partitioning, each replicated kinetochore is tethered bidirectionally to microtubules attached at opposing spindle poles. To ensure fidelity of division, segregation can occur only when equal bilateral tension is sensed at the kinetochores. Improper microtubule attachments that generate unequal tension may induce a SAC that arrests cells in the prometaphase until the errors are corrected. The SAC is a complex composed of many proteins, several located at the kinetochore, which include the mitotic arrest deficient (MAD) proteins (MAD1, MAD2 and MAD3), the budding uninhibited by benzimidazole (BUB) proteins (BUB1, BUB2 and BUB3), the monopolar spindle 1 protein, the ROD-ZW10-Zwilch complex and the microtubule motor centromere protein E [11]. Loss of SAC function can result in premature segregation and increase the ambient prevalence of aneuploidy.

A link between SAC and CIN comes from studies that show the emergence of aneuploidy in cells lost for SAC components. For example, a hereditary mutation in BUBR1 was uncovered in individuals with mosaic variegated aneuploidy, in which >25% of the cells in the body are aneuploid. Patients with a BUBR1 mutation are highly susceptible to childhood cancers, such as rhabdomyosarcoma and leukemia [12, 13]. In addition, up to 40% of the human lung cancers have cells which carry defects in mitotic checkpoint genes, including *mad1* and *mad2* [14, 15]. Collectively, these findings suggest that a loss in or a weakening of SAC could be causal for carcinogenesis.

Failed DNA Damage Response

Carcinogenesis generally requires multiple genetic alterations in cells. The spontaneous mutation rate in a normal human cell is approximately 1.4×10^{-10} per base pair per cell generation [16]. However, the number of mutations accumulated in premalignant and cancerous cells far exceeds that observed in normal cells. Hence, one theory proposes that malfunctions (either gains or losses of functions) in genes involved in DNA repair pathways are prerequisite for driving the tumorigenic process. Here, the thinking is that competent DNA repair contributes to the maintenance of genetic stability.

There are 3 major biochemical pathways employed to maintain genome integrity: the mismatch repair, the nucleotide excision repair (NER) and the base excision repair pathways [16]. Mismatch repair proteins correct errors that result from replication of misincorporated nucleotides, NER acts on base damage caused by exogenous agents such as mutagenic and carcinogenic chemicals and photoproducts derived from sunlight exposure, and base excision repair functions in the repair of mutations caused by reactive oxygen species, generated during aerobic metabolism. Defects in intracellular mechanisms of DNA damage repair have been inferred to play critical roles in genomic instability.

Relevant to the role of DNA damage repair and cancer, the most frequently observed genetic alteration associated with proliferative/survival/repair capability

involves mutation of the p53 tumor suppressor gene. The p53 gene is mutated in >50% of all human tumors. Currently, >15,000 mutant p53 alleles have been sequenced and have been found to carry various inactivating mutations [17]. In normal cells, p53 is responsible for arresting cell growth in response to certain types of molecular and biochemical damage until such damage is repaired. In other types of damage and physiologic stress, the p53 protein triggers a program of apoptosis which eliminates the damaged cell. Another well-characterized tumor suppressor gene, the retinoblastoma protein (pRB), is mutated frequently in cancers such as retinoblastomas, lymphoma, osteosarcomas and small-cell lung carcinomas. The pRB protein plays a central role in determining whether a cell will proceed through the G_1 phase of the cell cycle.

Many familial colon cancers are linked to germline mutations in the tumor suppressor gene, adenomatous polyposis coli. Adenomatous polyposis coli can bind β-catenin, a protein that functions in cell adhesion and WNT signal transduction [18]. β-Catenin thus participates in both cell-cell adhesion and the transcription of genes responsive to T cell transcription factor/lymphoid-enhancer-binding factor. Inactivation of adenomatous polyposis coli allows large amounts of β-catenin to accumulate in the cytosol and to then translocate into the nucleus which mediates the transcription of target genes such as c-*myc* and cyclin D1, which can contribute to cell proliferation, survival and transformation.

Telomere Dysfunction

Telomeres are specialized DNA protein structures located at the end of linear chromosomes. Telomeres of eukaryotic nuclear chromosomes typically harbor an array of simple-sequence repeats. In mammalian cells, telomeres have approximately 1,000 copies of a hexanucleotide motif, TTAGGG, which serves multiple functions, including prevention of fusion or degradation of chromosomes and facilitating chromosome segregation [19]. In humans, long telomeres are maintained in germline cells but shorten progressively in most somatic cells after each round of genome replication due to the lack of sufficient telomerase activity. When the telomeres reach a critical length, the cells stop dividing and enter senescence. At this stage, CIN is maximal and manifests itself with increased end-to-end telomere fusions. The role of telomeres in preventing chromosome fusion suggests that the status of the telomeric complex as well as the length of telomeric repeats contribute to chromosomal stability.

In most eukaryotes, replenishment of telomeres can be carried out by a nuclear ribonucleoprotein called telomerase, which adds a simple telomeric sequence to preexisting 3′ overhangs. In human cells, this activity is constituted by a catalytic component human telomerase reverse transcriptase (hTERT) associated with an RNA subunit h(TR). The expression of hTERT is highly regulated. Constitutive expression of telomerase has been detected in extracts of human cells in early embryogenesis and in the germline. On the other hand, inactivation of telomerase results in quiescence and death, which limit the renewal capacity of differentiated cells. Therefore, inactivation

of telomerase in somatic cells has been proposed to function as a tumor-suppressing mechanism. Indeed, the observation that 90% of the tumors exhibit an aberrantly heightened telomerase activity [19] highlights that maintaining the correct length of telomeres is a process required for the proliferative capacity of cells.

A growing number of proteins involved in the DNA repair machinery have been demonstrated to interact with telomeres. As discussed above, telomeres that are shortened can no longer protect the chromosome ends that can create cells with DNA end-to-end fusions, which may trigger senescence or apoptosis. Although most cells with DNA damage die, rare cells may survive the damage crisis and emerge to persist in proliferation. Hence, embryonic fibroblasts that lack telomerase activity ($mTERC^{-/-}$) show shortened telomeres, and a breakage-fusion-bridge cycle has been shown for $mTERC^{-/-}p53^{+/-}$ compound mice, which results in gains and losses of chromosome segments that drive epithelial carcinogenesis [20]. These findings are consistent with failed repair of structurally damaged DNA contributing to cancer development.

Human T Cell Leukemia Virus 1 Tax and Genetic Instability

Tax transgenic mice that target the mature T lymphocyte compartment show that the expression of Tax alone is sufficient for T cell leukemia in vivo [6]. In humans, the clinical features of ATL include leukemic cells with aneuploid multilobulated nuclei called 'flower cells'. Of relevance, Tax expression in cultured human cells frequently produces multinucleated cells [21, 22], suggesting a CIN phenotype for such cells. Below, we speculate how Tax may disturb chromosome stability by affecting cellular controls of the centrosome cycle, mitotic checkpoint, DNA damage response and telomere function (fig. 2).

Tax and the Centrosome Cycle

Centrosome hyperamplification is frequent in HTLV-1 transformed cells. Ching et al. [23] and Peloponese et al. [24] showed that this phenotype may be correlated with direct action of the viral Tax oncoprotein through 2 different mechanisms. First, a fraction of Tax protein that localizes to centrosomes interacts with TAX1BP2, a novel centrosome protein, which regulates centrosome duplication. Tax subverts TAX1BP2 function leading to supernumerary centrosomes. Second, Tax binds Ran-binding protein 1, which is located at spindle poles and causes abnormal centrosome fragmentation in the M phase [24]. In this setting, Tax does not affect the interphase duplication of centrosomes.

Tax and the SAC

The SAC guards against chromosome missegregation in mitosis. In cells with weakened SAC, aberrant mitoses with chromosome bridges and lagging chromosomes are

allowed to progress unchecked through the cell cycle to produce progenies with aneuploid genomes. In these cases, aberrant chromosomal content can manifest itself with micronuclei and aneuploid genomes, which are common findings in HTLV-1 transformed lymphocytes and Tax-expressing cells [22]. Jin et al. [21] first reported that MAD1, a component of the SAC, is a cellular target of Tax. Through direct protein-protein binding, Tax is thought to abrogate the checkpoint function of MAD1 in the M phase.

Recently, the CDC20-associated anaphase-promoting complex/cyclosome (APC/C) involved in the SAC regulation has been reported to be another cellular target for Tax in mitosis. APC/C is an E3 ubiquitin ligase that controls metaphase to anaphase transition by polyubiquitinating and degrading cyclin B1 and securin [25]. Through direct interaction with APC/C-CDC20 and CDC27-APC3 complexes, Tax is thought to promote premature mitotic exit. However, new findings suggest that the APC/C may not be prematurely activated by Tax [26]. The role of APC/C in HTLV-1 transformation requires further experimental clarification.

Tax and DNA Damage Response

Tax can repress NER. Indeed, the ability of Tax to inhibit NER correlates with PCNA (proliferating cell nuclear antigen) overexpression [27]. PCNA is a eukaryotic DNA polymerase processivity factor that is involved in both DNA replication and repair. Activated PCNA expression appears to reduce DNA repair. In addition to PCNA, Tax also downregulates the expression of human β-polymerase, a cellular DNA polymerase involved in host cell DNA repair [28].

Mutation in the tumor suppressor p53 gene is found in approximately 30% of ATLs [29]. In the 70% of ATLs that have nonmutated p53, the activity of this protein appears to be inactivated by the HTLV-1 Tax oncoprotein [30]. It is currently not fully understood how Tax represses p53 activity. However, there are suggestions that this occurs through either an NF-κB-associated pathway [31, 32] or through a CREB/CREB-binding protein pathway.

Tax can also inactivate pRB protein through activations of cyclin-dependent kinases [33]. The phosphorylation and/or degradation of pRB proteins free the E2F1 transcription factor, resulting in a dysregulated cell cycle and premature cellular entry from the G_1 into the S phase. Separately, Tomita et al. [34] have reported that in Tax-positive HTLV-1-infected T cell lines β-catenin protein was overexpressed in the nucleus and that β-catenin-dependent transcription was significantly enhanced. β-Catenin is highly expressed in several leukemias, and there is evidence that elevated β-catenin mediates enhanced survival of chronic lymphocytic leukemia cells. Activation of β-catenin by Tax may also be important to T cell transformation by HTLV-1.

Dysregulation of hTERT by Tax

Telomeres play a vital role in protecting the ends of chromosomes and preventing chromosomal fusion. While telomere shortening induces cell death and may lead to

tumor suppression, a failure to maintain telomeres can be an important inciting event for CIN. The telomerase (i.e. hTERT) enzyme is needed to maintain telomere length. Strikingly, hTERT is usually not expressed in normal cells but is prevalently expressed in most (85–90%) human cancers [19].

Chromosome end-to-end fusions and shortened telomeres are frequently seen in ATL cells. This observation can be explained by the ability of Tax to repress telomerase activity [35]. Thus inhibition of hTERT by Tax in the early phase of carcinogenesis might contribute to CIN [36]. However, late in ATL transformation, hTERT activity appears to be activated; interestingly, this event seems to be correlated with silencing of Tax expression in these leukemic cells [37].

Concluding Remarks

Despite the enormous progress that has been made in the last 2 decades in cancer research, a complete understanding of the causations of cancers and their vulnerabilities to treatment remains elusive. A major reason for this difficulty lies in the multitude of genetic and epigenetic changes that occur during carcinogenesis. Indeed, no 2 cancers are genetically or phenotypically the same. Moreover, there is a high degree of genetic heterogeneities even in the same tumor mass. This genomic instability may be an initial causal event for cancer development.

HTLV-1/ATL provides an excellent model for understanding the detailed course of oncogenesis. In this system, the expression of the HTLV-1 oncoprotein Tax alone is sufficient to induce tumors in mice. While additional insights await further research, currently, a preliminary summary of the steps that Tax employs to transform cells appears to involve the following. First, Tax promotes cell survival through activation of Akt and NF-κB [4]. Second, Tax enhances CIN by (i) interfering with the host DNA repair machineries, by repressing tumor suppressors p53 and pRB, and by activating β-catenin; (ii) Tax interferes with centrosome replication by interacting with TAX1BP2 and Ran-binding protein 1; (iii) Tax weakens SAC control through MAD1 interaction, and (iv) Tax deregulates hTERT expressions. Better understanding of these steps can advance our knowledge for treating ATL.

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