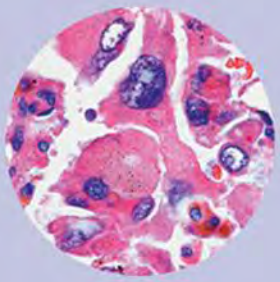

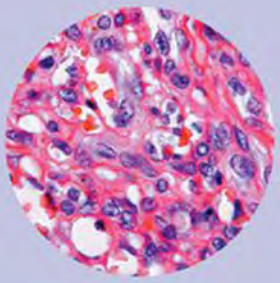

VIRAL ONCOLOGY

Basic Science and Clinical Applications

EDITED BY

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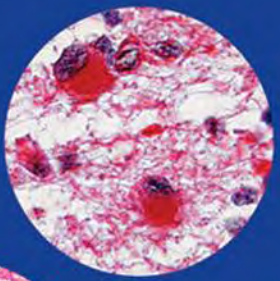

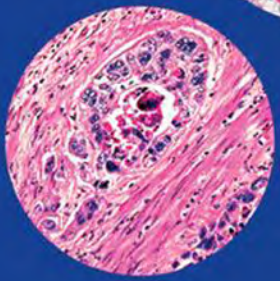

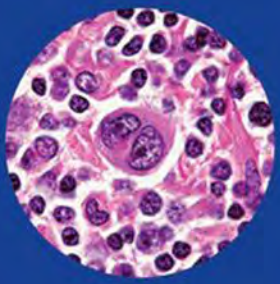

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VIRAL ONCOLOGY

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Basic Science and Clinical Applications

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FOREWORD

Cancer, in its several forms, has been and is one of the most common causes of death and disease in human populations. Primary prevention has been an effective means to decrease the incidence of several cancers. Programs for smoking cessation have lead to dramatic drops in cancer of the lung and other cancers and disease. The removal of carcinogenic toxins (i.e., asbestos) and other measures, such as diet control, have also had a positive effect. Prevention of cancers with antiviral vaccines has now been shown; this book will deal with issues related to the viral etiology of cancers and their prevention and treatment.

There have been significant improvements in treatment in recent decades, particularly of the hematological cancers of the young. However, for many cancers, particularly those with low survival expectations, even very effective treatments may only prolong life for a short time and the side effects of the treatments can decrease the enjoyment of life. Early detection and intervention have been effective for some cancers. The increased survival of patients with cancer of the breast has apparently been due, in large part, to early detection programs. Surveillance for polyps in the colon and rectum and their removal have helped decrease the incidence of clinical disease. However, not all early detection methods are effective. Recent research has questioned the value of screening for cancer of the prostate using prostate-specific antigen (PSA). Unnecessary surgery, radiotherapy, and other treatments may cause damage. Overall, PSA screening and intervention programs may not significantly decrease survival. Routine chest X-ray screening for the detection of early cancer of the lung was abandoned when it did not appear to be of benefit, although recent improvements in imaging may increase the value of surveillance.

It is obvious that preventing a disease is more desirable than becoming sick and then receiving treatment. The recent availability of viral vaccines that prevent cancer could usher in a new phase of effective cancer control. About 15% or more of human cancers are considered to be caused by viruses, and a discussion of these is a major goal of this book. Diseases have several causes, and it is likely that there are other cancers in which viruses have a role in causation, and where prevention of infection may favorably alter outcome.

The discovery of hepatitis B virus (HBV) in our laboratory was reported in 1967. We invented a method for a vaccine in 1969; in 1971 commercial production was being considered, and in 1975 it was agreed. The etiological association of HBV with primary cancer of the liver (hepatocellular carcinoma [HCC]) was postulated in 1969 by Smith and Blumberg and confirmed in the following years by striking epidemiological

evidence (Beasley in 1981, and others) and laboratory studies. HCC is the third most common cause of death from cancer in males and the seventh in females. There are probably about 1 million deaths a year from HCC and other diseases caused by HBV.

Following a convincing field trail of the hepatitis B vaccine by Szmuness and his colleagues (reported in 1980), the vaccine was approved in the early 1980s for use in the United States and later elsewhere. By 1990, universal or directed (to the newborn children of maternal HBV carriers) vaccination programs were in place in a large percentage of the national members of the World Health Organization and by 2005 in more than 75% of the nations. Compliance has, in general, been good, with recent fallback because of antivaccine sentiment in some locations. There has been a dramatic drop in the prevalence of HBV carriers in the vaccinated populations. On logical grounds, it would be expected that a fall in HBV prevalence (HBV is said to be the cause of about 70% of all primary cancers of the liver) would result in a fall in liver cancer incidence. After the vaccine had been in use for more than a decade, this inference was confirmed in a national study in Taiwan (1997) by Chang and colleagues. They found that the incidence of liver cancer had decreased by about two-thirds in the vaccinated cohorts. Soon afterward, Mark Kane, then the director of hepatitis programs at the World Health Organization, noted that vaccine prevention of HCC was one of the two most important cancer control programs along with smoking cessation projects. In less than two decades after the approval of the vaccine, it was in use worldwide and a common and deadly cancer had decreased in incidence.

About 25 years after the introduction of the first cancer prevention vaccine, a second was approved, and vaccination programs were initiated. In 2008, Harald zur Hausen was awarded the Nobel Prize in Medicine for identifying strains of the human papillomavirus (HPV) as the cause of cancer of the cervix of the uterus, and several other cancers. Painstakingly, zur Hausen had identified portions of the genomes of these strains in cancer cervical cancer cells and described the molecular pathogenesis of the cancer. Cancer of the cervix is the second most common cancer in women; worldwide, there are about half a million new cases annually and approximately 260,000 deaths.

In 2006, the U.S. Food and Drug Administration approved a vaccine that, based on a well-designed and well-executed field trial, protected against infection. Vaccination programs began soon afterward, and these are likely to become more widespread as the public health community learns more about the strategies for vaccination and the economics of national and regional vaccination programs.

An additional argument for national vaccination programs is the value of HBV and HPV vaccines in preventing diseases in addition to cancer. HBV vaccine prevents disabling and life-shortening acute and chronic hepatitis. HBV is also involved in the pathogenesis of kidney disease, polyarteritis nodosa, and other diseases that may be decreased by the use of the vaccine. (HBV infection is associated with cancer of the pancreas; the significance of these epidemiological observations requires further study.) HPV is associated with genital warts that can be disabling, as well as with several less common cancers. The use of the vaccines in large populations can be justified not only for the prevention of cancer but also for other more common diseases. HBV- and HPV-related pathologies are among the most common sexually transmitted diseases.

There are about 400 million people who are currently HBV carriers; some of them are at risk of developing chronic liver disease and HCC. Liaw (2004) and others have shown that antiviral treatment for relatively long periods can significantly decrease the risk of disease. This results need to be confirmed and the treatment modes determined in detail, including the problem of antiviral resistance with long usage. They hold promise for the treatment of early cancers with antivirals rather than cancer therapies that destroy normal cells along with cancer cells.

The widespread use of the vaccines has already and will in the future result in a dramatic drop in the prevalence of HBV and the strains of HPV affected by the vaccine. These viruses have affected large proportions of the world's population for many generations, possibly even before the emergence of *Homo sapiens*. For HBV, it is known that there are multiple polymorphic susceptibility loci (SNIPS) that affect the probabilities that the viral hosts will become carriers or, alternatively, develop protective antibody (anti-HBs). The implication is that there has been a balance, at a population level, of advantages and disadvantages that have maintained the virus in many populations at a high level. There are also other microbial agents related to HBV in that they are affected by the same susceptibility loci. It will be important to monitor the changes in prevalence of these and other microorganisms in the environment as the vaccines alter these dynamic relations.

The success of the research on viruses and cancer, and in particular, the major contributions of the two cancer prevention vaccines, have revived interest in viral oncology. The publication of this book, the establishment of viral oncology programs in research centers, and the convening numerous conferences are indications of this change. These activities could once more bring virus research to the forefront in the never-ending endeavor to understand how viruses maneuver and negotiate in a complex environment, including the environment of the human host. It should also give urgency to a concerted program to develop vaccines and other interventions that will help prevent additional cancers and decrease their burden on human populations.

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PREFACE

Oncogenic viruses are the known etiologic agents in 15%–20% of all human cancers. Their impact on global health is significant. The first recognition that cancer can be caused by a virus dates back to observations of Rous sarcoma virus in chickens almost 100 years ago. However, it was not until the 1970s that the mechanistic basis for retroviral transformation became clearer. That era was marked by the discovery of many viral oncogenes and counterpart cellular proto-oncogenes. Additionally, insights into cell growth factors, cell cycle regulation, checkpoints, and their operative protein factors followed rapidly. Today in 2009, there are six well-established human cancer viruses (HBV, HCV, HPV, HTLV, EBV, and KSHV). Besides these six viruses, there is mounting evidence, as presented in many of the chapters in the book, that illustrate a strong association of other viruses, most notably human polyomaviruses, including JCV, BKV, and SV40, with a variety of human cancers.

This book emerged from a desire to provide an up-to-date survey of human oncogenic viruses and their pathogenic properties. The 21 chapters in this book bring together teams of expert authors from all parts of the globe. As the editors, we have been fortunate to personally witness many pivotal advances during our scientific careers. It is therefore fitting that we should assemble these findings to share with a larger audience. Thus, editing this book represents our desire to capture and review current and past exciting discoveries on human viral oncology for colleagues and students.

A few important scientific concepts that are detailed in the various chapters include the epidemiology of human viral-induced cancers, cofactors for viral transformation, the multistepped process of viral transformation, and the common and varying mechanisms used by different types of viruses. Through such mechanistic understanding, we hope that safer and more effective therapeutics and additional effective cancer virus vaccines (such as the one developed for HPV) will ensue in the future. The latter subject areas are not major areas of focus for this volume; however, in future editions, we plan to expand in these directions.

This book will be of interest to graduate students, medical students, and to anyone engaged in the study of oncogenic viruses, their molecular biology, evolution, epidemiology, pathologic potential, and cancer research. It is our hope that this book provides information that helps better understand the pathogenesis of human cancers, particularly those that are associated with viruses, and assists in the development of more customized strategies toward cancer treatment and prevention.

An undertaking such as this book could not be accomplished without the combined efforts of many individuals. We are particularly grateful to the authors who have contributed thoughtfully written chapters, and we are indebted to Thomas H. Moore and Ian Collins at Wiley, and to Sandy Weiss who has provided us with excellent logistics and editorial assistance. The publication of this book is also accompanied by the development of an annual meeting on human oncogenic viruses organized by the International Committee on Viral Oncology Research (ICVOR).

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1

HUMAN PAPILLOMAVIRUS-ASSOCIATED CANCERS

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INTRODUCTION—HUMAN PAPILLOMAVIRUS (HPV) TYPES AND HPV LIFE CYCLE

HPV is a small, nonenvelope, double-stranded DNA virus of the Papillomaviridae family. Different HPVs are defined by their genotypes, with more than 100 described to date. HPV genotypes are clustered into genera (de Villiers et al., 2004) based on genetic relatedness. Within each genus, HPVs are categorized into species based on distinct genotypes; members of a species have similar biological properties or phenotypes. Differences of 10% or more in the viral capsid gene (L1) are noted as different HPV types, differences of 2%–10% as subtypes, and differences of less than 2% are variants (Fig. 1.1) (de Villiers et al., 2004; Doorbar, 2006).

The two main genera of HPV are the alpha and beta papillomaviruses, with nearly 90% of typed HPVs falling within these groups (Fig. 1.1). Genus alpha papillomaviruses are associated with genital and mucosal infections, although a few infect cutaneous epithelium. Genus alpha HPVs are further defined as high-risk or low-risk based on their association with anogenital cancers or benign genital warts, respectively. The genus beta papillomaviruses are associated with benign skin infections; however, in immunocompromised patients or patients with epidermodysplasia verruciformis (EV),

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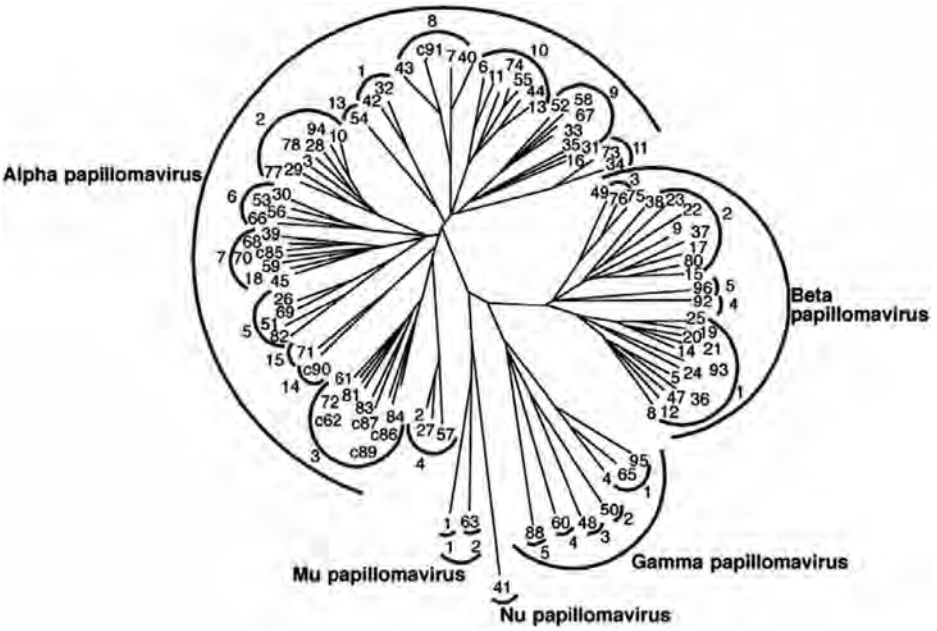

Figure 1.1. HPV cladogram. HPV types are categorized based on their genetic similarities. The genus alpha papillomaviruses primarily infect mucosal epithelium, and the genus beta papillomaviruses primarily infect the skin (figure from Doorbar, 2006).

a rare genetic disorder caused by mutations in the *EVER1* or *EVER2* genes (Ramoz et al., 2002; Keresztes et al., 2003; Kurima et al., 2003), squamous cell carcinomas (SCC) can develop.

HPV requires actively dividing and differentiating epithelium, typically the basal layer of stratified squamous epithelium, for its DNA replication, gene expression, and protein coat production. HPV reaches basal cells through epithelial microabrasions or at sites where the epithelium transitions from a monolayer to stratified squamous cells, such as at the cervical transformation zone or the anal verge (Fig. 1.2). Although these areas are more accessible to the virus for initial infection, they are poorer sites for viral production. This makes these anatomical transition areas sites where abnormal and inadequate viral gene expression, and ultimately HPV genome integration, are likely to occur. With HPV genome integration, regulatory viral genes may be lost, and epithelial cells are driven to immortalization, which can progress to cancer.

EPIDEMIOLOGY OF HPV INFECTIONS AND CANCER

Genus Alpha HPV Infection in Women

HPV is the most common sexually transmitted infection (STI). Seventy-five percent of men and women in the United States have evidence of a current or prior genus alpha

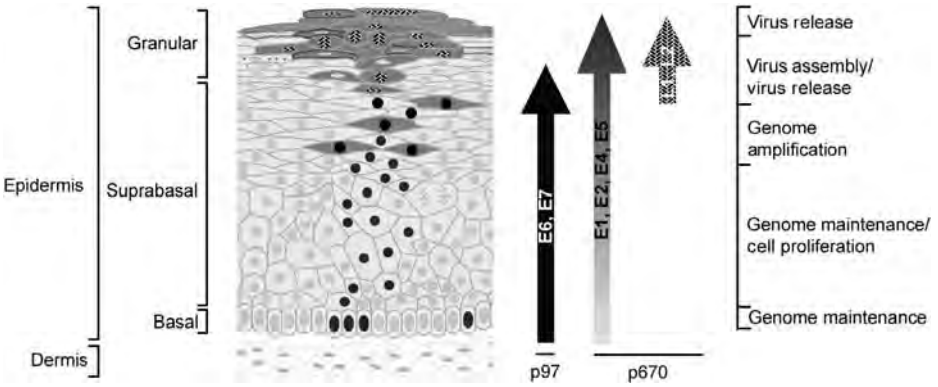

Figure 1.2. HPV life cycle. HPV infects the basal layer of stratified squamous epithelium. E6 and E7 drive continued proliferation as cells differentiate in the suprabasal layers and other early genes increase HPV gene expression and genome amplification. In the epithelial granular layers, the late L1 and L2 proteins are expressed, forming infectious virions that are released through desquamation (figure adapted from Doorbar, 2006).

TABLE 1.1. HPV-Associated Cancers

Cancer Type	HPV+ (%)
Cervical squamous cell carcinoma[1–3]	89.3–99.7
Cervical adenocarcinoma[1]	81.8
Vulvar[1]	89
Vaginal[1]	90.7
Anal[1,4]	84–93.8
Penile[1,5]	40–81.8
Head and neck[6]	25
Oropharynx/tonsil[7,8]	43.6–72

Note: The majority of anogenital cancers are associated with alpha genus HPV infections, and a significant subset of head and neck cancers are also associated with HPV.
[1]Carter et al. (2001).
[2]Munoz et al. (2003).
[3]Walboomers et al. (1999).
[4]Frisch et al. (1997).
[5]Gross and Pfister (2004).
[6]Gillison et al. (2000).
[7]Schwartz et al. (1998).
[8]D'Souza et al. (2007).

infection by serology, HPV DNA or RNA testing, cervical dysplasia, or genital warts (Koutsky and Kiviat, 1999). High-risk HPV infection has been identified as the key to cervical intraepithelial neoplasia and cancer (Table 1.1) (Durst et al., 1983; Koutsky et al., 1992; Munoz et al., 1992, 2003; Walboomers et al., 1999); however, most

high-risk infections do not become cancerous, so high-risk HPV is required but not sufficient for cancer progression.

Several natural history studies have been conducted to document the frequency and length of HPV infection in sexually active female adolescents and adults (Burk et al., 1996; Ho et al., 1998; Moscicki et al., 2001; Woodman et al., 2001; Richardson et al., 2003; Winer et al., 2003, 2005). A prospective longitudinal study of female university students found that with each new lifetime partner, the risk of HPV acquisition rose: 3% for virginal women, 7% for women with one lifetime partner, 33% for women with two to four partners, and 53% for women with five or more partners (Burk et al., 1996). Moscicki et al.'s (2001) longitudinal study of female adolescents found that their risk of HPV infection was also directly associated with the number of new partners as well as evidence of other STIs on exam. Winer et al. (2003) found that cumulative incident HPV infections in university women was 32.3% by 24 months, which is consistent with other studies (Ho et al., 1998; Richardson et al., 2003; Winer et al., 2003). Woodman et al. (2001) found a cumulative incidence of HPV infection in 15- to 19-year-olds at 44% by 36 months and 60% at 5 years.

In university women, new partners and smoking were risk factors for HPV acquisition (Winer et al., 2003), and the younger a female is at coitarche, the more likely she is to become infected with HPV (Kahn et al., 2002). Several studies have found that males' behaviors impact HPV infection in their female partners. The more lifetime partners a male has can increase the risk of HPV infection in a female partner; the briefer the relationship between a male and female can also increase the risk (Burk et al., 1996; Ho et al., 1998; Winer et al., 2003). So, the behavior of college-aged women and adolescents, as well as their partners, is an important risk factor for HPV infection. Young age at sexual debut, multiple partners, short relationships, smoking, and presence of other STIs all put teenagers and young women at risk for HPV.

The transition from a documented HPV infection to a low-grade squamous intraepithelial lesion (LGSIL) Pap smear is directly associated with a persistent infection as well as daily cigarette smoking in women (Moscicki et al., 2001). In a study of university women who had an HPV infection, nearly half of them developed cervical squamous intraepithelial lesions (SILs) and more than a quarter developed vaginal SILs within 36 months of their infection (Winer et al., 2005). These SILs regressed within 4.7–5.5 months on average (Winer et al., 2005).

LGSIL in adolescents also typically regress: 91% within 3 years (Moscicki et al., 2004). Women typically require a referral for colposcopy with the diagnosis of LGSIL; however, as adolescents and young women up to 21 years old have a greater likelihood of clearance of their HPV infection and regression of clinical disease, management of atypical cells of undetermined significance (ASCUS) and LGSIL Pap smears has been modified accordingly (http://www.asccp.org/consensus/cytological.shtml) (Wright et al., 2007). In contrast, older women are less able to clear infections and are more likely to have abnormal Pap smears, including high-grade squamous intraepithelial lesions (HGSIL) (Herrero et al., 2000; Castle et al., 2005), perhaps due to their relatively poor immune response to HPV infections (Garcia-Pineres et al., 2006).

Since the implementation of Pap smears for all sexually active women in the United States, rates of cervical cancer have fallen three-quarters since the 1950s. Within the

past 30 years, cervical cancer incidence has decreased from 14.2 per 100,000 in 1973 to nearly 3 per 100,000 in 1998, with a goal of 2 per 100,000 in Healthy People 2010 (http://www.ahrq.gov/clinic/3rduspstf/cervcan/cervcanrr.htm). However in the United States, women are still diagnosed with cervical cancer and die from this disease. In 2003, 11,820 women were diagnosed with cervical cancer and 3919 women died from it (U.S. Cancer Statistics Working Group, 2006). This makes understanding the infection and progression of disease critical to continued diagnosis and treatment.

More than 95% of cervical cancers contain high-risk HPV DNA, and HPV is also present in the majority of vulvar and vaginal cancers (Table 1.1) (Walboomers et al., 1999; Carter et al., 2001; Munoz et al., 2003). As the anal verge is similar to the cervix in transitioning from stratified squamous epithelium to columnar cells, HPV can also infect these cells and is associated with anal cancer in women. The rate of female anal cancer is increasing, likely representing a change in women's sexual behaviors (Frisch et al., 1993, 1999).

Genus Alpha HPV Infection in Men

HPV infection in men has been less well studied. HPV has been detected in 42%–80% of penile cancers (Table 1.1) (Gross and Pfister, 2004; Daling et al., 2005), the rate of which may reflect a greater difficulty of sampling for HPV from the penis, or the overlap of two distinct cancer types, one due to high-risk HPV infection and one not (Bleeker et al., 2006; Micali et al., 2006; Partridge and Koutsky, 2006). When compared with women, men have a less good antibody response to infection, which may be due to lower viral load or faster clearance of infection (Partridge and Koutsky, 2006). It has been shown that circumcision is protective against HPV acquisition in men (Hernandez et al., 2008), as well as cervical cancer in their wives (Castellsague et al., 2002); when circumcised, the amount of columnar epithelium on the glans of the penis is reduced, making it more difficult for HPV to infect.

Like in women, anal cancer in men is associated with HPV infection and is increasing in incidence (Table 1.1) (Frisch et al., 1993, 1997, 1999; Daling et al., 2004). Unlike women, men who have sex with men have a high (over 50%) and persistent HPV infection rate regardless of age (Chin-Hong et al., 2004), as well as a high and persistent level of abnormal anal cytology (Chin-Hong et al., 2005). The high level of infection and abnormal cytology put men who have sex with men at greater risk for anal cancer when compared with women and cervical HPV infections.

Patients who are human immunodeficiency virus (HIV) positive or have acquired immune deficiency syndrome (AIDS) are at increased risk for abnormal cytology on anal cytology (Palefsky et al., 1998; Frisch et al., 2000; Sobhani et al., 2004). Although regular screening for men who have sex with men has not been universally recommended, studies have shown screening programs for anal cytology similar to cervical Pap smears to be reliable and potentially helpful (Cranston et al., 2004; Mathews et al., 2004).

Genus Alpha HPVs and Head and Neck Cancers

Head and neck cancers are associated with genus alpha HPV infections (Table 1.1), although this is a weaker correlation than with anogenital cancers (Fakhry and Gillison,

2006). Also, although tobacco and alcohol use are risk factors for non-HPV-associated head and neck cancers, this is not true in HPV-associated head and neck cancers (Fakhry and Gillison, 2006; D'Souza et al., 2007; Gillison et al., 2008). HPV appears to be important, specifically in oropharyngeal cancers, such as the tonsils and the base of the tongue. The tonsils mimic the cervix and the anal verge as sites of metaplastic epithelium, making them an appropriate site for HPV infection. Studies have shown patients with anogenital cancers have a two-and-a-half- to fourfold increased risk of tonsillar cancer, and their partners are at increased risk of tonsillar cancer or cancer of the tongue (Frisch and Biggar, 1999; Hemminki et al., 2000).

A case-control study of patients with oropharyngeal cancer showed sexual behavior as a primary risk factor; a high lifetime number of vaginal-sex partners or oral-sex partners was associated with cancer (3.1 and 3.4 odds ratio, respectively) (D'Souza et al., 2007). Oral infection with high-risk HPV types were strongly associated with oropharyngeal cancer (12.3 odds ratio) (D'Souza et al., 2007). Again, understanding sexual behaviors is critical for predicting changes in oral cancer epidemiology, just as seen in anal cancer incidence.

Genus Beta HPVs and Skin Cancer

Nonmelanoma skin cancer is the most common cancer in the United States, with more than 1 million cases diagnosed annually (http://www.cancer.gov/cancertopics/types/skin). Nonmelanoma SCC accounts for 20%–30% of skin cancers, and SCC has been associated with genus beta HPV infection on sun-exposed skin. This cancer rate is even higher among two clusters of patients: organ transplant patients who are on immunosuppressive therapy and EV patients. Patients with EV develop lifelong warty lesions, 25% of which convert to SCC in sun-exposed areas (Orth et al., 1978, 1979; Ostrow et al., 1982; Orth, 2006). Genus beta HPVs are ubiquitously found in skin and hair follicle samples (Orth, 2006) and typically lead to benign lesions. However, the increasing number of transplant patients makes this infection a concern for future patients. The mechanism of genus beta HPVs leading to cancer is different than genus alpha HPVs and cancers, and it is the focus of ongoing research.

HPV EARLY AND LATE GENE EXPRESSION

The mechanism by which HPV binds to and enters basal epithelial cells is not known entirely, although both binding to laminin 5 and glycosaminoglycans are likely important (Joyce et al., 1999; Selinka et al., 2002; Culp et al., 2006). Once the virus infects cells, the HPV coat is removed and the HPV genome enters the nucleus. All HPV genomes have at least two promoters named early and late for their timed expression during the viral life cycle (Fig. 1.3). The E6 and E7 genes (designated E for early) are expressed from the early promoter (p97), and the E1, E2, E4, and E5 genes are expressed from the late promoter (p670) (Fig. 1.2) but utilize the early polyadenylation site. The early open reading frames drive viral DNA replication and expression, as well as dysregulate the normal epithelial cell cycle for the benefit of HPV viral production.

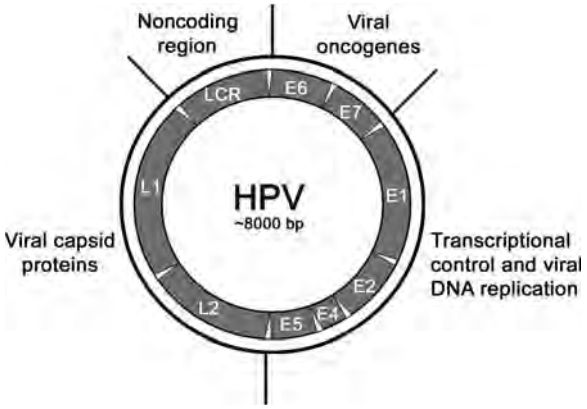

Figure 1.3. HPV genome. HPV is a double-stranded DNA virus with early (E) and late (genes) designated for their expression during the HPV life cycle. LCR=long control region.

The E2 protein has several critical roles in HPV genome expression and replication. It is expressed early in the viral life cycle and is found in basal and suprabasal layers of stratified squamous epithelium infected by HPV. E2 binds as a dimer to DNA and recognizes the palindromic motif AACCg(N_4)cGGTT in the noncoding region of the HPV genome 5′ of the early promoter (Hines et al., 1998; Masterson et al., 1998; Stubenrauch et al., 1998; Dell et al., 2003). E2 recruits the HPV viral helicase E1 to the viral origin and increases the DNA-binding affinity to the noncoding region (Masterson et al., 1998; Sun et al., 1998; Conger et al., 1999; Titolo et al., 2003). Both E1 and E2 together utilize cellular machinery for DNA replication and transcription (Masterson et al., 1998; Conger et al., 1999; Muller et al., 2002; Clower et al., 2006a,b). Although E2 is a transcriptional activator at low concentrations, high levels of E2 repress expression of E6 and E7 from the late promoter (Steger and Corbach, 1997; Francis et al., 2000). Finally, E2 also functions to segregate the HPV genome as cells divide by tethering the genome to cellular chromosomes during mitosis (You et al., 2004; McPhillips et al., 2005, 2006; Oliveira et al., 2006).

The E4 protein is found in the suprabasal and granular layers of stratified squamous epithelium. Without a functional E4 protein, HPV episomal DNA cannot amplify from their initial 50–100 copies per cell to the several thousands normally seen (Wilson et al., 2005).

E5 protein has effects on both cellular transformation and viral genome amplification. Although HPV E5 has little effect on monolayer undifferentiated keratinocytes *in vitro*, E5 does increase the number of suprabasal cells dividing in organotypic cultures grown to mimic stratified squamous epithelium (Genther et al., 2003). Additionally, in differentiated keratinocytes, E5 induces HPV genome amplification. HPV 16E5 can cause epithelial hyperplasia, abnormal cellular differentiation, and skin tumors when expressed in mice. This effect occurs through the epidermal growth factor receptor, although this may not be consistent across all HPV types (Fehrmann et al., 2003; Genther et al., 2005).

The viral coat proteins L1 and L2 are expressed from the late promoter after a change in splicing patterns and a transition to the late polyadenylation site. Three hundred sixty L1 proteins organize into 72 capsomers (Modis et al., 2002), with one L2 protein associated with each pentavalent capsomer (Trus et al., 1997). The capsomers self-assemble without HPV DNA *in vitro* as viruslike particles (VLPs) and in the cellular nucleus *in vivo* to encapsulate the HPV genome into infectious virus particles.

GENUS ALPHA HPV ONCOGENES

E6 and E7 expression is normally regulated by E2. In persistent HPV infections, HPV viral DNA can integrate into cellular DNA, and through this integration, the E2 gene is typically lost (Jeon et al., 1995). Unchecked, E6 and E7 mRNA and protein levels increase (Jeon and Lambert, 1995; Jeon et al., 1995), and this drives cell cycle dysregulation, cellular transformation (Munger et al., 1989), and immortalization (Hawley-Nelson et al., 1989). Cells with integrated high-risk HPV DNA grow faster, and for more populations doublings in culture, than do cells with extrachromosomal HPV DNA (Jeon et al., 1995). In transgenic mice expressing the early genes of HPV 16 that are exposed to estrogen, reproductive tract tumors develop (Arbeit et al., 1996; Elson et al., 2000; Riley et al., 2003). When high-risk E6 and E7 are coexpressed in epithelial cells in culture, they become immortalized (Kiyono et al., 1998), and when E6 and E7 expression is reduced, cervical cancer cell line's growth is arrested and tumor formation in nude mice is blunted (von Knebel et al., 1992; Francis et al., 2000). Both the E6 and E7 genes push the oncogenic potential of epithelial cells through their dysregulation of cell cycle progression, senescence, and apoptosis.

Genus Alpha HPV E6 Oncogene

The E6 protein is critical to the HPV life cycle (Fig. 1.4). Mouse models have been used to document the oncogenic potential of the genus alpha E6 gene. Transgenic mice expressing HPV 16E6 in their epithelium have both a promotion and a progression of skin tumors when compared with nontransgenic mice (Simonson et al., 2005). When mice expressing HPV 16E6 are exposed to estrogen, they develop cervical dysplasias, and with prolonged exposure to estrogen, they can develop cervical cancers (Shai et al., 2007a). This is strong evidence that the high-risk HPV E6 is an oncogene.

E6 binds an endogenous E3 ubiquitin ligase, E6-associated protein (E6-AP), found in epithelial cells (Huibregtse et al., 1991, 1993). E6 and E6-AP bind the critical cell cycle protein p53 and target it for degradation by the 26S proteasomal pathway (Scheffner et al., 1993), and there is evidence that HPV 16E6 itself can blunt the effect of p53 without E6-AP (Shai et al., 2007b). When p53 is lost, epithelial cells no longer recognize senescence or apoptosis signals and continue to divide throughout the stratified squamous epithelial layers. This is key to HPV production, but moreover, this is critical to the immortalization of epithelial cells and the stochastic collection of genetic and epigenetic mutations that lead to cancer.

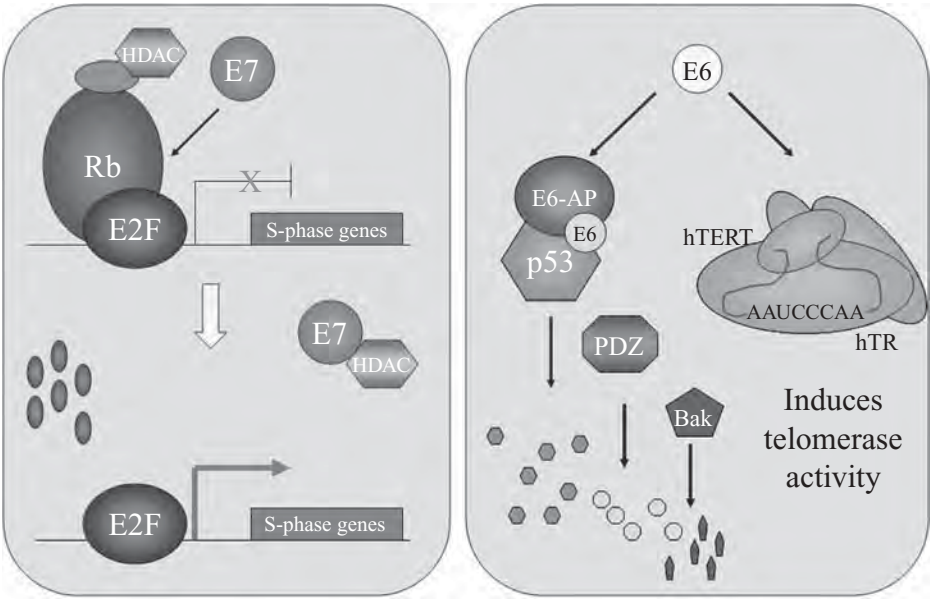

Figure 1.4. High-risk E6 and E7 roles in epithelial cells. E7 targets retinoblastoma protein, and other pocket proteins, for ubiquitin-mediated proteasomal degradation, allowing E2F to transactivate S-phase genes. E6 targets p53, PDZ domain-containing proteins, and Bak for degradation as well as activates the expression of hTERT, the catalytic subunit of telomerase. HDAC=histone deacetylase, a transcriptional repressor.

A second key role of high-risk HPV E6 in cell cycle dysregulation is the activation of telomerase (Fig. 1.4). Telomerase is a ribonucleoprotein that includes its catalytic subunit hTERT, its RNA component hTR, and dyskerin (Kilian et al., 1997; Meyerson et al., 1997; Nakamura et al., 1997; Counter et al., 1998; Cohen et al., 2007). Telomerase extends the repetitive telomeric DNA that caps the ends of chromosomes and protects genes within the chromosome from serial erosion with each round of DNA replication. Telomerase is normally expressed in stem cells, and telomerase activity is proportionate to the expression level of hTERT (Counter et al., 1998). In differentiated cells, hTERT expression is repressed. As these differentiated cells divide, their telomeres shorten; the age of a cell is therefore reflected in the inverse length of its telomeres. When the telomeres of a cell become critically shortened, it signals for that cell to go through senescence or apoptosis. In most cancers, telomerase is activated, so its expression is critical to the immortalization of cells and to oncogenic progression (Shay and Bacchetti, 1997).

E6 and E6-AP activate expression of hTERT in epithelial cells through transcriptional activation at the promoter (Gewin and Galloway, 2001; Gewin et al., 2004; Galloway et al., 2005; Liu et al., 2005). Additional regulators of hTERT that interact with E6 and E6-AP have been identified, such as c-myc, Mad, Max, NFX1-91, and NFX1-123 (Wang et al., 1998; Takakura et al., 1999; Wu et al., 1999; Oh et al., 1999,

2001; Veldman et al., 2001, 2003; McMurray and McCance, 2003; Gewin et al., 2004; Katzenellenbogen et al., 2007). Each of these endogenous protein partners is important in hTERT activation during HPV infection and HPV genome integration.

The loss of p53 and activation of telomerase are the two critical events driven by E6. These allow differentiated epithelial cells to continue to divide when they otherwise would not. They no longer recognize signals from DNA damage through the p53 pathway to go through apoptosis or senescence, and they no longer recognize their age as telomerase extends the ends of chromosomes. Loss of p53 and activation of telomerase are required in most cancers, so the role of E6 in oncogenic progression of HPV cancers is intuitive.

High-risk E6 proteins have roles beyond p53 degradation and telomerase activation (Fig. 1.4). High-risk E6 proteins have a four-amino-acid domain at their extreme C-terminus. This domain is required to bind proteins that contain a PDZ domain, such as *P*SD-95, *D*lg (disk large protein), and *Z*O-1 (hence the name PDZ), as well as human Scribble, MUPP-1, and MAGI-1, 2, and 3. These PDZ proteins are found primarily in the apicobasal region of epithelial cells, and their interaction with and degradation by E6 may be important in HPV-driven cell growth, metaplasia, malignant progression, and metastatic disease (Dobrosotskaya and James, 2000; Glaunsinger et al., 2000; Thomas et al., 2002; Watson et al., 2003; Lee and Laimins, 2004; Massimi et al., 2004). Transgenic mice expressing HPV 16E6 with the PDZ-binding domain deleted are unable to develop epithelial hyperplasia, induce DNA synthesis, or promote papillomas when compared with HPV 16E6 wild-type (WT) mice (Nguyen et al., 2003; Simonson et al., 2005). However, once tumors do develop, 16E6 WT and PDZ-binding-domain-deleted mice have equal numbers of carcinomas (Simonson et al., 2005). So, the PDZ-binding domain of high-risk E6 proteins may be critical to the initial steps of oncogenesis. Other PDZ-domain-containing proteins have been identified as targets for degradation by high-risk E6. E6-AP is required for the degradation of some PDZ-containing proteins, such as Scribble and Dlg4/SAP97 (Nakagawa and Huibregtse, 2000; Handa et al., 2007), but perhaps not others (Pim et al., 2000; Grm and Banks 2004; Grm et al. 2004), although there is conflicting data on this (Brimer et al., 2007; Kuballa et al., 2007). Finally, E6 and E6-AP can bind other cellular regulators such as Bak, a protein in the apoptosis cascade, and target it for degradation like p53 (Thomas and Banks, 1998). Therefore, the tumorigenic potential of high-risk E6 involves other cellular proteins beyond p53 and hTERT.

Genus Alpha HPV E7 Oncogene

Like E6, E7 expression is normally regulated by E2. Also, like E6, high-risk E7 is an oncogene (Fig. 1.4); keratinocytes expressing HPV 16E7 can become immortalized in culture, although coexpression of HPV 16E6 accelerates this process (Halbert et al., 1991). Transgenic mice that express the HPV 16E7 protein in their epithelium develop skin hyperplasia and, late in life, skin tumors (Herber et al., 1996). Those mice that express E7 at a high level can have stunted growth and early mortality from the severe epithelial hyperplasia of the esophagus causing dysphagia (Herber et al., 1996). When these same mice are exposed to estrogen, they quickly develop tumors

of the reproductive tract (Riley et al., 2003). All of these tissue culture and mouse model studies are strong evidence that high-risk E7 is an oncogene, driving epithelial cell proliferation and cancer.

E7 functions in tandem with E6 to drive differentiated epithelial cells to continue to divide. E7 is known to bind to many endogenous cell cycle proteins, including pocket proteins retinoblastoma protein (Rb), p107 and p130 (Dick and Dyson, 2002; Zhang et al., 2006), and cyclin-dependent kinase inhibitors p21 and p27 (Funk et al., 1997; Helt et al., 2002). The interaction of E7 and Rb has been the most studied of these interactions (Fig. 1.4). E7 degrades Rb through the ubiquitin-mediated proteasomal pathway (Boyer et al., 1996; Helt and Galloway, 2001; Dick and Dyson, 2002), and the degradation of Rb by E7 allows the transcription factor E2F to activate expression from S-phase promoters, driving DNA replication and cellular division. Transgenic mice expressing HPV 16E7 and a knocked-out Rb that cannot be bound by E7 have little skin hyperplasia, have a normal cell cycle arrest to DNA damage, and do not increase p21 protein levels (Balsitis et al., 2005). However, transgenic mice expressing HPV 16E7 and treated with estrogen develop cervical cancers whether or not Rb can be degraded (Balsitis et al., 2006). Therefore, the ability of high-risk E7 to degrade Rb is more important in skin tumorigenesis than in cervical cancer in mouse models. Other pocket proteins are bound by E7, and those interactions may be as important in the HPV life cycle as they are in oncogenic progression (Zhang et al., 2006). Finally, high-risk E7 can increase expression of DEK, a senescence inhibitory protein, which may be critical in HPV malignant progression (Wise-Draper et al., 2005).

High-risk E7 affects gene expression through its interaction with transcription factors and also chromatin remodeling proteins. HPV 16E7 can bind several factors from the AP1 family of transcription factors, which are likely important in driving the cell cycle (Antinore et al., 1996). E7 can bind to histone deacetylases (HDAC) (Brehm et al., 1999; Longworth et al., 2005), and this can allow continued cell growth (Brehm et al., 1999) and HPV DNA replication (Longworth et al., 2005) (Fig. 1.4). Combined, E7 can drive differentiated cells to continue to divide, activate genes normally quiescent in differentiated cells, and ignore DNA damage signals.

GENUS BETA HPV ONCOGENES

Like genus alpha HPV E6 and E7, genus beta HPV E6 and E7 have been increasingly identified as oncogenes. However, unlike genus alpha oncogenes in anogenital cancers, skin cancers do not universally have evidence of E6 or E7 DNA, and in an HPV-positive skin cancer, not all cells would have HPV DNA. So, a continued effect by genus beta HPV is not required in SCC development, and the cancer is not a clonal outgrowth from a genus beta HPV-positive cell, making their malignant progression fundamentally different from the classic high-risk HPV types. Despite these differences, genus beta HPV E6 and E7 are important in SCCs, especially in immunocompromised hosts, blocking apoptosis and activating telomerase. Their study is becoming increasingly important as the population at risk, including solid organ and bone marrow transplant patients, grows.

HPV 38 E6 and E7 expressed together in primary epithelial cells can drive DNA replication and the cell cycle, block senescence by Ras, and induce anchorage independence in fibroblast cultures, all of which point to transforming properties of beta HPV oncogenes (Caldeira et al., 2003). Organotypic epithelial cultures grown with several beta HPV E6 and E7 types show decreased differentiation of skin cells and basal cells within the suprabasal region (Boxman et al., 2001). Transgenic mice expressing the early genes of HPV 8 in the dermis develop skin tumors, and a subset develops SCC (Schaper et al., 2005), pointing to their importance in skin cancer progression.

Genus Beta HPV E6 Oncogene

Genus alpha HPV E6 binds E6-AP to degrade p53 and block apoptotic signals from DNA damage. Genus beta HPV E6 does not affect p53 protein levels, but instead, it targets proteins downstream of p53 for degradation. In the skin, the most common DNA damage seen is ultraviolet (UV) irradiation on sun-exposed skin; therefore, studies focus on this effect. In keratinocytes, p53 and p21 are induced after UV irradiation, as is the proapoptotic protein Bak. p53 activates the cascade of proapoptotic signals that lead to caspase release from mitochondria and cell death. In cultured and organotypic cells expressing genus beta HPV E6 types, p53 and p21 expression is increased after UV irradiation, but Bak is not induced. These cells do not undergo apoptosis but continue to divide despite double-stranded DNA damage (Jackson et al., 2000). Also, studies of HPV 77 E6 show disruption of proapoptotic gene activation by p53 after UV irradiation in epithelial cells (Giampieri et al., 2004). p53 levels may be important in E6 expression, as p53 can bind the HPV 8 E6 promoter, and it directly competes with HPV 8 E2 for binding and regulation of expression (Akgul et al., 2003). New evidence also points to a subset of genus beta E6 proteins that can activate telomerase and extend the life span of epithelial cells in culture (Bedard et al., 2008).

Genus Beta HPV E7 Oncogene

The genus beta E7 protein can function like genus alpha E7, triggering continued cellular proliferation while epithelial cells are terminally differentiating. Several genus beta E7 proteins have been reported to bind to Rb, and a subset of those can target Rb for degradation, although not all. However, in addition to the known cell cycle effects of E7, keratinocytes expression HPV 8 E7 protein can degrade the basement membrane, induce matrix metalloproteinase expression, and invade stromal tissues in organotypic cultures (Akgul et al., 2005; Smola-Hess et al., 2005). So, E7 alone may drive the oncogenic and metastatic potential of beta HPV-infected skin cells and push infected cells toward invasion even before collecting DNA damage from UV exposure.

FUTURE ISSUES

Although behavior modifications can decrease HPV infection rates, and consistent screening for and treatment of cytological abnormalities can decrease cancer rates and deaths, prophylactic vaccination against specific HPV types has the potential to

eliminate a significant burden of disease. The transition from HPV infection to anogenital cancers can take decades to occur, so the full impact of prophylactic vaccination will likely take a generation to be seen.

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2

MOLECULAR EVENTS ASSOCIATED WITH HUMAN PAPILLOMAVIRUS-INDUCED HUMAN CANCERS

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INTRODUCTION

Papillomaviruses have been isolated from a wide range of species, including humans. Their ~8-kb double-stranded DNA genomes are packaged into 55-nm nonenveloped particles. Their genomes can be divided into three regions: an early region, which encodes nonstructural proteins; a late region, which encodes the two viral capsid proteins; and a noncoding region (NCR). The early region open reading frames (ORFs) are designated "E" followed by a numeral; the late region ORFs bear the designation "L" followed by a numeral (Fig. 2.1). The numeral designates the relative length of the ORF; the lower the number, the longer the ORF. Although the third region is noncoding, it contains regulatory sequences, including the viral origin of replication and binding sites for cellular and viral transcription factors that control viral gene expression (reviewed in Howley and Lowy, 2007). This region is referred to in the literature as the NCR, "upstream regulatory region" (URR), or "long control region" (LCR). In this review, we will use the designation NCR. Only one of the two DNA strands is used for transcription of viral mRNAs. The viral ORFs are encoded within each of the three possible reading frames, and some are overlapping. Some early viral mRNAs encode proteins that are composed of portions of multiple ORFs through complex splicing

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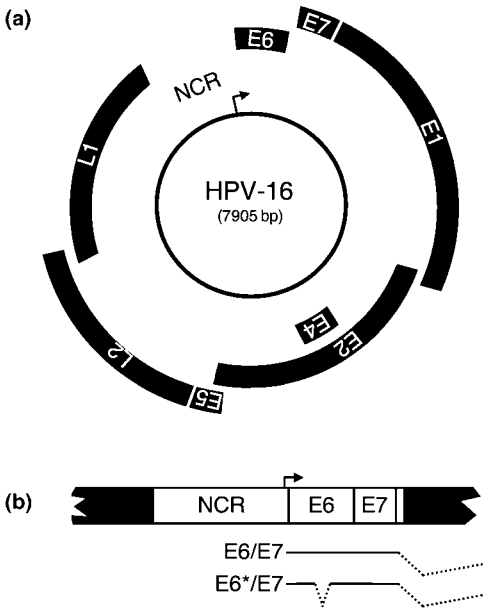

Figure 2.1. (a) Schematic representation of the HPV16 genome. The double-stranded circular DNA genome is represented by the central circle. Early (E) and late (L) genes are indicated. The major early promoter in the noncoding region (NCR) is represented by an arrow. See text for details. (b) Structure of the minimal HPV16 genome region retained after integration into a host chromosome. The major E6/E7 transcripts that are consistently expressed after viral integration are indicated. See text for details.

patterns (reviewed in Zheng and Baker, 2006). The early viral mRNAs encode regulatory proteins that reprogram the host cell to support the viral life cycle. Two early proteins, E1 and E2, are directly involved in viral genome replication. E1 is the viral origin binding protein, forms hexamers, and has ATPase and DNA helicase activity (reviewed in Stenlund, 2007). The E2 protein is a transcriptional regulator that binds $ACCN_6GGT$ sequences in the viral NCR. The full-length E2 protein forms dimers and can act as a transcriptional activator or repressor based on the context of its binding sites. E2 is composed of an amino-terminal transcriptional activation domain, a flexible hinge region, and a DNA-binding domain at its carboxyl-terminus. Some papillomaviruses encode multiple spliced and internally initiated versions of E2, which generally function as transcriptional repressors (reviewed in McBride et al., 1991). E2 is also necessary for viral replication; it associates with E1, and the E1/E2 complex associates with the viral origin. The origin sequence is flanked by E2-binding sites, and this arrangement ensures that the E1/E2 complex is efficiently bound to the viral origin (reviewed in Stenlund, 2007). E2 also plays an important role in viral genome segregation, and thus viral persistence; the amino-terminal E2 domain associates with mitotic host cell chromosomes through a protein/protein interaction, whereas the carboxyl-terminal domain associates with E2-binding elements on the viral genome. This results

in the segregation of viral genomes tethered to mitotic chromosomes during cell division (reviewed in McBride et al., 2006). A number of candidate cellular E2-binding proteins that mediate viral genome segregation have been reported (You et al., 2004; Parish et al., 2006). Alternative modes of papillomavirus genome segregation, whereby E2 associates with microtubules, have also been observed for some papillomavirus types (Van Tine et al., 2004). Other early papillomavirus proteins, including E6 and E7, contribute to the viral life cycle by ensuring the availability of cellular replication enzymes, which are critical for the synthesis of viral progeny. The late genes, L1 and L2, encode the viral major and minor capsid proteins, respectively. Some early genes, including E2 and E4, also importantly contribute to the productive phase of the viral life cycle (reviewed in Howley and Lowy, 2007).

Even though papillomaviruses have been isolated form a wide range of hosts, they are highly species specific. In addition, these viruses display a pronounced tropism for squamous epithelia.

THE HUMAN PAPILLOMAVIRUS (HPV) LIFE CYCLE—CHALLENGES AND IMPLICATIONS FOR CELLULAR TRANSFORMATION

The papillomavirus life cycle is coupled to the differentiation state of the infected epithelial cell. The skin is the largest organ of the human body and is constantly turned over. It contains a single layer of dividing cells, the basal cells. Basal cells undergo asymmetric mitosis; one daughter cell remains a basal cell, whereas the other daughter becomes a suprabasal cell. The suprabasal cell withdraws from the cell division cycle and undergoes a program of terminal differentiation while it is being "pushed" toward the surface of the epithelium. This ensures the mechanical stability of the skin and shields the basal cells from direct exposure to environmental mutagens, including ultraviolet irradiation. The terminally differentiated, denucleated cell bodies are eventually sloughed off. Papillomaviruses need to infect basal cells to establish a stable infection. HPVs enter these cells through mechanisms that remain poorly studied (reviewed in Streek et al., 2007). After the initial infection, the genomes are maintained at a low copy number in basal cells, and a persistent infection is established. The production of infectious progeny virus, however, occurs in the terminally differentiated layers of the epithelium. In these cells, large numbers of viral genomes are produced, and the capsid proteins are synthesized. Papillomaviruses are nonlytic, and viral progeny is shed along with the terminally differentiated skin flakes. These viruses are remarkably stable and retain infectious properties over extended periods of time (reviewed in Lee and Laimins, 2007).

The mechanistic interplay between HPVs and epithelial host cells at specific phases of the viral life cycle, and how they may contribute to cellular transformation, are discussed in the following sections.

Infection of Basal Cells

In intact skin, basal cells are shielded by a layer of terminally differentiated cells that also contribute to the remarkable mechanical stability of this tissue. Basal cells,

however, can become accessible to papillomavirus infection due to abrasions and other mechanical trauma. Cutaneous HPVs are likely transmitted through such mechanisms. Basal-like cells are also particularly easily accessible at squamocolumnar transformation zones, and these regions are exceptionally vulnerable to mucosatropic papillomavirus infections. Squamocolumnar transformations zones contain reserve cells, which can give rise to squamous or columnar epithelia. It is thought that reserve cells are physiologically relevant targets for infection with these viruses (Smedts et al., 1992; Tsutsumi et al., 1993; Martens et al., 2004).

Persistent Infection of Basal Epithelial Cells

One of the most remarkable properties of some HPVs is their ability to establish long-term, persistent infections of squamous epithelial cells that can last for decades. As described above, a squamous epithelium represents a dynamic equilibrium of cell division in the basal layer, which replaces the terminally differentiated skin flakes that are shed once they reach the top of the epithelium. Consequently, there is constant, rapid cellular turnover in a squamous epithelium. Moreover, basal cells, where HPVs establish a persistent infection, undergo constant cell division. Since the nuclear membrane breaks down at the end of mitosis, viral episomes may be located in the cytoplasm after cell division, where they can be degraded. Similar to herpesviruses, which often establish lifelong latency (reviewed in Pellet and Roizman, 2007), HPV genomes are segregated with the host cellular chromosomes to the nuclei of daughter cells. The viral E2 protein plays an important role in viral genome segregation by tethering the viral genomes to the host mitotic chromosomes (Bastien and McBride, 2000). Several models have been put forward to explain how viral genomes may be segregated during cell division (reviewed in McBride et al., 2006). However, it is not clear whether genome segregation is equal between the two unequal daughter cells, which result during the division of a basal cell. If segregation were equal, there would be a need for a constant, low level of papillomavirus genome synthesis in an infected basal cell to ensure that viral genomes are retained in basal cells. Alternatively, genomes may be segregated asymmetrically so that they preferentially remain in the daughter cell that retains its basal cell characteristics. The latter model is more plausible, as it would require that only a limited set of viral genes be expressed during a prolonged, persistent infection. The proteins that are most likely to be expressed in this scenario would certainly include E2. Expression of a minimal set of viral proteins would decrease the likelihood that infected basal cells would be recognized and eliminated by the host's immune system. There is no experimental evidence to date, however, that supports either model.

Regardless of the mechanism that accounts for HPV genome maintenance in basal cells, it is conceivable that papillomaviruses infect basal cells that have stem cell-like properties (Martens et al., 2004). High-risk mucosal HPVs preferentially infect reserve cells in the squamocolumnar transformation zone, a primitive, bipotential epithelial cell type that can develop into columnar or squamous epithelial progeny (Smedts et al., 1992; Tsutsumi et al., 1993). Papillomaviruses may, preferentially, infect such cells based on surface marker expression. Alternatively, some papillomaviruses may encode

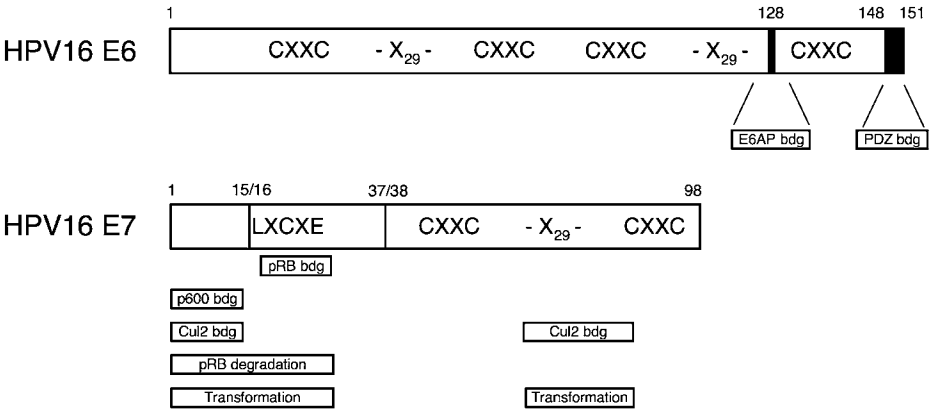

Figure 2.2. Schematic representations of the HPV16 E6 (top) and E7 (bottom) oncoproteins. Regions implicated in binding to the major cellular targets are highlighted. See text for details and references.

proteins that function to convert an infected cell to a more stemlike state. It was recently shown that altering the expression of a small number of genes that are epigenetically silenced is sufficient to convert normal human diploid cells to bonafide stem cells, which express telomerase (Takahashi et al., 2007; Yu et al., 2007). Interestingly, expression of the high-risk HPV E6 protein in primary human epithelial cells causes increased transcription of hTERT, the catalytic subunit of telomerase (Kiyono et al., 1998; Oh et al., 2001; Veldman et al., 2001), and coexpression of the E7 oncoprotein results in further activation of hTERT expression (Fu et al., 2003) (see Fig. 2.2).

Viral Genome Replication and Viral Progeny Synthesis in Terminally Differentiated Keratinocytes

With the exception of the E1/E2 complex, papillomaviruses lack essential enzymes for viral genome replication. Synthesis of viral progeny, which includes viral genome amplification and expression of late viral proteins, is restricted to terminally differentiated strata of the infected epithelium. Terminally differentiated cells, however, are intrinsically incompetent to support viral genome production. Consequently, papillomaviruses need to retain or reestablish a replication competent state in such cells (reviewed in Lee and Laimins, 2007). Many HPVs target the retinoblastoma tumor suppressor protein pRB and the related p107 and p130 proteins. These proteins are important cell cycle regulators; pRB controls G1/S-phase transition, whereas p130 appears to play a significant role in exit from quiescence (G0). Not all papillomaviruses, however, directly target pRB (reviewed in Münger et al., 2004).

The ability of HPVs to subvert cell cycle control is often regarded as key to the oncogenic activities of some HPV types. Given that low-risk HPVs are able to induce large hyperplastic lesions that commonly do not undergo malignant progression, it would appear that this model is too simplistic and needs to be revised. As described in

more detail below, there are important differences between how low-risk and high-risk HPVs target pRB and the related p107 and p130 proteins.

HPV-ASSOCIATED DISEASES AND CANCER

Approximately 200 HPVs have been characterized. Some HPVs preferentially infect cutaneous epithelia, whereas others target mucosal epithelia. As with all papillomaviruses, HPV infections give rise to epithelial hyperplasias. HPVs are divided into "low-risk" and "high-risk" HPVs based on the relative propensity for malignant progression of the lesions that they cause. Low-risk HPVs cause generally benign lesions, whereas lesions caused by high-risk HPVs are at some risk for malignant progression (de Villiers et al., 2004).

Cervical Carcinoma and Other Anogenital Tract Cancers

The concept of high-risk and low-risk HPVs has been particularly well established for mucosal HPVs. Low-risk mucosal HPVs, such as HPV6 and HPV11, cause genital warts, whereas high-risk mucosal HPVs, such as HPV16 and HPV18, cause squamous intraepithelial lesions (SILs) that are at risk for malignant progression. The vast majority of cervical cancers, as well as a fraction of other anogenital tract carcinomas, including vaginal, vulvar, and anal carcinomas, can be attributed to high-risk HPV infections (reviewed in zur Hausen, 2002; Schiffman et al., 2007) (see Fig. 2.3).

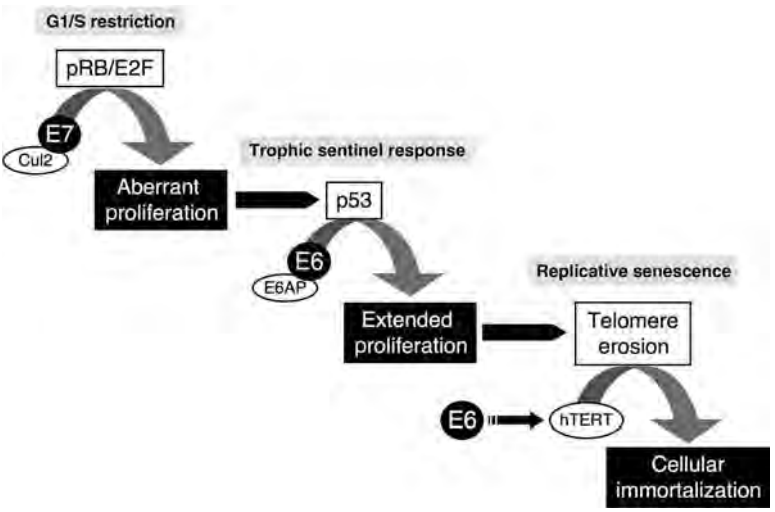

Figure 2.3. Simplified depiction of the major steps in HPV16-mediated cervical carcinogenesis. The steps illustrated here represent three of the five molecular alterations that have been shown to be minimally required for generating fully transformed cell lines *in vitro*. See text for details and references.

Malignant progression of a high-risk, HPV-associated, premalignant lesion is a relatively infrequent, and often slow, process. Hence, there is an extended a window of opportunity to detect such lesions. The Papanicolaou ("Pap") smear allows for cytological examination of exfoliated cervical cells and has dramatically diminished cervical cancer incidence. In countries without widespread Pap smear programs, cervical cancer is a leading cause of cancer death in young women; ~10%–25% of all cancers in females are cervical carcinomas. With approximately 500,000 new cases diagnosed each year, cervical cancer is the third most frequent cancer, and the second leading cause of cancer death in women worldwide, with approximately 288,000 deaths (Ferlay et al., 2001). According to the National Cancer Institute, 11,150 cervical cancers were diagnosed in the 2007, and more than 10 women succumb to cervical cancer every day (3670/year) (Ries et al., 2007). Cervical cancer mortality remains the second leading cause of cancer death in U.S. women 20–39 years of age (Jemal et al., 2005).

One of the hallmarks of malignant progression is the integration of HPV genome sequences into a host cell chromosome (see Fig. 2.1). Integration is relatively nonspecific (Ziegert et al., 2003) but frequently targets common fragile sites (Smith et al., 1992). Integration often leads to deletions of viral sequences, and only the NCR and E6/E7 coding sequences are consistently maintained (Schwarz et al., 1985; Baker et al., 1987). Since viral genome integration typically leads to deletion of the E2 transcriptional repressor, HPV E6/E7 transcription becomes dysregulated. Moreover, HPV E6/E7 mRNAs produced from integrated copies are spliced to cellular sequences, resulting in increased mRNA stability (Jeon and Lambert, 1995; Jeon et al., 1995).

Several lines of evidence suggest that high-risk HPV E6/E7 expression is rate limiting for cervical cancer development. First, expression of high-risk HPV E6/E7 in primary human epithelial cells causes life span extension and leads to cellular immortalization (Bedell et al., 1989; Hawley-Nelson et al., 1989; Münger et al., 1989a). Second, high-risk HPV E6/E7-expressing keratinocyte populations grown in "organotypic" raft cultures exhibit many histopathological abnormalities of high-risk HPV-associated premalignant lesions (McCance et al., 1988). Third, expression of HPV16 E6/E7 in basal epithelial cells in conjunction with continuous low-dose estrogen treatment results in the development of cervical carcinomas in transgenic mice (Arbeit et al., 1996). In addition, persistent expression of high-risk HPV sequences in cervical carcinoma cell lines is necessary for maintenance of the transformed phenotype (Thierry and Yaniv, 1987; Francis et al., 2000; Goodwin and DiMaio, 2000). Hence, cervical cancer is the only human solid tumor where the initiating oncogenic trigger, high-risk HPV E6/E7 expression, is known at a molecular level (reviewed in zur Hausen, 2002).

Mucosal HPVs are transmitted by sexual contact; therefore, the incidence of HPV-associated anogenital tract diseases is correlated to early onset of sexual activity and the number of lifetime sex partners (reviewed in Schiffman et al., 2007). It has been estimated that 26.8% of all women between the ages of 14 and 59 are infected with one or more HPVs; the highest rate of infection (44.8%) was found in the 20- to 24-year age group (Dunne et al., 2007). High-risk HPV infected males can develop penile intraepithelial lesions (PINs). Although these lesions are far less common than cervical intraepithelial neoplasias (CINs), they can undergo malignant progression to penile carcinoma. The incidence rates of cervical and penile carcinoma are correlated in many parts of the world (Franceschi et al., 2002).

High-risk HPV infections are also highly associated with other squamous cell carcinomas that arise in the anogenital tract, including vulvar and perianal carcinomas (reviewed in zur Hausen, 2002). HPV-associated anal carcinomas are particularly frequent in acquired immune deficiency syndrome (AIDS) patients (Berry et al., 2004).

Oral Squamous Cell Carcinoma

Approximately 34,360 cases of oropharyngeal cancer were diagnosed in the United States in 2007, causing ~7550 deaths (Ries et al., 2007). Most of these tumors arise in patients with a long-term history of alcohol and tobacco abuse, but approximately 25% of oral carcinomas are associated with high-risk HPV infections (Gillison et al., 2000). HPV-positive tumors occur more frequently in younger patients who lack the classical risk factors (Fakhry and Gillison, 2006). The mode of transmission has not been definitively determined, but oral sex practices have been implicated (Kreimer et al., 2004). Unlike with cervical cancers, there are no standard screening programs that would aid in the early detection of oropharyngeal premalignant lesions.

Epidermodysplasia Verruciformis (EV) and Nonmelanoma Skin Cancers (NMSC)

The cutaneous HPV5 and HPV8 types are sometimes referred to as high-risk HPVs because they are associated with EV, a rare genetically determined skin disease (Ramoz et al., 2000, 2002). EV patients do not efficiently clear infections with these HPV types and subsequently develop warts, which can progress to skin cancers later in life, particularly in sun-exposed areas of the body (reviewed in Majewski et al., 1997). Skin tumors in EV patients were the first cancer type that was definitively associated with HPV infection (Jablonska et al., 1966; Pass et al., 1977; Orth et al., 1978; Pfister et al., 1981). More recently, HPV5 and HPV8, as well as related HPV types, have also been linked to development of NMSC, particularly in immunosuppressed patients (Shamanin et al., 1994). Unlike with cervical carcinoma, HPV genomes are not detected in every cancer cell, suggesting that these HPVs may contribute to initiation but may not be necessary for maintenance of the transformed phenotype. Infections with cutaneous HPVs may also contribute to NMSC development in nonimmunosuppressed patients, but this remains a somewhat controversial issue (reviewed in Akgul et al., 2006).

Malignancies Associated with Low-Risk Mucosal HPVs

While lesions caused by low-risk mucosal HPVs have a low risk for malignant progression, it is important to note, however, that in rare cases, low-risk HPV infections can also cause serious disease. Two examples are recurrent respiratory papillomatosis (RRP) and the giant condyloma of Buschke–Lowenstein.

RRP is a rare disease and the particularly insidious juvenile form affects young children, with an average age of 3.8 years at diagnosis. The disease is caused by HPV6 or HPV11 infection, presumably at birth. There is most likely a genetic component to the development of this disease but the exact genetic locus is unknown. RRP patients

are unable to control or clear the virus and develop rapidly growing wartlike lesions in the larynx and around the vocal cords, which can spread to the trachea and lungs. These patients require very frequent, often monthly surgery to keep the airways clear. Consequently, this disease is associated with considerable morbidity and mortality (reviewed in Tasca and Clarke, 2006).

Giant condyloma of Buschke–Lowenstein is also caused by HPV6 or HPV11 infections, and similar to RRP, patients are unable to control or clear the infection. It is a slow-growing tumor that is extremely destructive to adjacent tissue. These tumors are most commonly located on the glans penis but can also occur on other anogenital mucosal surfaces, including the vulva, vagina, rectum, scrotum, and bladder. Unlike RRP, Buschke–Lowenstein condyloma can give rise to local and distant metastases (reviewed in Rhea et al., 1998; Frega et al., 2002).

TRANSFORMING MECHANISMS OF HIGH-RISK MUCOSAL HPVs

In their classic review article, Hanahan and Weinberg (2000) cataloged the major hallmarks of human tumors, which include unlimited proliferative potential, evasion of apoptosis, growth factor independence, insensitivity to cytostatic signals, tumor invasion and metastasis, and sustained angiogenesis. The authors point out that each of these characteristics has to be acquired through genomic alterations and results in oncogene activation and tumor suppressor inactivation (Hanahan and Weinberg, 2000). As is detailed below, the high-risk HPV E6 and E7 oncogenes target critical regulators of each of these processes. In addition, high-risk HPV E7 oncoproteins act as potent mitotic mutators, greatly increasing the incidence of mutations during each round of cell division. Hence, high-risk HPV oncoproteins are extraordinarily potent oncogenes whose mere presence in a cell can substitute for multiple oncogenic hits due to exposure of environmental carcinogens. Consistent with this model, an infection with a high-risk HPV type is a far more significant relative risk factor for the development of cervical cancer (RR = 50–100) than cigarette smoking is for developing lung cancer (RR = 10) (Franco and Harper, 2005).

Cell Cycle Dysregulation

One of the major challenges of the papillomavirus life cycle is to establish and/or retain a replication competent cellular milieu in differentiated keratinocytes, which normally have withdrawn from the cell division cycle. Strategies acquired by the papillomaviruses to overcome these challenges are discussed in the following sections.

Inactivation of the Retinoblastoma Tumor Suppressor Protein pRB. The retinoblastoma tumor suppressor pRB controls S-phase entry through association with E2F transcription factor family members. E2F bound to pRB acts as a transcriptional repressor complex, which controls expression of many genes that are rate limiting for S-phase entry and progression. In normal cells, S-phase entry is triggered by pRB phosphorylation through cdk4 and/or cdk6/cyclin D complexes. As a consequence,

pRB/E2F complexes dissociate, and E2F transcription factors are rendered active as transcriptional activators (reviewed in Dyson, 1998). The E7 proteins encoded by many HPVs contain LXCXE amino acid sequence motifs (L, leucine; C, cysteine; E, glutamate; X, any amino acid) (Phelps et al., 1989), which constitute the core pRB-binding domain (Münger et al., 1989b). HPV16 E7 preferentially associates with the growth-inhibitory, hypophsophorylated, E2F-bound form of pRB, and E7 binding results in aberrant E2F activation and S-phase entry (reviewed in Münger et al., 2001). The simian vacuolating virus 40 (SV40) large tumor antigen (TAg) and the adenovirus (Ad) E1A proteins contain similar sequences and also have evolved to target pRB family members (DeCaprio et al., 1988; Whyte et al., 1988), presumably for similar reasons (Chellappan et al., 1992). In addition, some E7 proteins have been reported to associate with E2F family members, resulting in enhanced transcriptional activity (Hwang et al., 2002).

Among the mucosal HPVs, it has been demonstrated that high-risk E7s bind to pRB more efficiently than low-risk E7s. This binding differential was mapped to the amino acid residue preceding the LXCXE motif; high-risk HPVs contain an aspartate residue, whereas low-risk HPVs contain a glycine (Heck et al., 1992; Sang and Barbosa, 1992). Interestingly, however, among the cutaneous HPVs, the presence of an aspartate residue and efficient pRB binding is not associated with a high-risk phenotype *in vivo*, and maybe more surprisingly, does not predict transforming potential *in vitro* (Ciccolini et al., 1994).

A hallmark of high-risk HPV E7s is their ability to target pRB for proteasome-mediated degradation (Boyer et al., 1996). For HPV16 E7, it has been shown that pRB is an ubiquitination target of an HPV16 E7-associated cullin 2 ubiquitin ligase complex. Biochemical studies have shown that HPV16 E7 associates with the cullin 2 complex through the elongin C subunit, and hence, HPV16 E7 may function as a substrate adapter for the cullin 2 ubiquitin ligase complex (Huh et al., 2007).

Inactivation of pRB by destabilization represents an enzymatic process, as opposed to a stoichiometric protein/protein binding mechanism. This discovery has two major important biological implications that are unique among the high-risk HPV E7 proteins. First, a nonstoichiometric mechanism ensures pRB inactivation even when E7 is expressed at low levels. This mechanism may have evolved from the necessity of low-level HPV protein expression during a long-term persistent infection so as to avoid detection and elimination of infected cells by the immune system. Second, HPV E7-mediated pRB degradation results in subversion of all the biological activities of pRB, not just those related to cell cycle regulation. This is significant as pRB has also been implicated in regulating cell death and cellular differentiation (reviewed in Du and Pogoriler, 2006).

Other Mechanisms of G1/S Cell Checkpoint Dysregulation. HPV E7 proteins also contribute to cell cycle dysregulation through other mechanisms. Most importantly, E7 proteins cause aberrant activation of cyclin-dependent kinase 2 (cdk2). Cdk2 activity is necessary for S-phase entry and progression. The enzymatic activity of cdk2 is regulated by association with the positive regulatory subunits, cyclins E and A, as well as negative regulators including cdk inhibitors (CKIs), most prominently $p21^{CIP1}$ and $p27^{KIP1}$. HPV E7 expression results in the dysregulated expression of cyclins E and

A through E2F-dependent pathways (Zerfass et al., 1995). In addition, high-risk and low-risk HPV E7 proteins can also inactivate the inhibitory activities of the CKIs $p21^{CIP1}$ and $p27^{KIP1}$ (Zerfass-Thome et al., 1996; Funk et al., 1997; Jones et al., 1997a). E7 also causes increased expression of cdc25, a cellular phosphatase that removes an inhibitory phosphorylation on cdk2 (Katich et al., 2001; Nguyen et al., 2002). Moreover, HPV16 E7 has been reported to directly associate with cdk2 and enhance its enzymatic activity (He et al., 2003).

Abrogation of Cell-Abortive Mechanisms

Each cell contains built-in self-destruction machinery. Apoptosis is a process that can be triggered by a number of cell autonomous and nonautonomous mechanisms. Cell autonomous initiation of apoptosis represents an important tumor suppressive mechanism. It ensures that aberrantly proliferating, potentially premalignant cells are permanently removed from the proliferative pool (reviewed in Lowe et al., 2004). Noncell autonomous mechanisms can be triggered by cytokines that are produced in response to viral infection and/or viral gene expression. In addition to apoptosis, several other abortive mechanisms exist. These include cellular senescence and terminal differentiation. It is conceivable that a certain trigger may induce different abortive responses in different cell types. This may be particularly important in a squamous epithelial cell where extensive apoptosis may lead to tissue damage and degradation of the barrier function of the skin.

Trophic Sentinel Signaling—Abrogation of p53 Tumor Suppressor Protein Activity. Induction of aberrant cell proliferation triggers the activation of the p53 tumor suppressor, resulting in cell cycle arrest and/or cell death. Indeed, HPV16 E7-expressing primary human fibroblasts is predisposed to cell death through a p53-dependent mechanism, particularly under conditions of limiting growth factors or when cells reach confluence. This concept was initially established by studies with Ad E1A and c-myc oncogene-expressing cells (Evan et al., 1992; Rao et al., 1992) and has been referred to as the trophic sentinel response (Evan and Vousden, 2001). In the case of HPV16 E7, mutational analyses have shown that trophic sentinel signaling is correlated to the ability of high-risk HPV E7 proteins to induce pRB degradation (Jones et al., 1997b). The mechanism of cell death is dependent on the integrity of the p53 tumor suppressor pathway, but does not involve transcriptional activation of canonical p53 transcriptional targets. Curiously, HPV16 E7 expression generally interferes with the transcriptional activity of p53 (Eichten et al., 2002). Moreover, the mechanism of cell death triggered by HPV16 E7 expression in primary human fibroblasts may be distinct from classic apoptosis. Although caspases are activated and DNA is degraded to nucleosomal fragments, cell death appears mostly caspase independent (Eichten et al., 2004). Even though low-risk HPVs associate with pRB and induce efficient activation of E2F-responsive promoters, this does not result in trophic sentinel signaling.

To avoid elimination through trophic sentinel signaling, high-risk HPV E6 proteins target p53 for proteasome-mediated degradation (Scheffner et al., 1990). E6 associates with the cellular ubiquitin ligase E6 associated protein (E6AP), and the E6/E6AP

complex binds and ubquitinates p53 (Huibregtse et al., 1991; Scheffner et al., 1993). The ability to target p53 for degradation is unique to E6 proteins encoded by high-risk HPVs; low-risk HPV proteins do not target p53 (Scheffner et al., 1990).

Anoikis Signaling. Anoikis is a form of apoptosis that is triggered when cells attempts to enter the S phase despite the loss of matrix attachment (reviewed in Frisch and Screaton 2001). Anoikis resistance is a key hallmark of cellular transformation, manifested by the ability of transformed cells to overcome density-mediated growth arrest and form multilayered structures ("foci") and to proliferate when suspended in soft agar ("anchorage-independent growth"). The p600 protein has been identified as a target of low-risk and high-risk HPV E7 proteins and associates with E7 through conserved amino-terminal amino acid sequences (Huh et al., 2005). In addition, p600 also associates with the bovine papillomavirus (BPV) E7 protein (DeMasi et al., 2005). The p600 protein is a very large, 5183-amino-acid protein (Nakatani et al., 2005) that functions as a ubiquitin ligase in the N-end rule pathway (Tasaki et al., 2005). While its biological activities remain enigmatic, p600 depletion causes loss of anchorage-independent growth in human cancer cells (Huh et al., 2005). Expression of papillomavirus E7 proteins results in anoikis resistance in a number of different cell types, and in the case of BPV1 E7, this is at least in part due to p600 association (DeMasi et al., 2007).

Subversion of Cytokine Signaling

Transforming growth factor-β (TGF-β) is an important negative growth regulator of epithelial cells. Acquisition of TGF-β resistance is a hallmark of epithelial tumors, and cervical carcinoma cell lines are TGF-β resistant (De Geest et al., 1994). Early studies showed that ectopic HPV16 E7 expression abrogates TGF-β-mediated growth inhibition (Pietenpol et al., 1990). Interestingly, acquisition of TGF-β resistance of HPV-positive cell lines is a multistep process since TGF-β can repress HPV16 E6/E7 expression (Woodworth et al., 1990; Creek et al., 1995). This effect is mediated though Nuclear Factor I (NFI) sites in viral NCR. The Ski oncogene links TGF-β signaling and NFI transcription factor activity on the HPV16 NCR. In untreated cells, the NFI/Ski complex activates HPV16 E6/E7 expression. Ski levels decrease upon TGF-β addition, leading to decreased expression of the HPV16 E6/E7 oncoproteins. The emergence of TGF-β resistance in such cells has been correlated to Ski overexpression, but the exact mechanism of this is not yet known (Baldwin et al., 2004).

HPV-positive cervical carcinoma cell lines are also resistant to the cytostatic effects of tumor necrosis factor α (TNF) (Villa et al., 1992). TNF is a major inflammatory cytokine in the skin and mucosa and causes G1 growth arrest and cellular differentiation in normal keratinocytes (Basile et al., 2001). This effect is keratinocyte specific and is not observed in fibroblasts, and it involves NF-kB-mediated induction of $p21^{CIP1}$ (Basile et al., 2003). In contrast, HPV16 E7-expressing keratinocytes are TNF resistant, and cells continue to proliferate (Villa et al., 1992; Vieira et al., 1996). TNF-related cytokines, such as Fas ligand and TNF-related apoptosis-inducing ligand (TRAIL), do not affect keratinocyte proliferation (Basile et al., 2001).

TNF treatment can also trigger apoptosis when co-administered with the protein synthesis inhibitor, cycloheximide. HPV E7-expressing keratinocytes exhibit increased apoptosis upon TNF/cycloheximide treatment (Stoppler et al., 1998; Basile et al., 2001), whereas apoptosis is inhibited in HPV16 E7-expressing diploid human fibroblasts at the level of caspase 8 activation (Thompson et al., 2001).

Interferons (IFNs) are cytostatic cytokines that are produced due to the innate immune response to viral infections. IFN expression is activated by IFN regulatory factors (IRFs). HPV16 E6 has been reported to associate with and inhibit the transcriptional activity of IRF-3 (Ronco et al., 1998). In addition, HPV16 E7 can associate with IRF-1 and impair its transcriptional activity (Park et al., 2000; Perea et al., 2000). Hence, HPV16 E6 and E7 may cooperate to subvert innate immune signaling. Moreover, HPV16 E7 can form a complex with p48 (the DNA-binding component of the transcription factor Interferon-stimulated gene factor 3 (ISGF3), a mediator transcriptional mediator of IFNs) and perturb its nuclear translocation upon stimulation (Barnard and McMillan, 1999; Barnard et al., 2000).

HPV16 E7 may also interfere with insulin-like growth factor (IGF) signaling, which regulates cell survival. HPV16 E7 can associate with IGF binding protein-3 (IGFBP-3) (Mannhardt et al., 2000), thereby accelerating its proteasome-mediated degradation (Santer et al., 2007). Particularly under conditions of growth factor depletion, HPV16 E7-expressing cells express increased IGFBP-2 and IGFBP-5 levels through an NF-kB-dependent mechanism (Eichten et al., 2004).

Transcriptional Activities of HPV Oncoproteins

As pointed out previously, HPV E6 and E7 oncoproteins lack specific DNA-binding activities. Nonetheless, E6 and E7 each can associate with transcription factor complexes and alter their transcriptional activities. The best-documented examples are the p53 and E2F transcription factor complexes. High-risk HPV E6 proteins can bind c-myc (Veldman et al., 2003) and the repressor of hTERT expression, NFX1 (Gewin et al., 2004; Katzenellenbogen et al., 2007), resulting in increased hTERT transcription (McMurray and McCance, 2003). HPV E7 proteins can associate with the forkhead transcription factor MPP2 (Luscher-Firzlaff et al., 1999), AP1 (Antinore et al., 1996), and interfere with NF-kB activation (Huang and McCance, 2002; Spitkovsky et al., 2002). HPV E6 and E7 proteins have also been reported to associate with components of the basal transcription factor machinery, although the potential functional relevance of these associations is not clear (Mazzarelli et al., 1995; Massimi et al., 1996).

Of particular interest are reports that have documented associations of HPV E6 and E7 proteins with histone-modifying enzymes and transcriptional cofactors that are in turn associated with such factors (Brehm et al., 1999; Patel et al., 1999; Zimmermann et al., 1999; Huang and McCance, 2002; Bernat et al., 2003). While the biological consequences of some of these interactions await further clarification, it has been shown that HPV16 E7 association is linked to cytoplasmic relocalization and functional inactivation of steroid receptor coactivator-1 (SRC1) (Baldwin et al., 2006). Collectively, these results suggest the exciting possibility that HPV oncoprotein expression may alter the epigenetic program of infected host cells.

Cellular Metabolism

The concept that HPV oncogene expression may cause alterations in cellular metabolism was established by reports that HPV16 E7 can associate with and alter the activity of the metabolic enzyme pyruvate kinase (PK) (Zwerschke et al., 1999). Presumably, as a consequence of this association, HPV16 E7-transformed cells contain high levels of a relatively inactive, dimeric form of PK. As a consequence, HPV-transformed cells appear to derive their metabolic energy mostly from glycolytic processes rather than from oxidative phosphorylation (Mazurek et al., 2001). Many human tumor cells undergo a similar metabolic shift, which is commonly referred to as the "Warburg effect" (reviewed in Warburg, 1936; reviewed in Aisenberg, 1961). Hence, HPV16 E7-expressing cells may constitute a useful model to determine the biochemical basis of this phenomenon, which has been shown to be critical for tumor maintenance (Fantin et al., 2006). HPV16 E7 may also act as an allosteric activator of α-glucosidase (Zwerschke et al., 2000), an enzyme that causes glycogen catabolism. Like many other tumors, HPV-associated cervical cancers contain low glycogen levels (Pedersen, 1975; Bannasch et al., 1997).

Another metabolic hallmark of transformed cells is a relatively high intracellular pH. HPV E7 expression has been shown to cause cytoplasmic alkalinization due to increased activity of the Na^+/H^+ exchanger protein, and inhibition of this process decreased cell proliferation and inhibited anchorage-independent growth of HPV16 E7-transformed NIH3T3 cells (Reshkin et al., 2000).

Cytoskeleton and Cell Polarity

Papillomavirus E6 proteins associate with the focal adhesion protein paxillin. Similar to E6AP and IRF-3, paxillin associates with E6 through a leucine/aspartate-rich alpha helical (LD) motif that normally mediates the association of paxillin with vinculin and focal adhesion kinase (FAK). Association of E6 with paxillin causes disruption5 of these associations and correlates with the ability of E6 to disrupt the cellular actin cytoskeleton, a hallmark of transformed cells (Tong and Howley, 1997; Tong et al., 1997; Chen et al., 1998; Vande Pol et al., 1998).

E6 proteins encoded by high-risk, but not low-risk, HPVs contain a (S/T)-X-V-I-L sequence motif at their extreme carboxyl-termini. This sequence acts as a ligand for cellular PDZ-domain-containing proteins (Kiyono et al., 1997; Lee et al., 1997). Such proteins act as scaffolds and spatially organize important cellular signal transduction processes, including cell polarity. A number of putative PDZ targets have been identified (Glaunsinger et al., 2000; Lee et al., 2000; Nakagawa and Huibregtse, 2000; Thomas et al., 2002; Jing et al., 2007; Storrs and Silverstein, 2007). Since PDZ protein- and E6AP binding are mediated by different E6 sequences, E6-associated PDZ proteins can be ubiquitination targets for the E6/E6AP ubiquitin ligase complex (Gardiol et al., 1999; Nakagawa and Huibregtse, 2000). Mutational analyses have shown that the integrity of the PDZ ligand sequence is important for E6 activities during the viral life cycle (Lee and Laimins, 2004) and for the transforming activities of E6 *in vitro* (Kiyono et al., 1997) and *in vivo* (Nguyen et al., 2003). Other viral oncoproteins including Ad

type 9 E4 ORF1 and HTLV-1 tax also associate with PDZ proteins (Gardiol et al., 1999), and in each case, association with PDZ proteins has been linked to transforming activities of these viral oncoproteins (reviewed in Tauber and Dobner, 2001; Hall and Fujii, 2005; Liu and Baleja, 2008).

Induction of Genomic Instability

HPV16 E6/E7 oncogene expression in primary human keratinocytes causes cellular immortalization, and such cells exhibit many hallmarks of premalignant lesions. Nonetheless, HPV-immortalized cells are not fully transformed in that they do not form tumors when injected into nude mice. Such cells, however, can acquire a fully transformed phenotype upon prolonged passaging in cell culture (Hurlin et al., 1991) or when additional oncogenes are expressed (Dürst et al., 1989; Pei et al., 1993). This situation is similar to the development of clinical lesions, where there is a long latency period between the initial infection and the development of invasive cervical carcinoma (reviewed in Schiffman et al., 2007). Hence, while HPV oncogene expression is necessary and sufficient for the initiation of cervical carcinogenesis, additional mutations in the host genome are required for malignant progression. Consistent with this notion, cervical cancer cells have accumulated a plethora of numerical and structural chromosomal aberrations (Mitelman et al., 2007).

Ectopic expression of HPV16 E6 and/or E7 in primary cells affects genomic stability (Hashida and Yasumoto, 1991; White et al., 1994). In contrast, cells expressing low-risk HPV E6 and/or E7 proteins remain genomically stable (White et al., 1994).

Careful investigation of these initial observations provided evidence for at least three types of mitotic abnormalities in HPV oncogene-expressing cells: lagging chromosomal material, anaphase bridges, and most prominently, multipolar mitoses (Duensing et al., 2000; Duensing and Münger, 2002).

The mechanistic basis of the formation of mitoses with lagging chromosomal material remains unknown, but progress has been made in deciphering the molecular mechanism that leads to the formation of anaphase bridges and multipolar mitoses. These mechanistic insights will be discussed in the following two subsections.

Anaphase Bridges. Anaphase bridges, the presence of chromosomal material that stretches between two anaphase nuclei, arise as a consequence of dicentric chromosomes, that is, fused chromosomes that contain two centromeres. Fused chromosomes can arise in cells undergoing telomere attrition, which results in the generation of "uncapped" chromosomal ends. These are recognized by the DNA repair machinery as DNA double-strand breaks and may be aberrantly fused to another chromosome that is also "uncapped" due to telomere erosion. This can cause chromosomal translocations. This mechanism is the cornerstone of the "big bang" model of chromosomal instability, which may ensue when cells continue to proliferate in the presence of critically shortened telomeres (reviewed in Chang et al., 2001). It is important to note that this model predicts that genomic instability is temporarily confined to a short period of tumor development prior to induction of telomerase activity to overcome senescence. Such a model fails to account for ongoing genomic instability, which is clearly required for

an emerging tumor to rapidly adapt to changes in the microenvironment (reviewed in Cahill et al., 1999). Moreover, "big bang" genomic instability is unlikely to arise in high-risk HPV infected lesions due to sustained telomerase activity as a consequence of HPV E6 oncoprotein expression (Kiyono et al., 1998). Alternatively, chromosomal fusions and formation of dicentric chromosomes can arise as a consequence of aberrantly repaired DNA double-strand breaks. In either case, dicentric chromosomes, once formed, will be broken again during the next round of mitosis. This results in breakage–fusion bridge cycles, as originally discovered by Barbara McClintock (1940). Anaphase bridges in HPV oncogene-expressing cells are caused by unrepaired DNA double-strand breaks and not by telomere attrition (Duensing and Münger, 2002). However, the exact mechanism remains unknown.

Interestingly, Fanconi anemia (FA) patients have a highly increased incidence of squamous cell carcinomas at sites that are infected by HPVs (reviewed in zur Hausen, 2002). It has been suggested that oral cancers arising in FA patients are more frequently HPV positive than in the general population (Kutler et al., 2003). The FA pathway is activated by DNA cross-linking agents and stalled replication forks (reviewed in Kennedy and D'Andrea, 2005). It was recently shown that the FA pathway is activated by HPV16 E7 expression, and that the capacity of HPV16 E7 to induce DNA repair foci is enhanced in FA patient-derived cell lines (Spardy et al., 2007).

Multipolar Mitoses. Abnormal, multipolar mitoses are histopathological hallmarks of high-risk HPV-associated premalignant lesions and cancers (Winkler et al., 1984) and are caused by supernumerary centrosomes (Duensing et al., 2000). Centrosomes function as microtubule-organizing centers (MTOCs) and form mitotic spindle poles. They consist of centrioles surrounded by pericentriolar material. To ensure bipolar, symmetric chromosome segregation during mitosis, centrosomes are duplicated in synchrony with the cell division cycle (reviewed in Stearns, 2001).

High-risk HPV E6 and E7 proteins cooperate to generate mitotic defects and aneuploidy through induction of supernumerary centrosomes in primary human epithelial cells, and characteristic, multipolar mitoses in cervical lesions represent a direct manifestation of HPV E6/E7 expression. Importantly, low-risk HPV E6/E7 proteins do not induce centrosome abnormalities (Duensing et al., 2000). Centrosome abnormalities and associated mitotic defects are apparent in cells that, similar to high-risk HPV-associated premalignant lesions, express episomal HPV-16 at a low copy number (Duensing et al., 2001b), and their incidence increases in cells with integrated HPV (Skyldberg et al., 2001; Pett et al., 2004). Centrosome abnormalities have also been documented in HPV16 E6- and/or E7-expressing transgenic mice that develop cervical (Balsitis et al., 2003; Riley et al., 2003) or skin lesions (Schaeffer et al., 2004).

Centrosome abnormalities can arise through two distinct mechanisms; directly through aberrant centriole synthesis and indirectly as a consequence of cytokinesis failure or through cell fusion (reviewed in Duensing, 2005). Centrosome abnormalities that emerge as a consequence of defects in cytokinesis and/or cell division or arise as a result of cell fusion are accompanied by nuclear abnormalities and/or tetraploidy (reviewed in Storchova and Pellman, 2004). It has been shown, however, that HPV16 E7 expression uncouples centriole synthesis from the cell division cycle and induces

supernumerary centrosomes through primary centrosome and centriole duplication errors in normal diploid cells (Duensing et al., 2001a; Guarguaglini et al., 2005). Evidence for aberrant centriole synthesis has also been detected in high-risk HPV-positive tumor tissues (Duensing et al., 2007a). HPV16 E7 induces supernumerary centrosomes through concurrent synthesis of multiple daughter centrioles from a single maternal template (Duensing et al., 2007b).

Even though it has been postulated that aneuploidy arises from a tetraploid intermediate (Fujiwara et al., 2005), it has in fact been documented that aneuploid cell populations can arise directly from diploid cells. This discovery was made in mammary epithelial cells where loss of $p16^{INK4A}$ gives rise to aberrant centriole splitting. This caused the formation of multipolar mitoses and aneuploid progeny (McDermott et al., 2006). Hence, even though HPV16 E7 induces centrosome duplication errors in diploid cells through a different mechanism, the presence of supernumerary centrosomes in diploid cells may contribute to the development of aneuploidy in the absence of a tetraploid intermediate.

Unlike normal centrosome duplication, the ability of HPV16 E7 to induce supernumerary centrosomes is strictly dependent on cdk2 activity. Cdk2 inhibition specifically interferes with E7-mediated induction of supernumerary centrosomes and decreases the incidence of centrosome abnormalities and aneuploidy in E7-expressing cells (Duensing et al., 2004; Duensing et al., 2006). As discussed above, HPV16 E7 expression causes aberrant cdk2 activation through multiple pathways, most of which are dependent on E2F as a consequence of HPV16 E7-mediated degradation of pRB and the related p107 and p130 proteins. Since HPV16 E7 expression augments the incidence of supernumerary centrosomes in mouse embryo fibroblasts that lack pRB, p107, and p130, the ability of HPV16 E7 to induce centrosome duplication errors is at least in part independent of the ability to target pRB family members (Duensing and Münger, 2003). A possible pRB/p107/p130-independent mechanism for the emergence of supernumerary centrosomes in HPV16 E7-expressing cells was recently documented. HPV16 E7 was shown to associate with the centrosomal regulator γ-tubulin through sequences that overlap the pRB core-binding site and interfere with γ-tubulin recruitment to centrosomes. HPV16 E7 association with γ-tubulin was pRB/p107/p130 independent, and binding correlated with the ability of HPV16 E7 to induce supernumerary centrosome abnormalities in pRB/p107/p130-deficient cells. Moreover, HPV16 E7 expression significantly inhibited γ-tubulin recruitment to the centrosome (Nguyen et al., 2007).

Epithelial to Mesenchymal Transition (EMT) and Angiogenesis

In addition to genomic instability, expression of high-risk viral oncoproteins has also been implicated in the dysregulation of other cellular pathways that are associated with malignant progression.

EMT is a process where epithelial cells acquire certain characteristics of fibroblasts. This reversible process is thought to play a key role in carcinogenesis and metastasis. It may reflect the final step of tumor dedifferentiation, which is generally associated with high invasion potential and chemoresistance. The molecular hallmarks

of EMT are down-regulation of the epithelial adhesion molecule E-cadherin, *de novo* expression of N-cadherin, nuclear translocation of β-catenin, and expression of mesenchymal proteins such as vimentin, smooth muscle actin, and fibronectin (reviewed in Moustakas and Heldin, 2007). HPV16-expressing cells have been shown to undergo EMT-related processes (Gilles et al., 1994), and this has been reported to be associated with E6 expression (Watson et al., 2003).

Angiogenesis is a critical process in tumor progression that ensures that rapid tumor growth can be sustained by an adequate supply of oxygen and nutrients. HPV16 E6 has been shown to induce the expression of vascular endothelial growth factor (VEGF) through a p53-independent transcriptional pathway (Lopez-Ocejo et al., 2000). Moreover, HPV16 E6 expression also resulted in increased expression of fibroblast growth factor-binding protein (FGF-BP), which has been implicated as a modulator of the angiogenic switch (Stoppler et al., 2001). Consistent with these results, transcriptional analyses of HPV16 E6/E7-expressing human keratinocytes demonstrated increased expression of VEGF and interleukin-8 and decreased expression of the two angiogenesis inhibitors thrombospondin-1 and maspin (Toussaint-Smith et al., 2004; Chen et al., 2007).

CONCLUDING REMARKS

Great strides have been made in unraveling the molecular mechanisms that contribute to cancer development. The availability of the sequence of the human genome, the development of high-throughput technologies that allow accurate enumeration of genetic and epigenetic abnormalities in tumors, technical advances such as small inhibitory (si)RNAs, as well as the emerging field of network biology have greatly contributed to the recent rapid advancements in this field. Since most human solid tumors cancers arise through an individual sequence of distinct oncogenic hits, one of the major challenges with these approaches is the ability to parse the massive amounts of data that are generated and to extract information on specific abnormalities that drive cancer development.

HPV-associated cervical cancers, however, almost uniformly arise as a consequence of HPV infections. Moreover, maintenance of the transformed phenotype is strictly dependent on the expression of only two viral proteins, HPV E6 and E7. There is no other human solid tumor type that can be modeled with similar molecular accuracy in cell culture or animal models. Hence, it is obvious that studies focused on delineating the molecular mechanisms of high-risk HPV E6/E7-associated carcinogenesis will continue to yield invaluable and generally applicable insights on human cancer development.

This notion has been impressively validated by the insight that the main targets of high-risk HPV E6 and E7 oncoproteins, the p53 and pRB tumor suppressor pathways, respectively, are rendered dysfunctional in the majority of human cancers (reviewed in Sherr and McCormick, 2002). In addition, subversion of these two tumor suppressor pathways as well as the capacity to retain telomere length, an activity that is provided through E6-mediated activation of hTERT expression, constitute

three of the five oncogenic hits that are minimally required to convert primary human epithelial cells to a full transformed phenotype *in vitro* (reviewed in Hahn and Weinberg, 2002).

Similarly, studies on the molecular mechanisms of genomic destabilization as a consequence of HPV oncogene expression have yielded important insights and lent, experimentally, support to the almost 100-year-old hypothesis that certain oncogenic hits can give rise to numerical centrosome abnormalities in normal cells, which then drive unequal chromosome segregation (Boveri, 1914) and chromosomal instability.

Since the recently introduced HPV vaccines are prophylactic and thus cannot target HPV infections that already exist, it has been estimated that it will take up to 25 years before cervical cancer rates decline (reviewed in Frazer, 2004; reviewed in Franco and Harper, 2005). The fact that approximately 10 women succumb to cervical cancer every day in the United States alone should provide sufficient motivation for researchers and granting agencies alike to continue to be vigorously engaged in research that addresses molecular mechanisms of HPV-associated carcinogenesis. If we cannot translate these insights to the benefit of patients, how can we expect to discover mechanism-based therapies for other human solid tumors that arise by a much more complex series of oncogenic events?

As detailed throughout this chapter, the transforming activities of high-risk HPVs reflect biological activities that are relevant to the viral life cycle. It is thus curious that not all mucosatropic HPVs encode transforming activities even when they are tested under standardized conditions. Why do high-risk HPVs encode E6 and E7 proteins that target p53 and pRB for degradation, whereas the homologous low-risk HPV proteins lack these activities? Similarly, why do high-risk HPV E6/E7 proteins induce genomic instability, whereas low-risk HPV encoded proteins do not? The latter question, in particular, is puzzling since E6/E7-induced genomic instability may contribute to HPV genome integration, which terminates the viral life cycle, and thus should provide a strong evolutionary disadvantage to high-risk HPVs compared with low-risk types.

Whereas there are no conclusive answers to this conundrum, it is important to keep in mind that there two fundamentally different paradigms for successful host–pathogen interaction. One strategy is based on rapid spread; the pathogen quickly replicates to high levels so that it can infect other hosts or reinfect the same host. The second strategy is to coexist with the host for extended periods of time. While there is no experimental proof, it is tempting to speculate that high-risk and low-risk HPVs each have evolved and perfected different strategies for successful host–pathogen interactions.

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3

THE ROLE OF THE HUMAN PAPILLOMAVIRUS E6 ONCOPROTEIN IN MALIGNANT PROGRESSION

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INTRODUCTION

The human papillomavirus (HPV) E6 and E7 oncoproteins contribute directly to the development and maintenance of the transformed phenotype in cervical cancer. The *E6* and *E7* genes are invariably retained and expressed in cervical cancers, and in the cell lines isolated from them, many years after the initial immortalizing events, thus indicating the importance of these proteins for the maintenance of the transformed phenotype. Indeed, blocking E6 and E7 expression in tumor-derived cell lines by various means has, in each case, resulted in the senescence or apoptosis of those cell lines. In a broad generalization, it could be said that the virus requires the E7 oncoprotein to enhance the replicative capacity of differentiating epithelial cells, which normally would have exited the cell cycle and shut down their DNA replication machinery, thus rendering them capable of supporting viral DNA replication (reviewed in Münger et al., 2001). The means used by E7 to do this, and its contribution to cellular transformation, are considered in detail in the chapter by Baldwin and Münger (Chapter 2). Equally broadly, it could be said that E6 is required to clear up the mess made by E7. The eukaryotic cell has a wide repertoire of defenses against unscheduled stimulation of cell cycle and DNA replication, which it attempts to deploy in response to E7's activities. Many of the functions of E6 are related to the

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need to circumvent these defenses and, since these are also defenses against malignant cellular transformation, this explains the continued requirement for E6 in cervical tumors and cell lines.

Although more than 100 different HPV types have been identified, only a small number of them have been associated with the development of human cancers. Understanding the differences between the "high-risk" virus types and the "low-risk" types has been one of the main challenges in HPV research for many years. In the following discussion, we will highlight those aspects of E6 function that we believe may be responsible for the differences in pathology observed between different HPV types, and which most probably contribute to the malignant progression of HPV-induced tumors.

HPV E6 AND CELL TRANSFORMATION

Over the years, a wide range of cell types has been used to study E6- and E7-induced cellular transformation, and work in primary rodent cells showed that E6, while possessing weaker activity than E7, could cooperate with an activated ras oncogene to induce transformation (Storey and Banks, 1993; Liu et al., 1994; Pim et al., 1994). In these assays, high-risk HPV E6 and E7 proteins showed strong transforming activity, whereas E6 and E7 from low-risk HPVs had little or no transforming activity (Storey et al., 1988; Phelps et al., 1988; Storey and Banks, 1993; Liu et al., 1994; Pim et al., 1994). These studies thus provided the earliest evidence of clear biological differences between the oncoproteins derived from high- and low-risk virus types.

The most meaningful *in vitro* assays of HPV transformation are obviously those using primary human keratinocytes, the target cells of the virus *in vivo*. Several studies have shown that E6 and E7 from high-risk HPV types can cooperate to immortalize human foreskin, oral, or cervical keratinocytes (Dürst et al., 1987; Kaur and McDougall, 1988; Schlegel et al., 1988; Sexton et al., 1993; Garner-Hamrick et al., 2004). It is also clear that, as in primary rodent cells, the low-risk HPV E6 and E7 proteins have no activity in such assays (Pecoraro et al., 1989; Woodworth et al., 1989). It is important to note, moreover, that the immortalized keratinocytes are not fully transformed by E6 and E7, and cannot form tumors in nude mice. The cells become tumorigenic only after extended passage in culture, or upon transfection of activated oncogenes, (DiPaolo et al., 1989; Dürst et al., 1989; Hurlin et al., 1991), thereby reflecting the multistep nature of cancer development *in vivo*.

More recently, further insights into the activities of the two major HPV oncogenes have been provided by an elegant series of studies in transgenic mice, in which E6 and E7 were expressed from the keratin 14 promoter. This promoter is activated in basal epithelium, and thus the E6 and E7 proteins are expressed in a physiologically relevant tissue (Arbeit et al., 1994; Herber et al., 1996; Song et al., 1999). Mice expressing E6 and E7 developed epithelial neoplasias at a high frequency, as well as cervical cancer when treated with estrogen. Upon detailed analysis, the individual roles of E6 and E7 in inducing such neoplasias in these transgenic mice proved to be most revealing. Individual expression of either E6 or E7 resulted in skin tumors, but with interesting differences.

Expression of E7 alone resulted in a high frequency of mainly benign differentiated tumors. In contrast, mice expressing E6 alone developed significantly fewer tumors, which were, however, much more aggressive and prone to progression into metastatic cancer (Song et al., 2000). Studies in these mice using chemical carcinogens indicate that E7 promotes immortalization, whereas E6 promotes progression into full malignancy (Song et al., 2000). Similar results were found in studies in the reproductive tract of mice expressing E6 and/or E7 from the keratin 14 promoter upon treatment with exogenous estrogen: In this case, E6 contributes to the increased size of tumors in estrogen-treated, doubly transgenic mice (Riley et al., 2003; Brake and Lambert, 2005).

Further work with transgenic mice also showed that the PDZ (PSD95/Discs Large /ZO-1)-binding motif of E6 (discussed in detail below), which is strictly specific for the high-risk mucosal virus types, is absolutely required for E6's induction of epithelial hyperplasia (Nguyen et al., 2003a; Shai et al., 2007a).

THE E6 POLYPEPTIDE

Despite much work over the years, the physical structure of the E6 protein is still not fully defined. However, a recent series of nuclear magnetic resonance (NMR) studies has defined more clearly the C-terminal half of the HPV-16 E6 protein (Nominé et al., 2003, 2005). Owing to the conserved nature of the E6 proteins, these data have allowed modeling of the characteristics of the N-terminal half, and thereby allowed predictions to be made about the overall structure of the E6 oncoprotein. This study has confirmed the absolute requirement for the C-x-x-C motifs, and the fact that they do indeed form zinc-binding domains, as had previously been postulated (Cole and Danos, 1987). Moreover, these reports show that the binding of zinc is highly important for the maintenance of the structural integrity of the E6 protein (Lagrange et al., 2005; Nominé et al., 2005, 2006; Zanier et al., 2005, 2007), and this is supported by data showing that the mutation of these residues results in a complete loss of E6 function (Kanda et al., 1991; Sherman and Schlegel, 1996). The structural integrity of the protein and its ability to bind zinc are, in turn, essential for the integrity of the p53/E6-associated protein (E6-AP) interaction site (Zanier et al., 2005). Indeed, both halves of the E6 protein are required to form this binding site, and many mutations in the body of E6 have been shown to disrupt it. Thus, at least one of the major functions of E6 is exquisitely sensitive to structural changes, and great care is obviously needed in interpreting the results of mutational studies, at least as far as they refer to the E6-AP/p53 interactions of E6. Similar analyses have now been performed on the E6 proteins from several other HPV types: the cutaneous HPV-5 E6, the low-risk HPV-11 E6, and E6 proteins from the high-risk types 18, 33, 45, 52, and 58 (Zanier et al., 2007). All these proteins exhibited similar zinc-binding activities in these assays, but only the high-risk mucosal HPV E6 proteins were shown to induce p53 degradation *in vitro*. Thus, the ability to bind zinc would seem to be an intrinsic characteristic of all E6 proteins, although the cellular targets of different HPV types may differ. When considering the results of these NMR studies it should be noted that, in order to obtain soluble proteins *in vitro*, each of the nonconserved cysteine residues was mutated to serine, and thus the interpretation of

these results should take that into account. The gold standard would obviously be structural studies and crystallization of the native proteins; this goal now appears less impossible than it once did. Some recent studies have also begun to focus in detail on the structure of the extreme C-terminus of E6, which in the NMR analysis appears to be completely unstructured (Nominé et al., 2005), and this aspect of E6 structure will be discussed in more detail below.

The E6 proteins from all HPV types are highly homologous, particularly in the central portion of the molecule, both in their primary amino acid sequences and in their three-dimensional arrangements, as we saw above, reflecting their many common activities. However, the extreme amino- and carboxy-terminal portions of the protein are more divergent, and, most importantly, the high-risk mucosotropic HPV types alone possess a PDZ-binding motif in the extended C-terminus. The implications of this will be discussed below.

THE CELLULAR TARGETS OF E6 PROTEINS

The E6 protein has been shown to interact with a large number of cellular proteins, and thus to impinge upon a wide range of cellular functions. All of these interactions are intended to increase the replicative efficiency of the virus, but in the case of the high-risk virus types, some have the additional and unintended consequence of promoting cellular immortalization and transformation.

Figure 3.1 is a representation of the E6 protein structure indicating the regions of the protein that are known to be involved with some of its target proteins. This

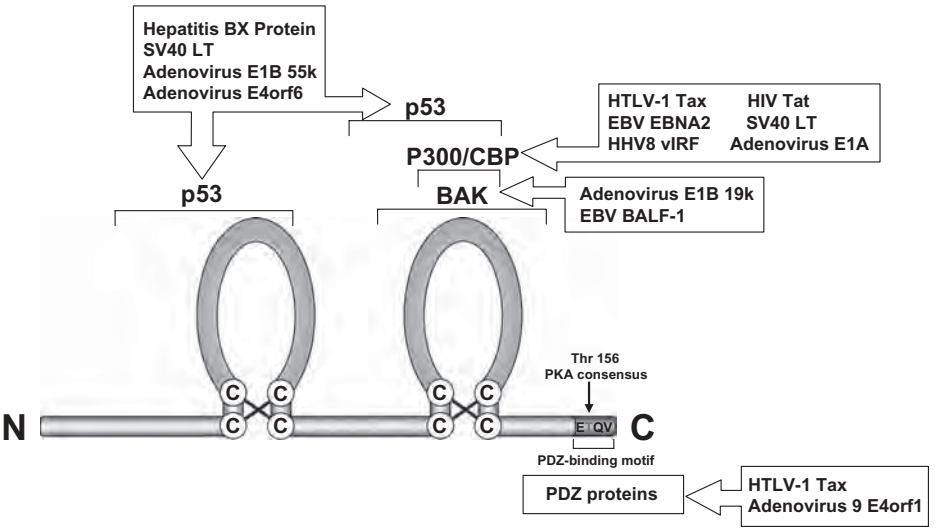

Figure 3.1. The overall structure of the E6 polypeptide, showing the regions of the proteins involved in its known interactions. Those target proteins that are also recognized by other virus proteins are indicated.

also highlights the targets of E6 that are recognized by other DNA tumor viruses, suggesting the existence of common pathways of virus-induced cell transformation, as well as common obstacles that must be overcome during the different virus life cycles.

p53 as a Target for Viral Oncoproteins

The p53 protein is an extremely well-studied and multifunctional protein (reviewed in Vousden and Lane, 2007). It is also probably the best-known target of high-risk mucosal HPV E6 (reviewed in Thomas et al., 1999) and is also targeted by a number of other viral oncoproteins. The SV40 large tumor antigen (TAg) (Lane and Crawford, 1979; Linzer and Levine, 1979) binds to the DNA-binding domain of p53, thus blocking its transactivation of target genes (Ruppert and Stillman, 1993). The adenovirus (Ad) E1B-55k protein (Sarnow et al., 1982) binds to the transactivation domain of p53 (Lin et al., 1994), while the hepatitis B virus X protein prevents p53 transactivation by sequestering it in the cytoplasm (Elmore et al., 1997). Each of these interactions results in the accumulation of p53 in stable and inactive complexes; however, in HPV-containing cells, no such complexes are seen, and little or no p53 protein can be detected, despite there being an abundance of p53 mRNA present (Matlashewski et al., 1986; Scheffner et al., 1991). *In vitro* studies eventually showed that HPV-16 and HPV-18 E6s are able to bind p53 and to induce its degradation via the ubiquitin pathway (Scheffner et al., 1990; Werness et al., 1990;). This activity of E6 explains why p53 levels in HPV-transformed cell lines are often so low (Band et al., 1991), and this also explains the observations that p53 is wild type in such cell lines, and at least in the early stages in HPV-positive cervical tumors, whereas mutant p53 is detected in over 50% of all other tumors (Crook et al., 1991; Scheffner et al., 1991).

It has since been demonstrated that, in addition to its ability to block p53 transactivation, Ad E1B-55k associates with the Ad E4orf6 gene product to induce p53 degradation through the proteasome pathway (Steegenga et al., 1998; Querido et al., 2001): yet another example of the conservation of function between DNA tumor viruses. In addition, both high- and low-risk HPV E6 proteins can form complexes with p53 in the absence of degradation (Li and Coffino, 1996), blocking p53's DNA binding activity and, hence, its ability to transactivate. (Lechner and Laimins, 1994; Pim et al., 1994; Thomas et al., 1995). In addition, E6 proteins from both low- and high-risk HPV types can bind to p300, inhibiting the acetylation of chromatin-bound p53 and core histones, and consequently repressing p53-mediated transactivation of downstream genes such as p21 (Thomas and Chiang, 2005). Thus, it appears that neither Ad nor HPV needs to degrade all of the p53 present in the cell. This would suggest that either there is a threshold level, below which it does not interfere with the virus, or that the presence of p53 might be beneficial at certain stages of the viral life cycle. In support of this hypothesis, p53 has been reported to associate with the replication complexes of herpes simplex virus (HSV) (Wilcock and Lane, 1991; Zhong and Hayward, 1997; Boutell and Everett, 2004) and cytomegalovirus (Muganda et al., 1994; Fortunato and Spector, 1998), and to enhance the replicative fidelity of the HIV-1 reverse transcriptase

(Bakhanashvili, 2001; Bakhanashvili et al., 2004, 2005), all of which suggests that p53 might be required for viral DNA replication. HPV does not encode its own replicative DNA polymerase; it uses the cellular DNA polymerase α, which has no 3′-5′ proofreading capacity (Kuo et al., 1994; Masterson et al., 1998; Conger et al., 1999). It is therefore possible that p53, which does have such a proofreading capability (Huang, 1998), is hijacked by the virus for this purpose. In addition, the HPV-16 E2, the origin binding protein, has also been shown to interact with p53 (Massimi et al., 1999), and this might serve to recruit p53 to centers of HPV replication in a manner analogous to the activity of the HSV major DNA-binding protein (Wilcock and Lane, 1991; Zhong and Hayward, 1997).

The low-risk HPV E6 proteins either do not induce p53 degradation or do so at much lower efficiency (Storey et al., 1998). The reason for this may be related to the finding that low-risk HPV types initiate the replicative cycle low in the stratified epithelium (Doorbar et al., 1997), where the keratinocytes might still express at least some of the proteins required for viral DNA replication. In contrast, the high-risk viruses appear to initiate replication higher in the differentiating epithelium, where the cells have completely switched off the expression of DNA replication proteins. Thus, the high-risk virus E7 proteins would need to exert stronger effects upon the cells to induce apparently inappropriate DNA replication (Sato et al., 1989; Banks et al., 1990), thus risking the activation of p53, which then would require a more effective response by E6.

For a long time, it was assumed that the E6–p53 interaction was central to the ability of E6 to induce cell transformation. However, there is now a significant body of evidence suggesting that other targets of E6 also play vital roles in E6-induced transformation and malignancy (Pim et al., 1994; Nakagawa et al., 1995; Pan and Griep, 1995; Liu et al., 1999). In particular, it has been shown in an elegant series of studies that both p53-dependent and independent pathways of transformation are disregulated in transgenic mice expressing HPV-16 E6 from the keratin 14 promoter (Nguyen et al., 2002; Shai et al., 2007a). Moreover, these studies showed that distinct contributions to tumorigenesis are made by the alpha helical domain (binding E6-AP, among other partners) and the PDZ-binding domain (Nguyen et al., 2002, 2003a,b; Simonson et al., 2005; Shai et al., 2007a,b). Although blocking the ability of E6 to target p53 for degradation should have a negative impact on the growth of HPV-transformed cells (Beer-Romero et al., 1997), it is not clear how relevant this would be to cancer. In many cells derived from cervical tumors, the blocking of E6-induced degradation of p53 is not alone sufficient to induce high levels of p53 expression, indicating that the signaling pathways upstream of p53 may be compromised in some cervical tumor-derived cell lines (Mantovani and Banks, 1999). High levels of wild-type p53 in cervical lesions at different stages of disease progression have also been reported (Cooper et al., 1993; Lie et al., 1999; Giannoudis and Herrington, 2000), suggesting that E6 does not degrade all p53s all of the time, again highlighting the possibility that the presence of some p53s might be beneficial to viral replication. This is possibly supported by the findings that although p53 expression is increased in the cells of all HPV-containing lesions, in comparison with neighboring HPV-negative tissue, the increased p53 expression is highest in cells containing low-risk

HPV (Giannoudis and Herrington, 2000). The means by which low-risk HPVs evade the vigilance of p53 is still a matter of debate, although some reports suggest that the low-risk HPV E6 proteins can induce a low level of p53 degradation (Storey et al., 1998), probably through their interaction with E6-AP (Brimer et al., 2007). In addition, as mentioned before, both the low- and high-risk HPV E6 proteins can inhibit the p300-mediated acetylation of p53, thus blocking its transcriptional activities (Thomas and Chiang, 2005). It has also been shown that low-risk HPV E6 proteins can induce the degradation of PDZ-containing targets when supplied chimerically with a PDZ-binding motif (Pim et al., 2002), indicating that they are intrinsically able to recruit the cellular proteasomal machinery, although their targets are not yet known.

E6-AP

The E6-AP is an E3 ubiquitin-protein ligase and is the prototype HECT (*h*omologous to *E*6-AP *c*arboxy-*t*erminus) domain protein. It is encoded by the *UBE3A* gene, mutations in which can result in the Angelman and Prader–Willi syndromes, both serious neurogenetic diseases (Kishino et al., 1997; Matsuura et al., 1997; Jiang et al., 1998a). Although E6-AP was originally identified by its association with high-risk E6 proteins in the degradation of p53 (Huibregtse et al., 1991, 1993), it is not thought to be involved in p53 degradation in the uninfected cell. It has recently been reported (Mishra et al., 2008) that E6-AP is involved in the ubiquitin-mediated degradation of misfolded polyglutamine proteins, neuronal aggregates of which are responsible for the pathogenesis of a number of inherited neurodegenerative diseases such as Huntington's, Parkinson's, and Alzheimer's diseases (Dikshit and Jana, 2008). Thus, E6-AP may be one of a number of ubiquitin-protein ligases that, together with molecular chaperones, are thought to protect against such toxic protein aggregates (Hinault et al., 2006).

In uninfected cells, p53 levels are normally controlled by proteasomal degradation using the MDM2 ubiquitin-protein ligase (Honda et al., 1997), but this pathway appears to be nonfunctional in HPV-positive cells (Hengstermann et al., 2001) and, indeed, the pattern of p53 ubiquitination induced by MDM2 and by E6/E6-AP appears to be completely different (Camus et al., 2003).

It has been reported that mice with mutations in E6-AP show increased levels of p53 in neuronal cells (Jiang et al., 1998b; Mishra and Jana, 2008), but in human cells E6-AP has not been shown to be involved in the degradation of p53 in the absence of E6. In contrast, E6-AP has been shown to recognize both Bak and c-Myc, and binding of E6 to E6-AP appears to enhance the normal pathways by which the levels of these proteins are controlled (Thomas and Banks, 1998; Gross-Mesilaty et al., 1998). Interestingly, E6's binding to E6-AP appears to enhance E6-AP self-ubiquitination (Kao et al., 2000), perhaps as a feedback control. A number of studies using dominant-negative mutants of E6-AP (Talis et al., 1998), or antisense oligonucleotides to block E6-AP function (Beer-Romero et al., 1997; Traidej et al., 2000), only detected increased p53 levels in HPV-positive cells.

E6-AP-Associated Targets

HPV E6 binds to E6-AP through a linear helical motif on E6-AP, with a hydrophobic patch along one side that is essential for E6 binding (Elston et al., 1998; Chen et al., 1998; Be et al., 2001). Transgenic mice expressing an E6 that is mutant in this motif show lower levels of spontaneous skin tumors and other skin pathologies than mice expressing a wild-type HPV-16 E6 (Nguyen et al., 2002). However, the use of synthetic peptides showed that this core motif only binds weakly to E6 and that the binding is stabilized by contacts made in other parts of the protein (Sterlinko Grm et al., 2004), which would correlate well with NMR analysis of the E6 protein structure mentioned above (Nominé et al., 2003, 2005). In addition, these E6-AP peptides blocked E6-induced degradation of p53 but did not affect E6-p53 binding, suggesting that E6-AP is not required for the physical interaction of E6 with p53. This was confirmed using E6 and p53 expressed in wheat germ extract, in which no E6-AP is present (Sterlinko Grm and Banks, 2004). The use of E6-AP-null mice showed that E6 can inhibit p53 induction by an E6-AP-independent pathway (Shai et al., 2007b) and that E6 can induce the proteasome-dependent degradation of p53 in the absence of E6-AP (Massimi et al., 2008). Equally, it has been shown that HPV-16 E6 can induce the degradation of p53 in the absence of ubiquitination (Camus et al., 2007). Thus, while E6-AP is obviously important in the pathogenic effects of E6, it is by no means the only player in the game. Intriguingly, recent studies also suggest that the stability of E6 is very heavily dependent upon E6-AP. Thus, in the absence of E6-AP, E6 degradation is increased at the proteasome (Tomaiü et al., in press). The molecular basis behind this is still unclear, but it does demonstrate that there is a much more complex interaction between E6 and E6-AP than was previously thought.

Targeting Apoptotic Effectors

Apoptosis, or programmed cell death, is a process that is hardwired in the cell. It is essential for the correct development and homeostasis of multicellular organisms (Strasser et al., 1994; Jacobson et al., 1997), and acts as a defense against infection and neoplasia (Thompson, 1995).

One of the major control points prior to irreversible apoptosis is controlled by the proteins of the Bcl-2 family. This is a large group of proteins with, variously, pro- or antiapoptotic activities, and their interactions determine whether or not the cell enters apoptosis (reviewed in Danial, 2007; Youle and Strasser, 2008). Bearing in mind the functional conservation between various DNA tumor virus proteins, it is perhaps not surprising that a number of DNA tumor viruses impinge on this pathway (D'Agostino et al., 2005). In particular, the Ad E1B-19k protein can act as an antiapoptotic member of the Bcl-2 family (Huang et al., 1997; White, 2001, for review), while the HPV E6 proteins target the proapoptotic Bak for degradation (Thomas and Banks, 1998, 1999). Mutants of Bak that cannot bind E6 are resistant to degradation and have a considerably extended half-life even in the absence of HPV E6; it therefore seems likely that E6 enhances the normal turnover of Bak in the cell. Interestingly, the equally proapoptotic Bax does not appear to be a target for degradation, probably because it is induced by p53 (Miyashita and Reed, 1995) via another Bcl-2 family member PUMA (Vogt et al., 2006) and is thus found at very low levels in E6-expressing cells. In addition, Bak,

rather than Bax, is expressed at high levels at the site of HPV replication in the upper epithelial layers, (Krajewski et al., 1994, 1996). It has been shown that the E6 proteins of cutaneous HPV types can also target Bak for degradation (Jackson et al., 2000), and that Bak levels do not rise in HPV-containing epithelium upon ultraviolet B (UVB) irradiation (Jackson et al., 2000, 2002; Leverrier et al., 2007). Thus, Bak is a target of high- and low-risk, mucosal and cutaneous, HPV types, indicating that this is a highly conserved function of the HPV E6 proteins. Recent work has also shown the down-regulation of Bak in HPV-positive laryngeal cancer cells (Du et al., 2004; Manjarrez et al., 2006; Liu et al., 2008). Since many of the Bcl-2 family proteins' functions are controlled by homo- or heterodimerization between the protein family members, it is clear that E6 activity, by slightly altering the balance of key equilibria, might have quite fundamental effects on the fate of the host cell.

E6 has also been shown to circumvent apoptotic signals independent of the Bcl-2 family. It can induce NF-κB activity, resulting in the up-regulated expression of cIAP-2, which binds and inhibits the executioner caspases, 3, 7 and 9 (James et al., 2006). It has also been reported that the HPV-16 E6 can induce the degradation of both the FADD (*F*as-*a*ssociated *d*eath *d*omain) death effector, to inhibit Fas signaling (Filippova et al., 2004), and the procaspase 8, to inhibit TRAIL signaling (Garnett et al., 2006; Filippova et al., 2007), suggesting that there may be multiple ways in which E6 inhibits apoptosis.

Targets of E6 with PDZ Domains

PDZ domains are stretches of 80–90 amino acids (Ponting and Philips, 1995; Fanning and Anderson, 1999) involved in protein–protein interactions. There are several classes of PDZ-binding ligands, the most common of which contain the class 1 PDZ-binding motif, which consists of four amino acids (x-T/S-x-L/V), usually found at the carboxy-terminus of interacting proteins (Songyang et al., 1997); although internal class 1 ligands have been described, they are rare (Harris and Lim, 2001).

The high-risk mucosotropic HPV E6 proteins have PDZ binding motifs at their extreme carboxy-termini and, of all the protein interaction domains of E6, this is the only one that is strictly high-risk specific: All the other activities of E6 can be detected, to some extent, in both high- and low-risk HPV E6 proteins. Indeed, a number of studies in different systems have shown that loss of the PDZ-binding motif severely impairs the cell transformation activities of E6 (Nguyen et al., 2003a; Lee and Laimins, 2004; James et al., 2006; Shai et al., 2007a,b; Spanos et al., 2008a). Figure 3.2 is a comparison of the carboxy-termini of representative low- and high-risk virus types.

The MAGUK family (*m*embrane-*a*ssociated *gu*anylate *k*inase homologues) is a group of large proteins containing multiple interaction domains, including PDZ domains, and are thought to act as molecular scaffolds to mediate the formation of multiple-protein complexes. MAGUKs are detected on the cell membrane, often at regions of cell-cell contact, including subapical tight junctions (Ide et al., 1999; Mino et al., 2000; Kawabe et al., 2001), lateral adherens junctions (Nishimura et al., 2000; Firestein and Rongo, 2001), and the apical junctions of *Caenorhabditis elegans* (Köppen et al., 2001; McMahon et al., 2001). Indeed, cells expressing E6 were shown to form weaker cell contacts and grow in a more disorganized fashion, whereas cells expressing E6 mutants defective for PDZ protein interactions retained normal aspects of cell polarity (Nakagawa and

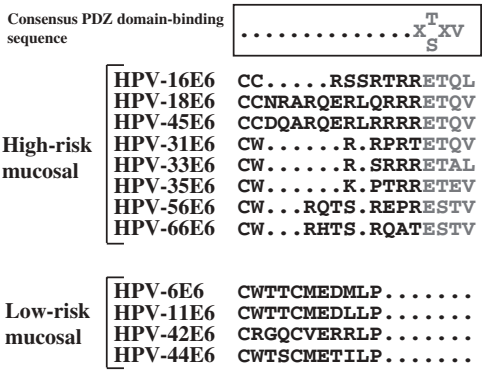

Figure 3.2. An alignment of the carboxy-terminal sequences of high- and low-risk HPV types.

Huibregtse, 2000). Because of their location, it is thought that they may be involved in both intercellular and intracellular signaling, particularly since some also have nuclear localization signals (Dobrosotskaya et al., 1997). Additionally, as well as high-risk E6, PDZ-binding motifs have been found in a number of other viral oncogenes, including the HTLV-1 Tax protein and the Ad9 *E4ORF1* gene product (Kiyono et al., 1997; Lee et al., 1997). Table 3.1 shows the PDZ-containing targets of E6 that have so far been identified; a number of these will be discussed below.

The MAGI Family. The MAGI family of proteins are *MAG*UKs with *I*nverted domain structure, having their guanylate kinase homology domains at the amino terminal end of the polypeptide. In addition, MAGI-1, MAGI-2, and MAGI-3 each have WW domains and at least five PDZ domains (Dobrosotskaya et al., 1997; Wu et al., 2000a,b), which gives them the potential to interact with a large number of other proteins, including β-catenin (Dobrosotskaya and James, 2000), mNET-1 (Dobrosotskaya 2001), and PTEN (Wu et al., 2000a,b; Kotelevets et al., 2005).

The MAGI proteins are targets for the Ad9 E4ORF1 and HPV E6 proteins (Glaunsinger et al., 2000; Pim et al., 2000; Thomas et al., 2001, 2002). In addition, MAGI-1 and MAGI-3, but not MAGI-2, have been identified as targets for the HTLV-1 Tax protein (Ohashi et al., 2004). The MAGI-1 is aberrantly sequestered in the cytoplasm by Ad9 E4ORF1 (Glaunsinger et al., 2000), as is MAGI-3 by HTLV-1 Tax (Ohashi et al., 2004). However, as with p53, the MAGI-1, MAGI-2, and MAGI-3 proteins are targeted by high-risk HPV E6s for ubiquitin-mediated degradation (Glaunsinger et al., 2000; Thomas et al., 2002). The functions of the MAGI proteins are not yet entirely clear, but they appear to be many and varied, depending upon the cell type studied. They have been reported to interact with the PTEN tumor suppressor (Wu et al., 2000a,b; Kotovelets et al., 2005), and MAGI-1 can suppress immortalization in an oncogene cooperation assay in primary rodent cells (Massimi et al., 2004). In addition, MAGI-3 is reported to bind the lysophosphatidic activated receptor LPA(2)

TABLE 3.1 PDZ-Dependent Activities of E6

Protein	Function	Result of E6 Interaction	Reference
Dlg	Polarity/tumor suppressor. **AJ**	Ubn & degradation	Gardiol et al. 1999
Scribble	Polarity/tumor suppressor. **AJ**	Ubn & degradation	Nakagawa & Huibregtse 2000
MAGI-1 **MAGI-2** **MAGI-3**	Polarity/tumor suppressor. **TJ**	Ubn & degradation	Glaunsinger et al. 2000 Thomas et al. 2002
MUPP1	Signalling complex scaffold. **TJ**	Ubn & degradation	Lee et al. 2000
PATJ	**TJ** formation and integrity	Ubn & degradation + E6*-directed degradation	Latorre et al. 2005 Storrs & Silverstein 2007
PSD95	Signalling complex scaffold. **SJ**	Ubn & degradation	Handa et al. 2007
PTPH1/ PTPN3	Protein tyrosine phosphatase	Ubn & degradation	Jing et al. 2007 Töpffer et al. 2007
PTPN13	Non-receptor phosphatase	?	Spanos et al. 2008b
TIP-1	-ve regulator of PDZ interactions	?	Hampson et al., 2004
TIP-2/ GIPC	TGFβ signalling	Ubn & degradation	Favre-Bonvin et al. 2005
CAL	Intracellular trafficking	Ubn & degradation	Jeong et al. 2007

AJ, adherens junction; TJ, tight junction; SJ, synaptic junction; Ubn,ubiquitination.

to enhance Erk and RhoA activation (Zhang et al., 2007a), with potential downstream effects on cell survival signaling. The interaction of HPV-18 E6 with MAGI-1 appears to be the strongest E6–PDZ interaction.

MUPP1. MUPP1 is another PDZ-containing target of Ad9 E4ORF1 and HPV E6 (Lee et al., 2000). MUPP1 is a *mu*ltiple *P*DZ-containing *p*rotein, having 13 PDZ domains (Ullmer et al., 1998), and it is thought to be involved in the selective targeting and assembly of signaling complexes (Becamel et al., 2000). In polarized epithelial cells, it is localized exclusively at tight junctions through the coxsackie and adenovirus receptor (CAR) (Coyne et al., 2004), where it interacts with members of the claudin and JAM (*j*unctional *a*dhesion *m*olecule) families and is thought to be involved in cell polarity signaling, tight junction barrier function, and responses to osmotic stress (Hamazaki et al., 2001; Kimber et al., 2002; Jeansonne et al., 2003; Lanaspa et al., 2007).

hDlg and hScrib. Perhaps the most important of E6's known PDZ-containing targets are Dlg and hScrib (Gardiol et al., 1999; Nakagawa and Huibregtse, 2000; Massimi et al., 2004; Thomas et al., 2005). These proteins are both classified as potential tumor suppressor proteins, based on their growth suppressor activities in *Drosophila* and in mammalian cells (Goode and Perrimon, 1997; Bilder, 2004; Massimi et al., 2004). Dlg and Scribble function together with the Lgl protein as a tripartite complex

involved in the control of cellular apicobasal polarity (reviewed in Humbert et al., 2003; Assémat et al., 2007). This complex has been demonstrated genetically in *Drosophila* (Bilder et al., 2000; Albertson and Doe, 2003; Tanentzapf and Tepass, 2003).

Dlg is classified as a tumor suppressor since its deletion in *Drosophila* results in aberrant cytoskeletal organization and disruption of polarity in columnar epithelium (Goode and Perrimon, 1997; Bilder et al., 2000). Its role in maintaining cell polarity has been separated, by mutational analysis, from its role in controlling cellular proliferation (Woods et al., 1996). In complex with the adenomatous polyposis coli (APC) protein, human Dlg represses the G1 to S transition (Ishidate et al., 2000), and its overexpression results in the down-regulation of β-catenin, and hence cell growth, via an APC-independent pathway (Hanada et al., 2000). Dlg also colocalizes with APC at the tips of cellular protrusions in epithelial cells in culture (Iizuka-Kogo et al., 2005). HTLV-1 Tax protein has also been shown to bind Dlg and to counteract these activities (Suzuki et al., 1999). Unlike the Tax and E4ORF1 proteins, E6 is expressed at very low levels and cannot sequester Dlg; however, it can target Dlg for ubiquitin-mediated degradation (Gardiol et al., 1999). Dlg possesses three PDZ domains and, interestingly, the ability of E6 to target Dlg for degradation requires PDZ domain 2, which is the PDZ domain recognized by APC (Gardiol et al., 2002). This raises the possibility that E6, by degrading Dlg, might interfere with the normal functioning of the APC pathway which, together with p53, is one of the most common pathways to be perturbed during the development of colon cancer (Kinzler and Vogelstein, 1996; Morin et al., 1996). Work on transgenic mice shows that expression of HPV-16 E6 in the ocular lens results in multilayering and disorganization of the epithelium and an increased number of undifferentiated, nucleated cells; an effect similar to that seen when mutant Dlg is expressed in the same system (Nguyen et al., 2003b). Interestingly, E6 appears to preferentially target nuclear phosphorylated forms of Dlg (Mantovani et al., 2001; Massimi et al., 2004, 2006), which suggests that a signaling subset of Dlg may be the true target of E6, whereas the disruption of the polarity control complex is a, fortunately rare, side effect. Indeed it would appear that the role of the PDZ ligand of E6 is directed toward a function regulating cell proliferation since both transgenic mice and keratinocytes expressing E6 mutated within the PDZ ligand exhibit greatly reduced proliferation compared with those expressing wild-type E6 (Nguyen et al., 2003a; Lee and Laimins, 2004; James et al., 2006; Shai et al., 2007a; Spanos et al., 2008a). Intriguingly, PDZ binding appears also to play a critical role in the normal viral life cycle, but in a very unexpected manner. In keratinocyte cultures, HPV DNA can be maintained as stably replicating episomes. However, this ability is lost if mutations are made in the HPV E6 PDZ binding motif, in the context of the whole viral genome (Lee and Laimins, 2004). This suggests that E6 interaction with at least one of its PDZ-containing targets is required for episomal maintenance.

Dlg plays a part in a highly complex series of protein interactions that regulate the development and control of epithelial cell polarity (Bilder and Perrimon, 2000; Bilder et al., 2003; Tanentzapf and Tepass, 2003; Bilder, 2004). Proteins of this group are highly conserved in higher eukaryotes and the mammalian homologues of Dlg, Scribble, and Lgl have been shown to complement their lack in mutant *Drosophila* (Thomas et al., 1997; Dow et al., 2003; Grifoni et al., 2004).

The high-risk HPV E6s can also induce the degradation of hScrib (Nakagawa and Huibregtse, 2000), the human homologue of *Drosophila* Scribble. hScrib is a member of the LAP (*l*eucine-rich *a*nd *P*DZ domain) family of proteins, having four PDZ domains and a leucine-rich domain that is required for its basolateral localization in epithelial cells (Kim, 1997; Bilder and Perrimon, 2000; Bryant and Huwe, 2000; Legouis et al., 2003). Studies in *Drosophila* have shown that Scribble and Dlg can at least partly complement each other (Bilder et al., 2000) and that, with the Lgl protein, they form a complex that is required for the maintenance of epithelial polarity (reviewed in Humbert et al., 2003; Assémat et al., 2007), and expression of both proteins is altered in HPV-induced tumors (Watson et al., 2002, 2003; Cavatorta et al., 2004; Nakagawa et al., 2004). Interestingly, similar alterations in the expression patterns of these proteins have been correlated with the degree of malignant transformation in colon and endometrial cancers (Schimanski et al., 2005; Gardiol et al., 2006; Kamei et al., 2007; Tsuruga et al., 2007) and in melanomas (Kuphal et al., 2006), all of which are unrelated to HPV, again showing that the tumor viruses can act as models for mechanisms of transformation in general. Although Dlg and hScrib are both required, with Lgl, for the fully effective functioning of the polarity-determining complex that they form (Bilder et al., 2003; Humbert et al., 2003), there appear to be differences in their recruitment. In synaptic development, which is very similar to epithelia in many respects, Dlg localizes correctly to the region just basal to the zonula adherens where the Dlg/Scribble/Lgl complex forms (Johnson and Wodarz, 2003; Tanentzapf and Tepass, 2003). Scribble, on the other hand, can localize to the basolateral membrane, but cannot colocalize with Dlg in the absence of the GuKholder protein, which binds the Scribble PDZ2 and the GuK domain of Dlg and brings them together in the complex (Mathew et al., 2002: Qian and Prehoda, 2006). Thus, in the absence of Scribble (equivalent to the presence of 16E6), Dlg can form a partial complex, whereas in the absence of Dlg (equivalent to the presence of 18E6), no complex can form. If the complex forms similarly in epithelial cells, it throws new light on studies indicating that HPV-18-containing cervical tumors are more prone than HPV-16-containing tumors to recurrence and to metastasis (reviewed in Franco, 1992). This finding correlates different clinical phenotypes with the defined biochemical activities of proteins from different, but closely related, HPV types, and indicates that the E6-induced control of PDZ protein levels within the cell could be fundamental in the progression to malignancy.

Other PDZ-Containing Targets of E6. In addition to the PDZ-containing targets of E6 discussed above, a number of other targets have been more recently reported, and these are summarized in Table 3.1. The majority of studies performed on the E6–PDZ interaction have concentrated upon the E6 proteins of HPV-16 and HPV-18, and it is probable that this is, as yet, an incomplete record since the PDZ domains of other high-risk HPV types appear to be diverse in their structure, and this may be reflected in the nature of their targets.

PDZ Ligand Specificity. As mentioned above, it is clear that the binding of HPV E6 proteins to PDZ-containing targets is not uniform. Indeed, the PDZ-binding motifs of the E6s from different HPV types are quite varied, as can be seen in Figures 3.2 and 3.3.

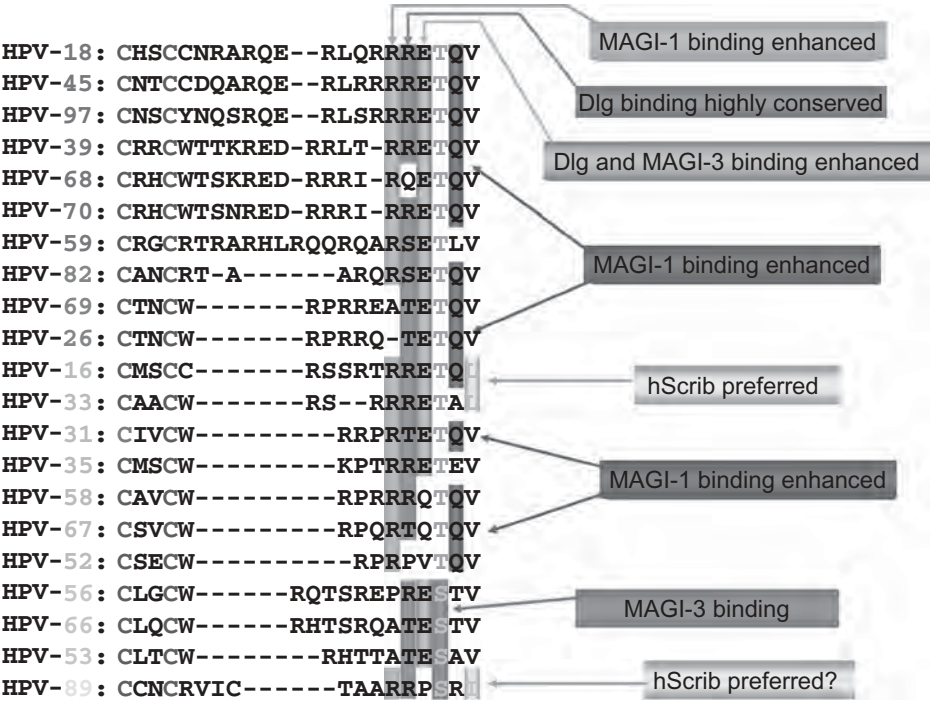

Figure 3.3. An alignment of the PDZ domains of a number of high-risk HPV types, together with the suggested target preferences.

It has long been known that HPV-18 E6 binds more strongly than HPV-16 E6 to Dlg and to MAGI-1, and induces their degradation with greater efficiency (Pim et al., 2000; Thomas et al., 2001). Exchanging the last amino acid residue of each E6 protein from the HPV-18 ETQV to the HPV-16 ETQL (and vice versa) reverses this. It is also clear that the HPV-16 E6 binds more strongly than HPV-18 E6 to hScrib and, equally, is more effective in inducing its degradation (Thomas et al., 2005); thus, the exact sequence of the PDZ-binding motif is instrumental in substrate selection. An exciting extension to this line of research came with the description of the crystal structures of the MAGI-1 PDZ1 and the Dlg PDZ2 and PDZ3 bound to a peptide ligand homologous to the HPV-18 E6 C-terminus (Zhang et al., 2007b). The Dlg PDZ3 is not actually a target of HPV-18 E6 *in vivo*, and it was interesting that it had a bare minimum of binding contacts with the peptide, in contrast to the MAGI-1 PDZ1 and Dlg PDZ2, which showed a number of additional contacts, including those with amino acid residues *outside* the canonical PDZ-binding motif. Based on these findings, we have made a series of mutants through the carboxy-terminus of 18-E6 and performed a number of biochemical and modeling analyses of their binding activities (Thomas et al., 2008). The results of this study give insights into each of the two interlocking sides of the interaction story. One side of this story depends, obviously, on the type of PDZ-binding motif, but in addition the exact sequence, not only of the motif itself, but also

of the upstream and noncanonical amino acid residues, makes a considerable contribution to the specificity of substrate selection. On the other side of the story, by comparing different but very closely related PDZ domain-containing substrates (the Dlg PDZ2 and PDZ3, and the PDZ1 domain of MAGI-1 and MAGI-3), we have shown how the ligand selection can be determined and modulated by the amino acid sequences of the PDZ domains themselves. By integrating different experimental approaches, we have begun to unravel the complicated means by which two molecules, already mutually predisposed by their binding sites, select their appropriate partners for the molecular dance in progress.

A Question of Specificity. It is clear from the discussion above that there are a number of constraints on the specificity of the E6–PDZ interaction. Analysis of the PDZ domains from E6 target proteins showed that only one domain per protein is recognized by HPV E6 (Lee et al., 2000; Thomas et al., 2001, 2002, 2005; Gardiol et al., 2002). Specific binding to PDZ domains has also been shown with β-catenin, which binds MAGI-1 PDZ5 (Dobrosotskaya and James, 2000), with the tumor suppressor PTEN, which binds to PDZ2 of MAGI-2 and MAGI-3 (Wu et al., 2000a,b), and with mNET1, which binds to PDZ1 of MAGI-1 (Dobrosotskaya, 2001). The GuKholder also binds specifically only to the PDZ2 domain of the Scribble protein (Mathew et al., 2002) This indicates that very specific blocking of E6's PDZ binding might be possible without affecting other PDZ domain interactions. Indeed, exogenously expressed MAGI-3 PDZ1 has been shown to block the E6-induced degradation of Dlg, MAGI-2, and MAGI-3, thereby confirming the feasibility of this approach (Thomas et al., 2002).

The recent structural analyses of the E6 protein have confirmed the importance of the overall protein structure and the conservation of the zinc-binding fingers (Lagrange et al., 2005; Nominé et al., 2005; Zanier et al., 2005, 2007). Meanwhile, studies focused on the C-terminal PDZ-binding region of E6, and its ability to form interactions with both target and nontarget PDZ domains of Dlg (Zhang et al., 2007b), have shown that, in addition to the conventional four amino acid binding motif (von Ossowski et al., 2006), E6 uses additional amino acid residues for selective PDZ domain binding (Liu et al., 2007; Zhang et al., 2007b).

The importance of these peripheral amino acid sequences, both within the PDZ domain itself and in the E6 ligand, has since been confirmed by structural and biochemical assays and by modeling (Zhang et al., 2007b; Thomas et al., 2008). As a result of such analyses, it may be possible to predict the preferred PDZ-containing targets of different high-risk HPV types, and possibly to correlate this with the various pathologies of these different types. In Figure 3.3, we show an initial attempt at such an analysis.

Other Specificity Controls

There are other factors that can determine the specificity of E6 interactions. We discussed earlier the possibility that a certain amount of p53 might be required during the replication of viral DNA; obviously, if that is the case, there must be a mechanism for

preventing certain E6 interactions. The E6* proteins provide a potential means of doing this; they are abundant, alternatively spliced transcripts of the high-risk *E6* gene (Smotkin et al., 1989). They can bind to E6-AP or heterodimerize with E6, blocking their interaction (Pim et al., 1997; Pim and Banks, 1999), and thus preventing their inducing, or enhancing, the degradation of E6/E6-AP target proteins, of which p53 is one.

The E6* Proteins. Low-risk mucosotrophic HPVs transcribe their early regions from two promoters, one transcript encoding E6, the other E7, whereas high-risk types have only one early promoter and transcripts from this are multiply spliced and encode both E6 and E7. All high-risk mucosal HPVs have a highly conserved splice donor site within the body of the E6 open reading frame (ORF), and most, although not all, also have a conserved splice acceptor site within the E6 ORF that lies before the start of E7 and leads to the removal of a small intron within the E6 ORF. Removal of this intron has been proposed to enhance E7 translation (Tang et al., 2006). However, the conserved first splice acceptor is absent from the E6 of some high-risk types, suggesting that this splicing event may have other functions, including expression of the so-designated E6* mRNAs as protein. In Figure 3.4, we show the splicing and protein structure of E6*. Analysis of many cell lines derived from cervical tumors has shown that the majority of early transcripts are of this spliced form and encode E6* (Schneider-Gädicke and Schwarz, 1986; Smotkin and Wettstein, 1986), and transient transfection of plasmids expressing HPV-16 or HPV-18 E6 into mammalian cells typically results in the expression of very high levels of spliced mRNA. When tagged at the amino terminus, high levels of E6* protein are detectable, indicating that E6*, as well as E7, is likely to play a role in the viral life cycle. Previous studies have shown that HPV-18 E6* interacts *in vitro* with full-length HPV-18 and 16 E6 and with E6-AP, but not with p53 (Pim et al., 1997). As a consequence, E6* can inhibit the E6-directed degradation of p53 *in vitro*, and *in vivo* when all three proteins are overexpressed in p53-null human and mouse cells (Pim et al., 1997). This observation suggests a possible mechanism

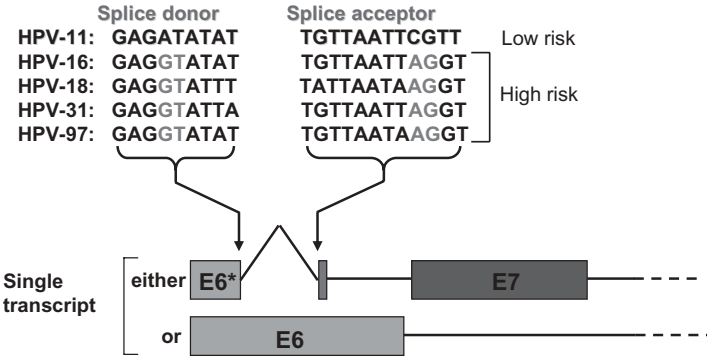

Figure 3.4. The splicing of the E6ORF and the structure of the E6* protein. Full-length E6 is included for comparison.

for blocking the E6-directed degradation of p53 during the viral life cycle, although the consequent increase in p53 levels might also activate p53-dependent apoptosis (Pim and Banks, 1999).

Other functions of E6* have been suggested, including the activation of proteolytic degradation of PDZ protein PATJ in an E6-AP-independent manner (Storrs and Silverstein, 2007). Interestingly, PATJ is also a target of the full-length E6 and E6-AP (Latorre et al., 2005; Storrs and Silverstein, 2007), and it has been suggested that separate cellular subsets of the protein might be targeted either by E6 or by E6* (Storrs and Silverstein, 2007).

Our recent studies would also suggest that E6* and E6 may localize to different cellular compartments, at least under certain conditions (Pim et al., in press). We have identified certain targets of E6* that are not recognized by E6, such as the Ser/Thr kinase Akt, and we have also identified a number of proteins that are targeted by both E6 and E6*, including Dlg. No direct interaction between E6* and the degraded targets has yet been demonstrated, but we have shown that Dlg, when phosphorylated along the p38 MAPK pathway, is resistant to E6-directed degradation, but is still sensitive to degradation in the presence of overexpressed E6*. However, the precise pathway involved in E6*-dependent degradation is, as yet, unclear.

It is apparent that the functions of both E6 and E6* need to be considered when discussing their target proteins and their activities within the cell. Alterations in the ratio between the two proteins, at specific times, could markedly affect the choice of target proteins and the outcome of the targeting. It is clear that there are cell cycle differences in the E6 : E6* ratio in CasKi cells, with E6* levels rising at G2/M (Guccione et al., 2004). It is also probable that the E6 : E6* ratio (or cell cycle patterns thereof) may be very different in a productive infection and in a transformed cell. It could be speculated that the long-term, persistently infected cell, which is thought to be likely to proceed to malignancy, might exhibit an E6 : E6* ratio that is midway between the two, or that might alter during malignant progression; such changes could conceivably be used as markers of malignancy. Systematic studies in this area are only just beginning, but they have the potential to give important insights into the biology of malignancy.

The importance of the E6* proteins also underlies a point made earlier with respect to the structure of E6. We said that care was needed in interpreting the effects of mutations in E6 since they might have global effects on E6 structure, and hence its activities. In this case, we would emphasize that mutations affecting either splice donor or splice acceptor sites could alter the ratios of full-length and spliced E6 in ways that are only just beginning to be appreciated.

CONCLUSIONS

Huge numbers of studies have been performed over the years to investigate the cellular pathways whose disruption leads to disease, in the hope that through understanding will come solutions. In that effort, the DNA tumor viruses have been immensely

powerful tools. These organisms, whose evolutionary thrust has been to use cellular mechanisms for their own reproduction, have acted fortuitously as signposts, indicating the cellular pathways that are required for cellular growth control. The study of DNA tumor viruses for their own sake has thrown new light onto the mechanisms of cell cycle control, apoptosis, polarity signaling, and many other fundamentals required for the maintenance of a healthy cell in a healthy organism. The data discussed here suggest that targeting HPV E6 might allow this information to be used in the treatment of a major human cancer.

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4

JC VIRUS ASSOCIATION WITH BRAIN TUMORS: THE ROLE OF T ANTIGEN AND INSULIN-LIKE GROWTH FACTOR 1 IN DNA REPAIR FIDELITY

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HUMAN NEUROTROPIC VIRUS JC VIRUS (JCV): STRUCTURE AND BIOLOGICAL FACTS

The family of polyomaviruses includes more than 13 members. Among them, three viruses, JC virus (JCV), BK virus (BKV), and simian virus 40 (SV40), are known to infect and cause disease in humans. JCV, in particular, has been associated with brain pathology since its discovery and characterization in the mid-1950s and early 1960s, respectively. JCV is a small, nonenveloped virus approximately 38–40 nm in diameter and covered by an icosahedral capsid. It is a DNA virus with a circular, closed, supercoiled, double-stranded genome that consists of 5130 nucleotides. The genome can be divided into three regions, a viral regulatory region, which contains the sites for initiation of replication and transcription, and is located between an early transcriptional region and a late transcriptional region. The early region encodes for two proteins, small and large T antigens, and the late region encodes for the capsid proteins, VP1, VP2, and VP3, as well as a small accessory polypeptide denominated agnoprotein (Frisque et al., 1984). With 360 copies, VP1 is the most common of the capsid proteins and is arranged in 72 pentamers, which gives the capsid an icosahedral structure (Salunke et al., 1986).

Primary infection with JCV is usually asymptomatic and occurs in early childhood as demonstrated by seroepidemiological studies, in which specific neutralizing

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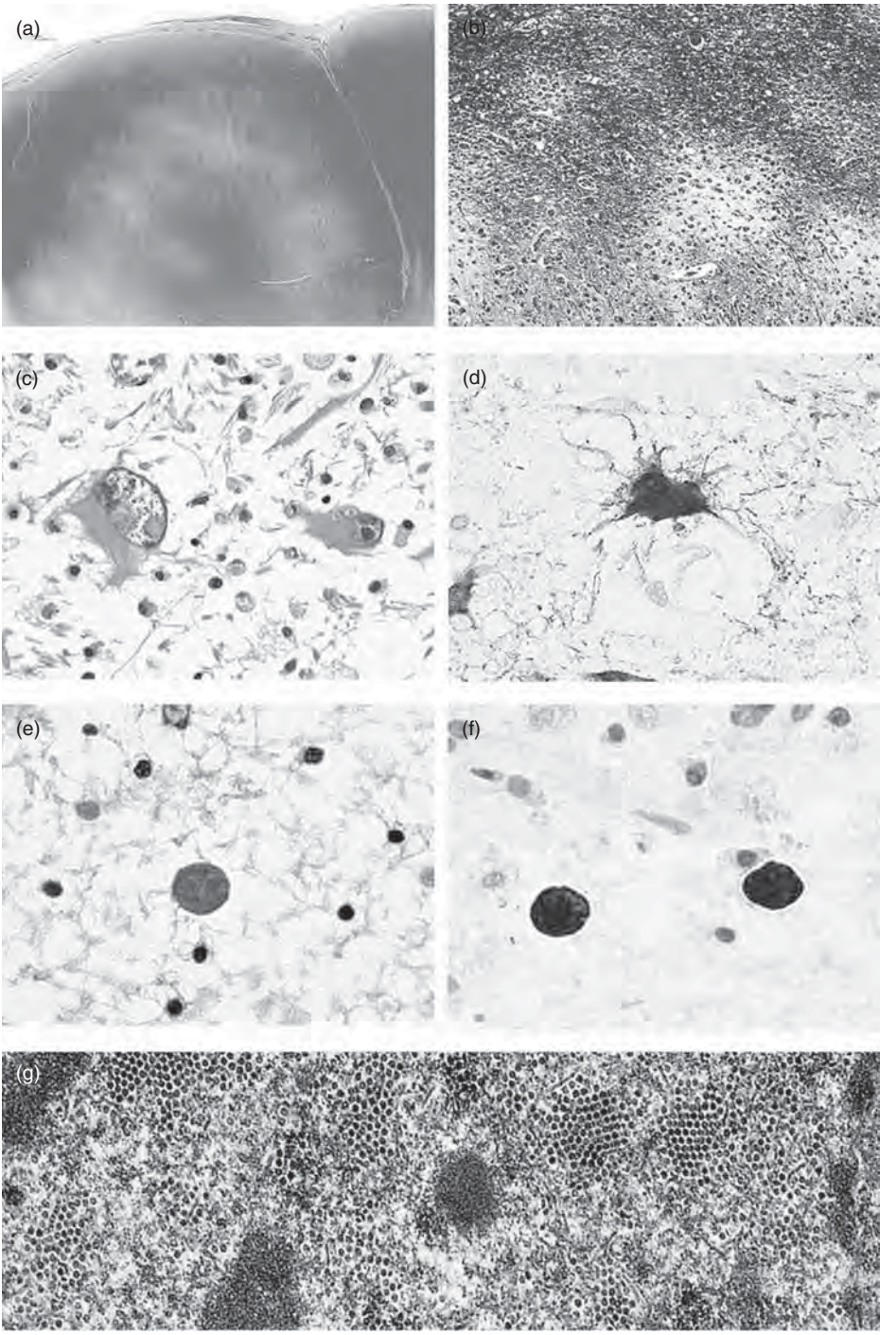
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antibodies can be detected in approximately 10% of children by age 5, but in as many as 65% by age 16. In adults, the percentage of seropositive individuals increases up to 85%–90% (Walker and Padgett, 1983; Del Valle et al., 2001b; Khalili and Stoner, 2001), indicating the widespread scale of infection. Two routes of infection have been postulated: respiratory, based on the finding of JCV genomic sequences in epithelial cells of the respiratory tract and in lymphocytes of the Waldeyer's ring (Monaco et al., 1998; Wei et al., 2000); and gastrointestinal, where genomic sequences have been isolated from epithelial cells of the upper and lower digestive tract (Laghi et al., 1999; Ricciardiello et al., 2001). The primary infection is subclinical, and it is accepted that the virus remains in a latent state in the kidney until immunosuppressive states result in viral reactivation, as demonstrated by the detection of viral particles in the urine of pregnant women or after renal transplant (Gardner et al., 1971; Coleman et al., 1977). However, under severe immunosuppressive conditions, the reactivation of JCV results in the demyelinating disease progressive multifocal leukoencephalopathy (PML) (Berger and Concha, 1995; Clifford, 2001). This fatal disease is the result of the productive infection and lytic destruction of oligodendrocytes. From the histopathological point of view, PML is characterized by three specific characteristics: first, multiple foci of myelin loss, denominated plaques, which are frequently confluent and in advanced stages of the disease can result in cavitations; second, enlarged oligodendrocytes harboring intranuclear inclusion bodies, which represent the site of active viral replication as demonstrated by electron microscopy studies; and third, bizarre astrocytes, which have an atypical appearance with pleomorphic cytoplasms and hyperchromatic, often multiple, nuclei (Del Valle and Piña-Oviedo, 2006). Figure 4.1 illustrates the histopathology of PML.

Once JCV was successfully isolated in culture (Padgett et al., 1971), its transforming properties *in vitro* have been well documented (Brodsky and Pipas, 1998; DeCaprio, 1999). Cell cultures exposed to JCV T antigen undergo distinct morphological changes, frequently become multinucleated, replicate at a high speed, have prolonged life spans, and possess the ability to form dense foci in culture—all characteristics of transformed phenotypes. Transformation of primary hamster brain cells by various strains of JCV, including Mad-1 and Mad-4, results in rapidly growing cells with several characteristics of malignancy, including growth in low-serum conditions, enhanced production of plasminogen activator, and anchorage-independent growth.

Figure 4.1. Histopathology of progressive multifocal leukoencephalopathy (PML). (a) Luxol fast blue, a special staining for myelin, demonstrates multiple and confluent foci of demyelination, one of the characteristic features of PML. (b) Plaques of myelin loss are shown at higher magnification (H&E). (c) Bizarre astrocytes with pleomorphic shapes and multiple nuclei are frequently seen within the demyelinated plaques mixed with foamy macrophages (H&E). (d) Immunohistochemistry for the JCV capsid protein VP1 demonstrates active viral activity in bizarre astrocytes. (e) The pathognomonic feature of PML is the enlarged oligodendrocyte harboring intranuclear eosinphillic inclusion bodies (H&E). (f) Expression of VP1 also demonstrates active viral replication. (g) Electron microscopy images from the nuclei of these inclusion-bearing oligodendrocytes shows characteristic icosahedral viral particles. See color insert.

JCV ONCOGENESIS IN ANIMAL MODELS

After the original description of PML (Åstrom et al., 1958) and the discovery of JCV particles in the nuclei of infected cells in cases of PML by electron microscopy (Silverman and Rubinstein, 1965; Zu Rhein and Chow, 1965), several series of animal experiments were carried out with the objective of characterizing PML as an infectious disease. The aim of these experiments was to reproduce demyelinated lesions after intracerebral inoculation of JCV particles in rodents and primates. Surprisingly, inoculation with JCV resulted, not in PML, but in the development of brain tumors of neuroectodermal and glial origin, providing the first clues onto the oncogenicity of JCV. For example, intracerebral inoculation of newborn Golden Syrian hamsters with JCV induced a broad range of tumors, including medulloblastomas, ependymomas, astrocytomas, and glioblastomas (Walker et al., 1973; Varakis et al., 1978; Zu Rhein and Varakis, 1979), and injection of JCV into the brains of newborn rats caused undifferentiated neuroectodermal tumors in 75% of animals (Ohsumi et al., 1986). Finally, intracerebral inoculation of JCV into owl and squirrel monkeys results in the development of astrocytomas (London et al., 1978, 1983). Unlike BKV and SV40, JCV is the only member of the polyomavirus family capable to induce tumors in nonhuman primates.

Later, to further reinforce the potential oncogenicity of JCV, and specifically of the early product T antigen, several lines of transgenic mice were created. Expression of T antigen in these mice is associated with the presence of neuroblastomas and mesenteric primitive neuroectodermal tumors (PNETs) (Small et al., 1986; Franks et al., 1996). In our laboratory, we have created two colonies of transgenic mice containing the early transcriptional region under the control of two promoters. In one colony, T antigen is driven by the regulatory region of the archetype strain (CY), and in the second by the promoter of the Mad-4 strain. A significantly high number of mice developed neuroblastomas (Small et al., 1986), and PNETs that resemble human medulloblastomas in the founder of the CY line (Krynska et al., 1999b). Progeny of these mice develop several phenotypes of brain tumors, all of neural tube origin, including PNETs, adrenal neuroblastomas, malignant peripheral nerve sheath tumors (MPNSTs), pituitary tumors, and glioblastomas (Del Valle et al., 2001b; Shollar et al., 2004; Piña-Oviedo et al., in press). Importantly, immunohistochemical studies demonstrate the expression of T antigen in neoplastic cells of all phenotypes of tumors, but not in any other tissue or organ, where the transgene is still present. Furthermore, in some of these tumors, we have characterized the binding and sequestration of p53 by T antigen. Figure 4.2 exemplifies some of these tumors developed in JCV transgenic mice.

JCV AND HUMAN BRAIN TUMORS

Even before the discovery of JCV, several reports demonstrated the association between PML and brain tumors. In one of the first original publications by Richardson, in which he characterized PML, one of the cases describes the postmortem examination of a 58-year-old man with chronic lymphocytic leukemia and PML, revealing the presence of an oligodendroglioma (Richardson, 1961). Later, a PML case was associated with

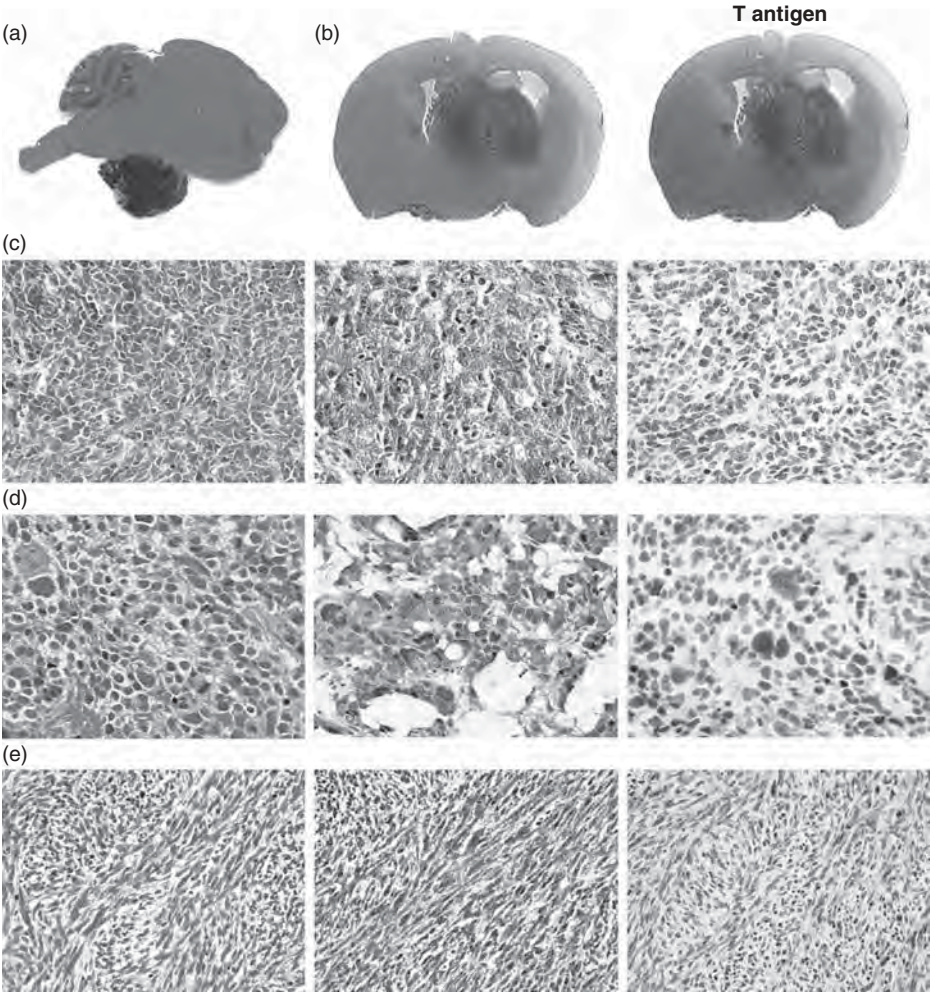

Figure 4.2. JCV-induced tumors in transgenic animals. Transgenic mice containing the early, T antigen-coding region of JCV driven by its own promoter develop several varieties of neural tube origin tumors, including PNETs, pituitary tumors, GBMs, and MPNSTs. (a) Montage of a mouse brain with a large pituitary tumor (H&E). (b) Montage of a transgenic mouse brain with a PNET located in the area of the basal ganglia, infiltrating the septum and protruding into the lateral ventricles (H&E). Immunohistochemistry for T antigen in a consecutive section demonstrates extensive expression of T antigen in the tumor but not in the normal adjacent brain. (c) PNETs are composed by numerous small cells with slightly elongated nuclei and scant cytoplasm (H&E). Immunohistochemical studies demonstrated the expression of class III β-tubulin and T antigen. (d) Pituitary tumors are composed of large pleomorphic cells with abundant cytoplasm and frequent giant nuclei (H&E). These tumors express prolactin. T antigen was detected in the nuclei of neoplastic cells. (e) MPNSTs are characterized by spindle cells arranged in interdigitating fascicles (H&E). Neoplastic cells express S100. Immunohistochemistry for T antigen demonstrates the activity of the transgene. See color insert.

multiple astrocytomas (Sima et al., 1983), and another with numerous foci of anaplastic astrocytes (Castaigne et al., 1974). More recently, Shintaku et al. (2000) reported a case of dysplastic ganglion like-cells in association with PML. Further detailed immunohistochemical studies of this case revealed that neoplastic cells that have differentiated to a neuronal phenotype can express JCV T antigen in the absence of capsid protein VP1 (Shintaku et al., 2000).

JCV has been also found in human brain tumors in the absence of PML lesions. In one of our more extensive works (Del Valle et al., 2001a), 85 clinical specimens of brain tumors from the United Kingdom, Greece, and the United States were examined for their possible association with JCV. Gene amplification was performed using three sets of primers that recognize JCV DNA sequences from the early and late regions and demonstrated presence of the viral early sequences in 49 out of a total 71 samples (69%). These multiple samples represented a wide variety of human brain tumors of glial origin, including oligodendroglioma, astrocytoma, pilocytic astrocytoma, oligoastrocytoma, anaplastic astrocytoma, anaplastic oligodendroglioma, glioblastoma multiforme (GBM), gliomatosis cerebri, gliosarcoma, ependymoma, and subependymoma. Importantly, results of immunohistochemical analyses demonstrated expression of JCV T antigen in the nuclei of tumor cells in 28 out of the 85 cases (32.9%). In a separate study, we have detected the presence of JCV in paraffin-embedded samples from medulloblastomas, the most common solid brain tumor in children (Del Valle et al., 2002c). JCV early gene sequences were amplified in 9 out of 20 (45%) cases of medulloblastoma. Polymerase chain reaction (PCR) revealed also the presence of viral DNA sequences of the agno region in 11 out of 16 samples (69%), and immunohistochemical analysis demonstrated the presence of T antigen and agnoprotein in the nuclei and cytoplasm of 11 out of 20 samples (55%), respectively. In another study, performed in a collection of oligodendrogliomas from Mexico and the United States, we obtained similar results with PCR amplification of genomic sequences from different regions of JCV, including the control region. Sequencing of these regions revealed that the tumors from the United States contained genomic fragments of the Mad-4 strain, while the tumors from Mexico contained fragments from the archetype region (Del Valle et al., 2002a). These observations provide new insights into the geographical distribution of JCV and make the possibility of laboratory contamination very unlikely.

Recently, JCV has been detected in a case of the most malignant intracranial neoplasm and one of the most aggressive in humans, GBM (Piña-Oviedo et al., 2006). In this case, the GBM showed regions with two patterns of differentiation, a classic GBM mixed with areas of a small cell tumor expressing PNET markers such as class III β-tubulin. Results from PCR in laser capture microdissected cells from the tumor revealed the presence of DNA sequences corresponding to the early, late, and control regions of the JCV genome in both phenotypes, and the expression of JCV proteins T antigen and agnoprotein, also in both phenotypes, but in a much higher number of cells in the primitive part of the tumor.

Although presented studies show association of JCV genome with human brain tumors, and the detection of JCV T antigen in human tumor cells (Del Valle et al., 2001a; Del Valle et al., 2002a), it is still a subject of debate whether indeed JCV contributes to the development of brain tumors in humans. This debate arises from ongoing

discrepancies in the percentages of positivity in DNA amplification and protein expression in different brain tumor studies, which range from 1% up to 45% (Reiss 2002; Delbue et al., 2005; Gordon et al., 2005; Rollison et al., 2005). We believe that the number of cases per study, which has been as low as five samples, as well as differences in the methodology, are contributing factors for these discrepancies (Rollison et al., 2005; White et al., 2005).

All together, however, the observations from clinical samples (Del Valle et al., 2001a, 2002a; Lassak et al., 2002), along with the data from cell culture and experimental animals (Krynska et al., 1999, 2000; Wang et al., 2001; Del Valle et al., 2002), provide solid evidence to support the involvement of JCV early proteins in the process of cellular transformation, and suggest a possibility that this human neurotropic polyomavirus may play an important role in the development and/or progression of human brain tumors. Figure 4.3 depicts some examples of the detection of JCV T antigen in human brain tumors.

JCV LARGE T ANTIGEN: INTERACTION WITH CELLULAR PROTEINS

While the mechanism or mechanisms responsible for cellular transformation by JCV are still under investigation, it is already well documented that large T antigen strongly contributes to this process via a direct interaction with host regulatory proteins (Sullivan et al., 2000). The high homology between SV40 T antigen and JCV T antigen was critical in the initial evaluation of JCV T antigen transforming properties. Similar to SV40 T antigen, the JCV T antigen has multifunctional activities, including ATP-ase, helicase, DNA binding, and α-polymerase (Pipas, 1992; Sullivan et al., 2000). Furthermore, results from immunoprecipitation analyses revealed that similar to SV40 T antigen, JCV T antigen has the capacity of interacting with and inactivating p53, as well as the members of the pRb family (Bollag et al., 1989; Haggerty et al., 1989; Del Valle et al., 2001). JCV T antigen has also been associated with other cellular proteins such as YB-1 and Purα (Gardner et al., 1971; Gallia et al., 1998). In one study characterizing MPNSTs in a JCV transgenic animal model, T antigen was found to also associate with the neurofibromatosis (NF) family of proteins (Shollar et al., 2004). Other possible mechanisms involve the binding of the J domain of JCV T antigen with cellular chaperon hsp70 (Sullivan et al., 2000), and the direct interaction between JCV T antigen and insulin receptor substrate 1 (IRS-1) (Lassak et al., 2002), which is the major signaling molecule for the type I insulin-like growth factor receptor (IGF-IR) (Baserga, 1999).

The requirement for a functional IGF-IR in the process of cellular transformation has been well documented. Overexpression of IGF-IR leads to IGF-dependent cellular growth in serum-free medium and may cause transformation (Kaleko et al., 1990; McCubrey et al., 1991; Sell et al., 1993; Coppola et al., 1994). Conversely, in the absence of IGF-IR, cells become quite resistant to transformation triggered by different oncoproteins, which are usually capable of transforming cells with a physiological level of the IGF-IR. Oncogenic proteins that fail to transform cells with targeted disruption of the IGF-IR gene (R cells) include SV40 T antigen (Sell et al., 1993); JCV T antigen (Del Valle et al., 2002); activated Ras, or a combination of SV40 T antigen and Ras

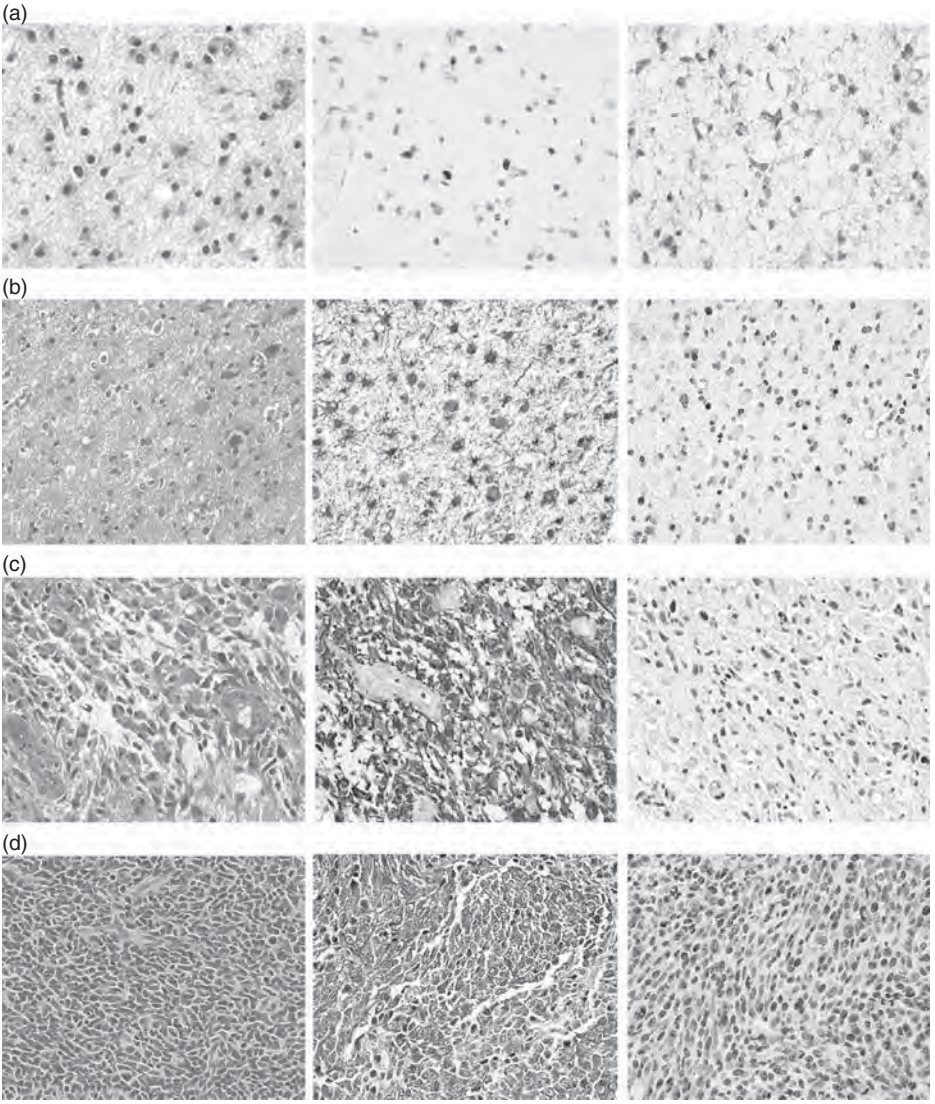

Figure 4.3. Detection of JCV T antigen in human brain tumors. JCV genomic sequences have been amplified in a variety of human brain tumors. In addition, expression of T antigen has been demonstrated in a high percentage of such tumors. (a) Oligodendrogliomas, characterized by round neoplastic cells with a clear halo (H&E) show nuclear expression of T antigen and the cytoplasmic presence of agnoprotein. (b) Low-grade diffuse astrocytomas, with small neoplastic cells, some of them gemistocytic (H&E), demonstrating cyoplasmic glial fibrillary acidic protein (GFAP), show nuclear positivity for T antigen. (c) Immunohistochemical detection of T antigen has also been found in cases of glioblastoma multiforme, a highly aggressive tumor characterized by very pleomorphic and atypical cells, which still retain the ability to express GFAP. (d) Medulloblastomas, characterized by sheaths of numerous small neoplastic cells with a slightly elongated nucleus. Although poorly differentiated, these cells show early neuronal markers such as synaptophysin. Expression of T antigen has been detected in the nuclei of neoplastic cells in approximately half of the cases studied. See color insert.

(Sell et al., 1993, 1994); bovine papilloma virus E5 protein (Morrione et al., 1995); and activated c-src (Valentinis et al., 1997). Taken together, these multiple observations make the IGF-IR particularly attractive as a potential therapeutic target against those malignances in which either IGF-I supports growth and survival of tumor cells or in tumors which arise as a result of cellular transformation via uncontrolled expression of viral oncogenic proteins.

Another interesting pathway disrupted by T antigen has emerged recently, with potential implications for oncogenesis and cancer. In cases of PML and in primary glial cultures infected by JCV, T antigen has been shown to activate the promoter of an antiapoptotic protein, survivin, which is normally expressed during embryonic development but should be completely silent in fully differentiated tissues. Activation of survivin by JCV leads to inhibition of apoptosis, even after the induction of apoptosis in different conditions (Piña-Oviedo et al., 2007). The JCV-mediated activation of survivin may contribute to prolonged cellular life spans, time in which T antigen-expressing cells may accumulate mutations and acquire the transformed and atypical phenotype seen in bizarre astrocytes.

THE ROLE OF IGF-IR IN DNA REPAIR OF DOUBLE-STRAND BREAKS (DSBs) BY HOMOLOGOUS RECOMBINATION

DNA DSBs are usually formed after cell exposure to ionizing radiation, endogenous free radicals, anticancer drugs, including cisplatin and mitomycin C, and can be inflicted spontaneously during DNA replication, when replication forks encounter other DNA lesions, including single-strand breaks and intrastrand cross-links (Hoeijmakers, 2001; Khanna and Jackson, 2001; Wozniak and Blasiak, 2002; Nowicki et al., 2004). Importantly, even a small number of DSBs can initiate a strong proapoptotic signal when damaged DNA is left unrepaired. Therefore, in addition to antiapoptotic pathways, cell survival relies also on the efficiency of DNA repair (Khanna et al., 2001). Early events in the detection of DSBs include activation of protein kinases ATM (ataxia-telangiectasia mutated), ATR (ATM related), and DNA-dependent protein kinase (DNA-PK), which all have been shown to phosphorylate histone H2AX (γ-H2AX) within mega-base pair regions surrounding DSBs, "attracting" different components of the DNA repair machinery (Paull et al., 2000; Burma et al., 2001). To prevent apoptosis induced by DNA damage, DNA breaks must be repaired. In replicating cells, homologous recombination DNA repair (HRR) seems to predominate, while quiescent cells utilize nonhomologous end joining (NHEJ) (Hoeijmakers, 2001). The choice between DNA repair mechanisms can be controlled, at least partially, by the availability of a DNA template. Cells that proliferate have the advantage of using newly synthesized templates supplied in the process of DNA synthesis. Cells arrested in G1 could potentially utilize HRR; however, the homology search is much more complicated and requires access to the template on the homologous chromosome. Alternatively, quiescent cells may simply link DNA ends without any template using DNA-binding Ku70/Ku80 complex and DNA-PK, followed by DNA ligation with XRCC4-ligase 4

(Pierce and Jasin, 2001; Lundin et al., 2002). This quick fix, however, may cost the cell a gain or loss of several base pairs. Therefore, DNA repair by NHEJ can be considered as an error-prone mechanism, which may lead to the accumulation of spontaneous mutations after DNA damage (Hoeijmakers, 2001; Wozniak and Blasiak, 2002; Nowicki et al., 2004).

The major enzymatic component of HRR in eucaryotic cells is Rad51. Rad51 is a structural and functional homologue of bacterial RecA recombinase (Baumann and West, 1998; Tombline and Fishel, 2002; Van Komen et al., 2002). Following detection of DSBs, the breast cancer susceptibility gene product (BRCA2) is suspected to mediate translocation of Rad51 to the sites of damaged DNA (Davies et al., 2001; Lu et al., 2005). In parallel, the ATM-activated 5′-3′ endonuclease complex (Rad51–MRE11–NBS1) exposes both 3′ ends of DNA at the point of break (Petrini, 2000; Zhu et al., 2000). The ends are initially protected by the ssDNA-binding protein replication protein A (RPA), which is subsequently replaced by Rad51 in a process that involves the initial binding of Rad52 into the ssDNA–RPA complex (Chen et al., 1999; Essers et al., 2002). As a result, newly formed Rad51 nucleoprotein filaments are directly involved in homology search and strand invasion, which in eukaryotic cells require ATPase activity from Rad54 (Sigurdsson et al., 2002; Tombline and Fishel, 2002; Tombline et al., 2002).

Environmental factors, cellular metabolism, as well as viral proteins, can interfere with the efficiency and fidelity of DNA repair. Although it is still difficult to draw the line between faithful and unfaithful DNA repair mechanisms, and their contribution to genomic instability, inherited genetic defects in HRR pathways provide strong evidence in support of genomic instability in high predisposition to cancer (Thompson and Schild, 2002). Some of these cancer-prone human genomic disorders targeting HRR include ataxia-telangiectasia (AT), where a mutated ATM kinase affects early events of HRR, including phosphorylation of histone H2AX, BRCA1, NBS1, and RPA (Spring et al., 2001; Thompson and Schild, 2002); and Nijmegen breakage syndrome (NBS), which is similar to AT with respect to clinical manifestation, involves the mutation of the NBS1 protein, which forms a functional complex (Rad51-Mre11–NBS1) responsible for the initiation of HRR by exposing both 3′ ends of DNA at the DSBs (Maser et al., 1997; Digweed et al., 1999). Different members of the RecQ family of DNA helicases, such as BLM, WRN, and RecQL4, are mutated in Bloom syndrome (BML), Werner syndrome (WRN), and Rothmund–Thomson syndrome (RTS), respectively. Considerable evidence suggest that BML helicase plays a role in HRR by repairing damage at stalled replication forks, and partially colocalizes with Rad51 and RPA at the site of DSBs (Ellis et al., 1995; Thompson and Schild, 2002).

During cellular transformation, predisposition to accumulate mutations may develop as a result of dysregulation of normal mechanisms controlling faithful and unfaithful DNA repair mechanisms. In the past, several reports suggested a potential involvement of the IGF-IR in DNA repair. These include enhanced radioresistance, which was found proportional to the IGF-IR protein level in mouse embryo fibroblasts and breast tumor cells (Turner et al., 1997); enhanced DNA repair via the IGF-I-activated p38 signaling pathway in response to ultraviolet (UV)-mediated DNA damage

(Heron-Milhavet and LeRoith, 2002); and delayed UVB-induced apoptosis via IGF-I-mediated activation of Akt, resulting in enhanced repair of DNA cyclobutane thymidine dimers in keratinocytes (Decraene et al., 2002). Although these few studies indicate that IGF-IR signaling may contribute to the development of drug resistance and/or DNA repair, it was unclear whether IGF-IR affects DNA repair directly, or whether IGF-IR antiapoptotic signals (Sell et al., 1995; D'Ambrosio et al., 1997; Valentinis et al., 1998) simply increased cell survival after DNA damage.

Our recent studies indicate that activated IGF-IR enhances HRR by a mechanism that controls translocation of Rad51 to the sites of damaged DNA (nuclear foci) (Trojanek et al., 2003). This effect involves a direct cytosolic interaction between Rad51 and the major IGF-IR signaling molecule, IRS-1. The binding is direct, occurs within the perinuclear region of the cell, involves the N-terminal portion of IRS-1, and was attenuated by IGF-I-mediated IRS-1 tyrosine phosphorylation. Importantly, cells with low levels of IGF-IR, or cells expressing IGF-IR mutants that fail to phosphorylate IRS-1, retain Rad51 within the perinuclear compartment and show significantly less DNA repair by HRR (Trojanek et al., 2003). These results indicate a novel function for the IGF-IR/IRS-1 signaling axis, which involves intracellular trafficking of Rad51 from cytosol to the sites of damaged DNA, a crucial step in the process of DNA repair by homologous recombination.

POLYOMAVIRUS LARGE T ANTIGENS AND GENOMIC INSTABILITY

After viral infection with polyomaviruses, JCV in particular, cells are forced to reenter the S phase of the cell cycle, so that the DNA replication machinery of the cell becomes available to the virus. Only the early viral genome is active at this time of infection. It transcribes a common precursor RNA, which is differentially spliced yielding several viral products, among which the tumor antigens (large and small T antigens) predominate (Khalili and Stoner, 2001). Polyomaviruses infect humans, monkeys, rodents, and birds with a restricted host and tissue specificity. The infection of tissues in which the virus does not replicate efficiently may lead to partial activation of the virus, expression of an early viral genome (Imperiale, 2001), dysregulation of cell growth mechanisms, and cellular transformation (Reiss and Khalili, 2003). The transforming properties of SV40 and JCV T antigens are well established in literature (Khalili and Stoner, 2001). The major known cellular targets for T antigen-mediated cell cycle dysregulation are p53 and pRb (Chen et al., 1992; Kao et al., 1993; Saenz-Robles et al., 2001). Although inactivation of these nuclear proteins by T antigen is a critical step in the reactivation of DNA replication in differentiated cells, it does not necessary confer malignant transformation. As previously reported, T antigens are strongly suspected to compromise the integrity of the genome. Several studies documented a substantial chromosomal instability with no consistent patterns and often with many new karyotypes emerging at each consecutive passage of T antigen-positive cells (Hunter and Gurney, 1994; Woods et al., 1994; Ramel et al., 1995; Kappler et al., 1999; Ricciardiello et al., 2003). It has also been shown that peripheral lymphocytes develop chromosomal damage when exposed to JCV T antigen (Lazutka et al., 1996; Neel et al., 1996). The

diversity in chromosomal defects found in cells carrying T antigen suggests a random nature for its action. This could also suggest that T antigens affect the stability of the genome at a very basic level. One possibility that could explain the appearance of random mutations in T antigen expressing cells is an obstruction of DNA repair of DSBs. As previously mentioned, DSBs must be repaired to ensure cell survival, especially in cells replicating DNA. To sustain cell proliferation after DNA damage, and to avoid apoptosis, at least one of the two major DNA repair mechanisms must be activated: usually, the faithful HRR, or less, the faithful NHEJ. The first documentation of a possible interaction between T antigen and DSBs repair was demonstrated by experiments in which the formation of MRE11 nuclear foci was significantly disrupted by the presence in SV40 T antigen (Digweed et al., 1999, 2002). Since the attenuation of the foci formation was found in both T antigen immortalized cells and cells transiently expressing this viral oncoprotein, it was concluded that the effect on DNA repair was direct and did not rely on secondary mutations. Similarly, another DNA repair protein, Nbs1, which forms an early DNA repair complex with MRE11 and Rad51, was shown to interact with SV40 T antigen, disrupting DNA replication control mechanisms (Wu et al., 2004).

THE INTERACTION BETWEEN IRS-1 AND JCV T ANTIGEN IN THE PROCESS OF DNA REPAIR AND THE DEVELOPMENT OF GENOMIC INSTABILITY

The involvement of IRS-1 in T antigen-mediated cellular responses was demonstrated for the first time in cells expressing SV40 T antigen. In these experiments, T antigen and IRS-1 coprecipitated with each other, and this protein–protein interaction has been functionally linked with cellular transformation *in vitro* (Zhou-Li et al., 1997). Our recent studies confirmed this interaction in medulloblastoma cell lines expressing JCV T antigen (Lassak et al., 2002; Trojanek et al., 2006a,b). Further molecular analysis revealed the nuclear translocation of IRS-1 in JCV T antigen-positive cell lines (Lassak et al., 2002) and in JCV T antigen-positive samples from patients diagnosed with medulloblastoma (Del Valle et al., 2002). The IRS-1 domain responsible for JCV T antigen binding was located in the N-terminal portion of the IRS-1 molecule, and the binding was independent from IRS-1 tyrosine phosphorylation and was strongly inhibited by IRS-1 serine phosphorylation.

Once we determined that a fraction of IRS-1 translocates to the nucleus in the presence of JCV T antigen, we asked whether nuclear IRS-1 could still bind Rad51, but this time at the sites of damaged DNA, and whether this nuclear IRS-1–Rad51 complex could affect the efficiency and fidelity of HRR. By utilizing a GFP-based HRR reporter system developed several years ago by Maria Jasin (Pierce et al., 1999), we have demonstrated that expression of JCV T antigen caused the inhibition of HRR, resulting in an accumulation of mutations in different cell types, including medulloblastoma (Trojanek et al., 2006a,b). In this process, JCV T antigen did not interfere with HRR directly, and instead contributed to the translocation of IRS-1 to the nucleus. In this

potentially wrong cellular compartment, IRS-1 has been found to bind Rad51 at the site of damaged DNA (Fig. 4.2a); in the absence of T antigen, IRS-1–Rad51 interaction was efficiently reproduced in cells expressing IRS-1 mutant with artificial nuclear localization signal (Fig. 4.2c), T antigen did not colocalize directly with Rad51 after DNA damage (Fig. 4.2b), and finally, the inhibition of HRR by T antigen did not function in cells lacking endogenous IRS-1 (Trojanek et al., 2006). Figure 4.4 shows the interaction between IRS-1–Rad51 and JCV T antigen in the process of DNA damage and repair.

CONCLUDING REMARKS

In summary, these multiple findings and mounting evidence prompt us to suggest a model that could explain how JCV T antigen contributes to the development of genomic instability (Fig. 4.5). This model involves the interaction between JCV T antigen and IRS-1, resulting in the translocation of IRS-1 to the nucleus. In this "wrong" cellular compartment, hypophosphorylated IRS-1 binds to Rad51 at the site of damaged DNA and attenuates faithful DNA repair. Virally damaged cells unable to repair DNA by HRR may be forced to use an alternative and nonfaithful pathway, NHEJ. Importantly, the indirect inhibition of HRR by JCV T antigen was associated with a dramatic increase in the number of cells that accumulate mutations during DNA repair (Trojanek et al., 2006a, b), suggesting that DNA damage inflicted to cells carrying JCV T antigen may initiate a selection process, which in turn may result in the development of cellular transformation. Other pathways affected by JCV T antigen may also contribute to this scenario, for example, T antigen activation of the survivin promoter results in inhibition of apoptosis and could prevent DNA damaged cells from undergoing apoptosis, allowing time for the disruption of the DNA repair mechanisms described and for the accumulation of mutations.

Although the role of T antigens in cellular transformation is already well established, at least in cell culture and experimental animal models, the question remains whether these viral oncoproteins contribute to the development of human cancer. Several recent publications illustrate how the scientific community is still divided with respect to this important issue. For instance, some of the reports provide a strong support (Reiss, 2002; Khalili et al., 2003; Reiss and Khalili, 2003; Wang et al., 2004; Cristaudo et al., 2005; Del Valle et al., 2005), while some others disagree (Lopez-Rios et al., 2004; Rollison et al., 2005) with the involvement of polyomavirus T antigens in human cancer. Although this perennial question remains still controversial, our results summarized in Figure 4.5 attempt to provide an explanation of how the expression of JCV T antigen in combination with the IGF-IR-IRS-1 signaling system could compromise genomic integrity, a strong prerequisite in the development of malignant transformation, and eventually, in the development of tumors.

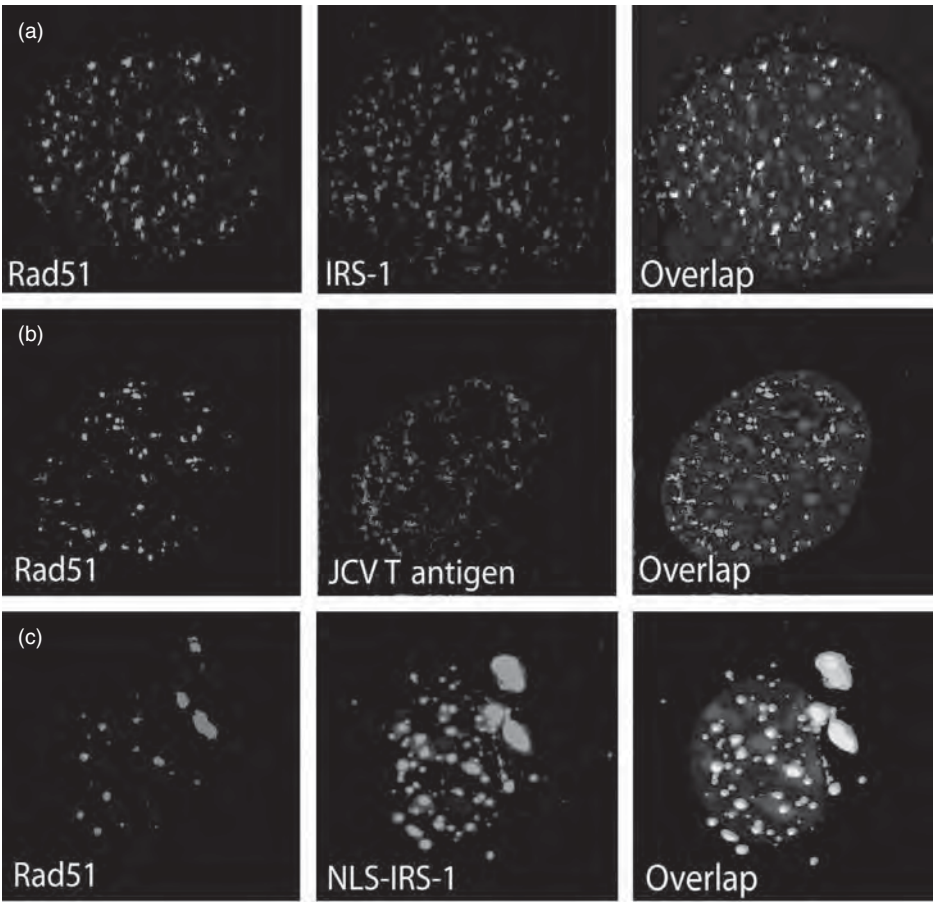

Figure 4.4. Interaction between IRS-1, Rad51, and JCV T antigen after DNA damage. (a) Double immunolabeling with anti-IRS-1 (rhodamine) and anti-Rad51 (fluorescein) was performed in JCV T antigen expressing R503 fibroblasts (R503/T clone #10) 6 h after the treatment with cisplatin (1 μg/ml). Digital images of 2 μm were collected with an inverted fluorescent microscope equipped with motorized *z*-axis and a deconvolution software (SlideBook4). The image marked "Overlap" represent a superimposition of two images depicted in the same row and demonstrates the nuclear colocalization between IRS-1 and Rad51 in JCV T antigen-positive cells (yellow fluorescence). Blue fluorescence represents 4′-6′-diamidino-2-phenylindole (DAPI) nuclear counterstaining. (b) Double labeling with T antigen (rhodamine) and anti-Rad51 (fluorescein). In contrast to the interaction between IRS-1 and Rad51 depicted in (a), in the same experimental setting, the interaction between JCV T antigen and Rad 51 is absent (lack of yellow fluorescence). (c) Detection of NLS-IRS-1 was carried out in T antigen-negative thymidine kinase negative (TK^-) cells at 48 h following transient transfection with the pCMV/myc/nuc/IRS-1 expression system. The cells transiently expressing NLS–IRS-1 were treated with cisplatin for 6 h and analyzed for the possible interaction between Rad51 and NLS–IRS-1 at the sides of DNA damage (nuclear foci). Double labeling of IRS-1 (fluorescein) and Rad51 (rhodamine) demonstrates a strong nuclear colocalization between both proteins, shown as yellow fluorescence after overlapping corresponding images. The nuclei are counterstained with DAPI. See color insert.

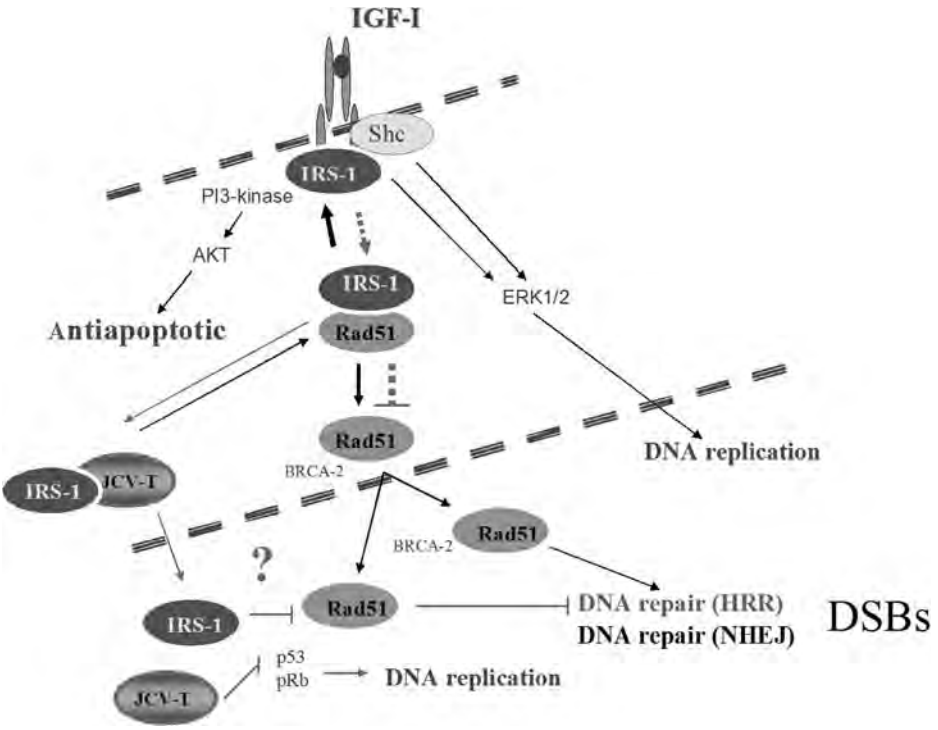

Figure 4.5. IGF-IR–JCV T antigen interplay and DNA repair of DSBs. In normal circumstances, the signal from activated IGF-IR supports cell proliferation and faithful DNA repair, which requires both homologous recombination directed DNA repair (HRR) and nonhomologous end joining (NHEJ). In contrast, factors capable of translocating IRS-1 to the nucleus, such as JCV T antigen, are strongly suspected to shift the balance from HRR to NHEJ by the inhibitory interaction between IRS-1 and the major enzymatic component of HRR, Rat51. This, in turn, may compromise the fidelity of DNA repair in cells replicating DNA. The rate of DNA damage and unfaithful DNA repair could be amplified in cells surviving genotoxic treatment (γ-irradiation, cisplatin, mitomycin). In such a scenario, accumulated mutations may lead to new cellular adaptations and the selection of cells capable of disseminating central nervous system. See color insert.

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5

INVOLVEMENT OF THE POLYOMAVIRUS, JC VIRUS, IN COLORECTAL CANCER

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THE PATHOGENESIS OF COLORECTAL CANCER (CRC)

CRC is caused by altered genes, and it evolves through a progressive, multistep continuum. Most CRCs are aneuploid and have experienced gains, losses, and large-scale rearrangements at the chromosomal level. In fact, aneuploidy in cancer has been recognized for over a century, is seen in most varieties of cancer, and each CRC appears to have a unique constellation of scrambled chromosomes. The heterogeneous pathological appearance of different CRCs, and the apparent randomness of the chromosomal alterations, generated the concern that it might not be possible to develop an integrated sense of how these defects are related to carcinogenesis.

However, the relatively easy access to early neoplastic tissues in the human colon and rectum (using endoscopes), followed by genetic analysis of the DNA extracted from them, has provided insight into the specific genes and processes involved in colorectal carcinogenesis. A common theme in colorectal carcinogenesis is the very early loss or inactivation of both alleles of the *APC* gene, followed by an activating mutation in the *K-Ras* oncogene, followed much later by losses or inactivation of both alleles of the *p53* gene. In fact, the loss of *APC* appears to occur at the transition from normal colon to benign neoplasm (the adenoma), the activating mutations in *K-Ras*

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typically occur during the growth of the adenoma, and the loss of *p53* occurs at the transition from adenoma to carcinoma. From this framework, the first integrated pathway of carcinogenesis was developed (Fearon and Vogelstein, 1990). Because of the dominance of aneuploidy in this pathway, which provided the mechanism for widespread allelic losses, it eventually was termed chromosomal instability (CIN) (Lengauer et al., 1997).

Within a few years of the development of the initial multistep carcinogenesis model, additional pathways of colorectal carcinogenesis were discovered. In some CRCs, numerous mutations were found throughout the genome, particularly at microsatellite sequences (Aaltonen et al., 1993; Ionov et al., 1993; Thibodeau et al., 1993). The term microsatellite instability (MSI) was coined to describe these tumors. These tumors tended to be near diploid, but had approximately 10^5 deletion mutations at microsatellite sequences. Differences were noted pathologically and clinically between these tumors and those without MSI. What became more apparent was that there was more than one pathway to CRC, and there were very large numbers of genetic alterations in these tumors, perhaps far more than what would be minimally necessary for a tumor to develop (Boland and Ricciardiello, 1999).

Before long, another pathway to CRC was discovered, which involved the silencing of tumor suppressor genes through a CpG island methylation pathway, which came to be called CIMP (Toyota et al., 1999). This epigenetic mechanism of tumor development further expanded the carcinogenetic repertoire, and again, many more alterations were found than what seemed necessary for tumor development. Interestingly, there is an inverse relationship between CIN and CIMP in CRC (Goel et al., 2007).

At least two critical concepts emerged from these findings. First, there are multiple distinct pathways to CRC. Second, there appeared to be types of destabilizing mechanisms underlying these pathways in which many more mutations were generated than what would be minimally necessary for tumorigenesis. Therefore, a reasonable interpretation of this was that the genomic or epigenetic instability was the fundamental process required for carcinogenesis, and that alterations would occur in many genetic targets depending on the DNA sequence and the nature of the destabilization process. Alterations of specific target genes would lead to evolutionary selection in favor of cells that had gained a growth, or survival, advantage necessary for clonal expansion. It also became apparent that CRCs with MSI (about 15% of all CRCs) came from two different backgrounds. About 20%–25% of these (or 3%–4% of all CRCs) came from patients with Lynch syndrome, a hereditary disease caused by germ line mutations in one allele of a DNA mismatch repair gene. The other allele was lost, usually through CIN, and the tumor developed MSI, which dominated the tumor and the mutational signature in the tumor DNA. The other 75%–80% of MSI CRCs (or 10%–12% of the total) occurred as a consequence of methylation of the promoters of both alleles of the *MLH1* DNA mismatch repair gene (Kane et al., 1997; Herman et al., 1998). These tumors therefore developed from a background of CIMP, which is evident in the mutational signatures of the DNA of these tumors. Therefore, MSI is a secondary phenotype in CRCs, and is actually derived from either the CIN or CIMP pathways (Goel et al., 2007). However, there has never been a definitive mechanistic explanation for CIN or CIMP.

THE PRESENCE OF A POLYOMAVIRUS IN CRC

It had long been appreciated that the simian polyomavirus SV40 is oncogenic in laboratory animals and can be used *in vitro* to transform mammalian cells (Butel and Lednicky, 1999; Gazdar et al., 2002). When this occurs, the diploid parental cells become aneuploid. Furthermore, Neel et al. (1996) made the observation that aneuploid lymphocytes in otherwise healthy individuals, which he termed "rogue cells," were significantly associated with increased antibody titers to JC virus (JCV), but not to the closely related human polyomavirus BK virus (BKV). Moreover, JCV DNA and T-Ag expression were detected in a brain tumor of an immunocompetent individual (Rencic et al., 1996). Thus, it was a reasonable hypothesis that a polyomavirus could potentially provide a mechanistic explanation for the generation of CIN and aneuploidy in human tumors such as CRC.

In 1994, we began a series of experiments to look for the presence of a polyomavirus in human CRC. We examined resection specimens collected and frozen at the time of surgery for CRC. The samples were thawed, DNA extracted, and the polymerase chain reaction (PCR) was performed for JCV. Our initial results were not always consistent, but Laghi et al., (1999) made a series of observations that overcame the technical hurdles involved in the detection of JCV from human tissues. First, the number of copies of JCV was quite low in the DNA extracted from both normal colon and colon cancers. Nested PCR helped increase the sensitivity of the amplification. Second, microheterogeneity of DNA sequence was anticipated, so degeneracy was introduced in the 3′ end of the PCR primers to increase the likelihood that minor variations in sequence would be amplified. Third, it was recognized that, if JCV were present as an intact virus, its circular supercoiled configuration might be an impediment to efficient amplification. The addition of topoisomerase I prior to amplification increased the sensitivity further (Laghi et al., 2004).

Using these modifications (topoisomerase I-sensitive PCR amplification [TISPA]), we were able to consistently detect JCV DNA in most CRCs and in most samples of nonneoplastic colonic mucosa adjacent to the tumor, (see Fig. 5.1) (Laghi et al., 1999). In our initial publication, we reported JCV DNA in 89% of CRCs and nonneoplastic colonic tissues. Without TISPA, the sensitivity of detection was 26%. Other laboratories have subsequently confirmed the presence of JCV in CRCs. Enam et al. (2002) found JCV early region DNA in 81.5% of the samples, but late region DNA in a smaller proportion. This group also detected an interaction between T-Ag and β-catenin, which provided insight into a possible pathogenetic mechanism of action in CRCs (Enam et al., 2002). Subsequently, two more groups confirmed the presence of JCV DNA in CRCs (Hori et al., 2005; Theodoropoulos et al., 2005). However, some groups have reported negative attempts to amplify JCV DNA from the colon using unique assays, which reflects the technical challenges involved (Boland et al., 2004; Newcomb et al., 2004). We confirmed that the PCR products amplified from the colonic tissues had come from JCV using Southern blot hybridization and by cloning and sequencing the PCR products (Laghi et al., 1999). In each instance, the cloned sequence was JCV, and never BKV or SV40. Different PCR targets in the T-Ag sequence and the transcription

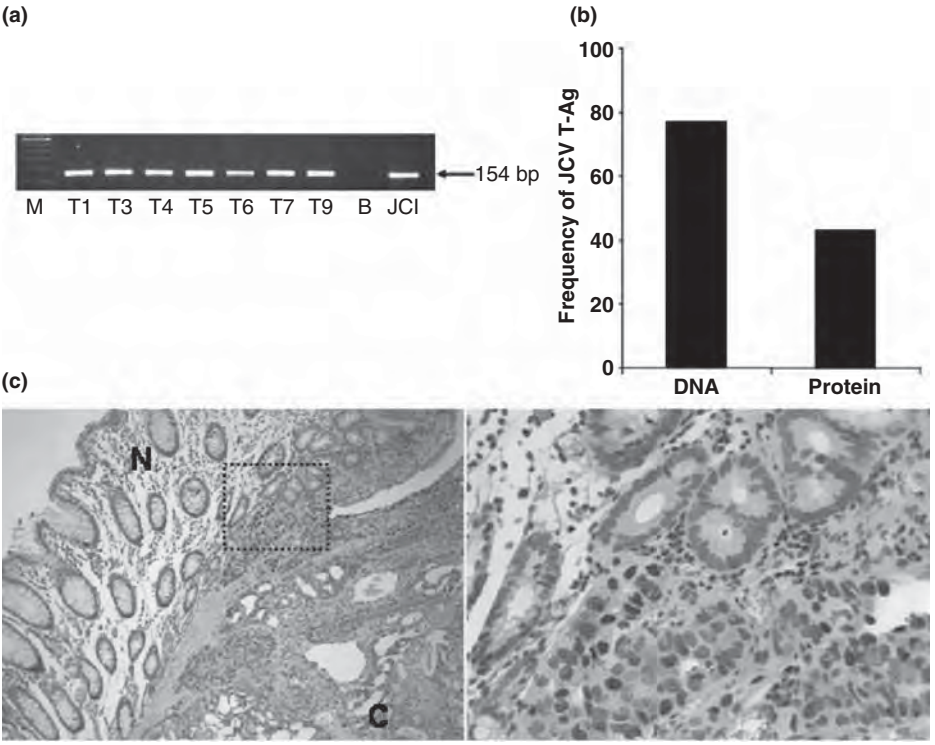

Figure 5.1. JCV DNA amplified from CRC specimens. Panel (a) shows the PCR amplification of JCV DNA from seven different CRCs, with positive (JCI) and negative (B) controls and a size reference marker (M). Panel (b) indicates the frequency of finding T-Ag DNA (77%) or protein (43%) in 100 consecutive CRCs. Panel (c) demonstrates a low-power immunohistochemical photomicrograph of staining for T-Ag on the left, in which the cancer (labeled C) is undermining the normal mucosa, labeled N, on the right lower half of the image. On the right is a higher-power image taken from the box outlined on the left, showing that the T-Ag is exclusively expressed in the tumor cell nuclei (taken from Goel et al. [2006]).

control region (TCR) were also amplified to mitigate the possibility that the PCR products were the result of contamination in the laboratory.

The TCR regions were examined to identify the viral strains present in the human colon (Ricciardiello et al., 2001). We isolated 285 clones from colons (cancer and nonneoplastic samples) that had been found to carry JCV and screened the TCRs for sequence variations. The Mad-1 strain was exclusively present in the colon, but the archetype strain was never found. Also, in the cancer specimens, rearrangements of the Mad-1 TCR were found in 17.2% of the clones, usually consisting of duplications or deletions of a 98-base pair (bp) tandem repeat sequence that encodes multiple binding sites for transcription factors (Raj and Khalili, 1995). Similar rearrangements were found in 1/156 clones isolated from the nonneoplastic colon

TABLE 5.1. Distribution of JC Virus TCRs in Colon Cancer Tissues and Corresponding Nonneoplastic Tissues (Ricciardiello et al., 2003)

Clone Group	Number (%) of Positive Samples	
	Colon Cancer	Nonneoplastic Epithelium
Clones (n = 285)	128	157
Archetype strain	0	0
Mad-1 strain	106 (82.5)	156 (99.3)
Mad-1 variants	22 (17.2)	1 (0.6)
Single 98-bp sequence	18 (14)	0
Triple 98-bp sequence	4 (3.1)	0
Quadruple 98-bp sequence	0	1 (0.6)
DNA samples (n = 16)	8	8
Mad-1	8	8
Mad-1 variants	8	1

specimens. All eight CRC specimens harbored at least one rearranged Mad-1 TCR, whereas this was rare in nonneoplastic colonic mucosa (Table 5.1).

JCV IN THE NORMAL HUMAN GASTROINTESTINAL (GI) TRACT

We looked for JCV in the DNA of mucosal biopsies from healthy volunteers undergoing endoscopic procedures. A total of 129 biopsies were taken from the esophagus, stomach, and colon. DNA was extracted and analyzed for JCV. Viral DNA was found in 81.2% of the colons, and in 70.6% of the upper GI tracts of these individuals, as illustrated in Figure 5.2 and Table 5.2. Again, the identity of the PCR products was confirmed by cloning and sequencing, and all were JCV. Also, each individual who had a prior history of a colorectal neoplasm (either a cancer or an adenomatous polyp) had evidence of JCV DNA in the normal colonic mucosa (Ricciardiello et al., 2000).

VIRAL COPY NUMBERS IN COLONIC TISSUES

We initially used a semiquantitative approach to estimate the relative copy number of viral DNA in the specimens of normal colon and CRC. This was done by using identical amounts of total DNA from specimens of matched normal colon and CRC from each individual. PCR was performed on each sample and repeated on serial 10-fold dilutions until it was no longer possible to obtain a PCR product. In each instance, the tumor specimen contained 10–100 times more copies of JCV DNA than the matched normal colon specimen (Laghi et al., 1999).

Theodoropoulos et al. (2005) used quantitative PCR to determine the number of viral DNA copies in CRCs, adenomatous polyps, the normal colon adjacent to the tumor, and the normal colonic mucosa from individuals unaffected by neoplasia. As

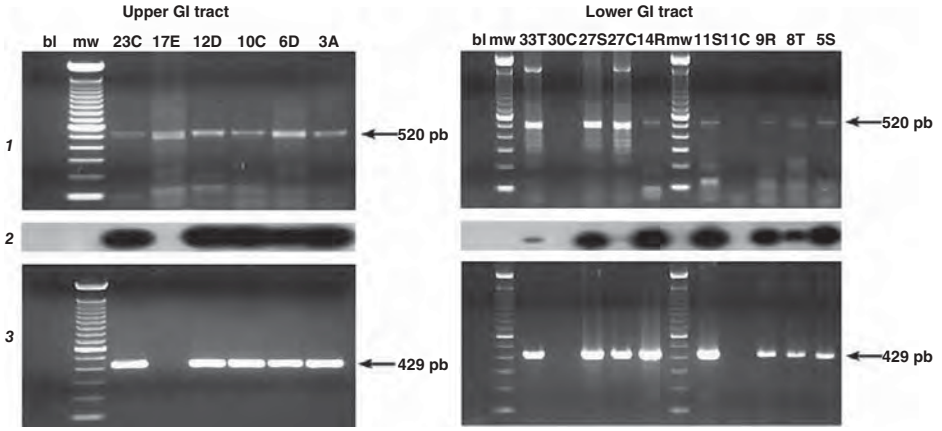

Figure 5.2. JCV sequences are present in GI mucosal DNA. Panel 1 (top left and right for the upper and lower GI tracts, respectively) shows ethidium bromide-stained 2.5% agarose gels demonstrating the amplification of 520 bp of DNA corresponding to the N-terminus of the JCV Mad-1 T-Ag. Panel 2 (middle, left and right) shows Southern blots of the 520-bp amplicons using a radiolabeled nested primer, confirming the amplification shown in panel 1. Panel 3 (bottom, left and right) shows ethidium bromide-stained 2.5% agarose gels showing amplification of a 429-bp nested DNA fragment. (bl indicates a blank template control, and mw is a size marker lane.)

TABLE 5.2. JCV in the Normal GI Tract

	n	%
Total samples	129	—
Amplified with topoisomerase I	36/129	27.9
Topoisomerase I alone or + degenerate primers	76/129	58.9
Upper GI tract		
Total	66	—
Positive	37	56
Esophagus	—	66.6
Gastric corpus	—	52.9
Gastric antrum	—	47
Duodenum	—	58.8
Lower GI tract		
Total	63	—
Positive	39	61.9
Cecum—ascending	—	66.6
Transverse colon	—	68.7
Sigmoid colon	—	50
Rectum	—	62.5
Positive patients		
Total	25/33	75.8
Upper GI endoscopy	12/17	70.6
Lower GI endoscopy	13/16	81.2

DNA was extracted from 129 biopsies of normal-appearing mucosa and assayed for JCV by PCR (Ricciardiello et al., 2000).

TABLE 5.3. JCV Viral Load in Colorectal Tissues (Theodoropoulos et al., 2005)

Tissue	Mean Copy Number (copies/μg DNA)	Range (copies/μg DNA)
CRC	14,298 ± 4,150	9,100–20,000
Adenoma	13,997 ± 3,053	9,000–20,000
Adjacent to CRC	242 ± 127	50–450
Normal colon	147 ± 50	100–250

demonstrated in Table 5.3, CRCs contain about 14,000 copies of JCV per microgram of DNA extracted from the specimen. Interestingly, adenomatous polyps contain virtually the same number of viral DNA copies as found in the CRCs. The samples of nonneoplastic mucosa adjacent to the cancer contained about 242 copies per microgram of DNA, and normal colonic mucosa contained about 147 copies, confirming the initial semiquantitative data (Theodoropoulos et al., 2005).

Thus, JCV is present in the normal colon, but in very low copy number, whether or not the colon harbors a simultaneous neoplasm. The similarities of the copy number between colonic mucosa adjacent to a CRC and normal colon suggests that this does not represent virus shed from the tumor. The fact that both adenomatous polyps and adenocarcinomas have ~100-fold more JCV copies than are found in the normal colon is compatible with the interpretation that the virus is involved with neoplasia from the earliest stages. What remained to be demonstrated was whether the virus is etiologically involved in carcinogenesis, or alternatively, a nonspecific passenger that is permitted in a neoplastic lesion.

JCV IN ESOPHAGEAL, GASTRIC, PANCREATIC, AND LUNG CANCERS

The presence of JCV throughout the GI tract (as shown in Table 5.2) raised the possibility that this virus might be involved in other cancers in the gut. Several groups have reported that this is the case.

Del Valle et al. (2005) reported that JCV DNA could be isolated from 11/13 esophageal biopsy specimens and from 5/5 esophageal carcinomas. JCV T-Ag was expressed at immunohistochemistry in 10/19 (53%) of the esophageal cancers, including 2/9 adenocarcinomas and 8/10 squamous cell carcinomas.

Shin et al. (2006) reported that JCV DNA could be detected in 21/37 (57%) of gastric cancers and in 11/37 (30%) of the adjacent nonneoplastic gastric tissue. Viral capsid DNA sequences were also detected in 24% and 19% of the neoplastic and nonneoplastic tissues, respectively. The strain of the JCV was Mad-1, the cancers had rearranged Mad-1 sequences, and the archetype TCR sequence was not seen, all similar to what had been observed in the colon. T-Ag was expressed at immunohistochemistry in 9/23 (39%) of the gastric cancers, but in none of the nonneoplastic tissues (Shin et al., 2006).

Murai et al. (2007) have confirmed this observation in Japanese specimens, and using nested PCR, found T-Ag DNA sequences in 86% of gastric cancers and adjacent

noncancerous tissue and in 100% of the normal gastric tissues not associated with a tumor. Interestingly, they were able to detect VP (capsid) DNA sequences in only 55% of the samples, and agnoprotein DNA in 9%. Yields were generally better from the frozen samples compared with that from paraffin-embedded material. Viral copy numbers were approximately 10-fold higher in cancer and the paired adjacent tissues (about 5000 viral copies/μg DNA) than in normal gastric tissue (about 500 viral copies/μg DNA) (Murai et al., 2007).

JCV DNA and T-Ag expression have also been found in pancreatic adenocarcinoma (Fuerst et al., 2005).

In addition, Zheng et al. (2007) have reported that JCV DNA can be detected in 68% of lung cancers and in 11% of normal lung tissues using nested PCR and Southern blot. Using quantitative PCR, this group reported that the cancers harbored significantly more copies of the virus compared with normal tissue. Tumors from men had significantly higher copy numbers compared with tumors from women, but there was no significant correlation between viral copy number and tumor size, patient age, or several other clinical and pathological features (Zheng et al., 2007).

THE MECHANISTIC ROLE OF JCV IN THE INDUCTION OF CIN IN COLON CANCER

There is now abundant data that JCV is present throughout the GI tract and in many cancers of the gut. There is a 100-fold increase in viral copy number in cancers of the colon compared with nonneoplastic colon. There are important theoretical reasons why a polyomavirus is a candidate oncogenic virus, including the behavior of this virus *in vitro* and in experimental animals.

However, it has been more challenging to prove convincingly that JCV is involved in the pathogenesis of human cancers. First, as many as 90% of the population have antibody titers to JCV, and yet only a minority of people develop GI cancer. The very high prevalence of seropositivity for JCV antibodies makes it difficult to prove associations between antibody titers and the development of cancer (Lundstig et al., 2007), even if accurate ascertainment of cancer prevalence over a lifetime were possible.

To deal with this problem, our group developed *in vitro* models in which to study the ability of JCV to induce the types of genomic or epigenetic instability observed in human CRCs. One approach to this is to determine whether JCV can induce CIN, MSI, and CIMP in colorectal epithelial cells. Models of cultured CRC cells that are free of CIN, MSI, and CIMP are available. As mentioned earlier, MSI in CRC is a consequence of either CIN (in the case of the hereditary disease called Lynch syndrome), which accounts for about 3%–4% of CRCs, or of CIMP (where the MSI is acquired as a consequence of methylation of the promoter of the *MLH1* gene), which accounts for about 10%–12% of CRCs. Thus, all CRCs can be attributable to either CIN or CIMP (Goel et al., 2003, 2007).

RKO cells are near-diploid colon cancer cells that have come from a CIMP background. These cells have wild-type *APC*, *p53*, and *β-catenin* genes, and do not have CIN. Full-length genomes of the Mad-1 strain of JCV, and another JCV strain

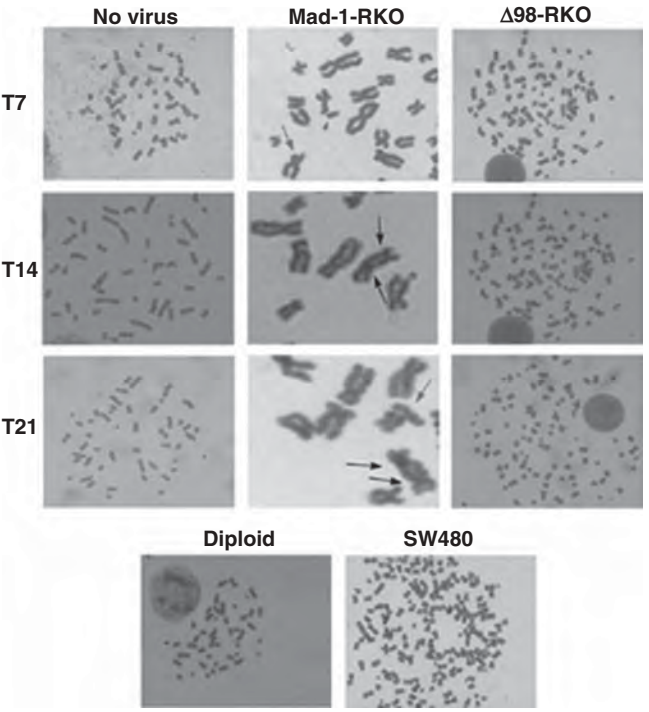

Figure 5.3. Induction of CIN in RKO cells by transfection of the JCV genome in a plasmid. CIN, characterized by chromosomal breakage, dicentric chromosomes, increasing chromosomal number, and other gross abnormalities, occurred in Δ98- and Mad-1-transfected cells between days 7 and 21 posttransfection. These abnormalities were not observed in nontransfected or control (pUC)-transfected cells. Arrows show chromosomal breaks and dicentric chromosomes. Human fibroblasts were used as a diploid control, while SW480 was employed as a prototype of CIN.

associated with CRC (Δ98), were inserted into a pBR322 vector and transfected into RKO cells. CIN (or aneuploidy) developed within a week, as illustrated in Figures 5.3 and 5.4, and Table 5.4.

ROLE OF JCV IN THE INDUCTION OF ABERRANT DNA METHYLATION

Viruses have traditionally been known to trigger a host defense mechanism involving transcriptional silencing of transposons and retroviruses that have accidentally accumulated in the mammalian genome (Doerfler, 1992; Bestor, 1998). In fact, a number of studies have demonstrated that methylation of the genomes of pathogenic human retroviruses, including human T-cell lymphotropic virus-I (HTLV-I) human immunodeficiency virus (HIV), and herpesviruses such as Epstein Barr virus (EBV), play an important role

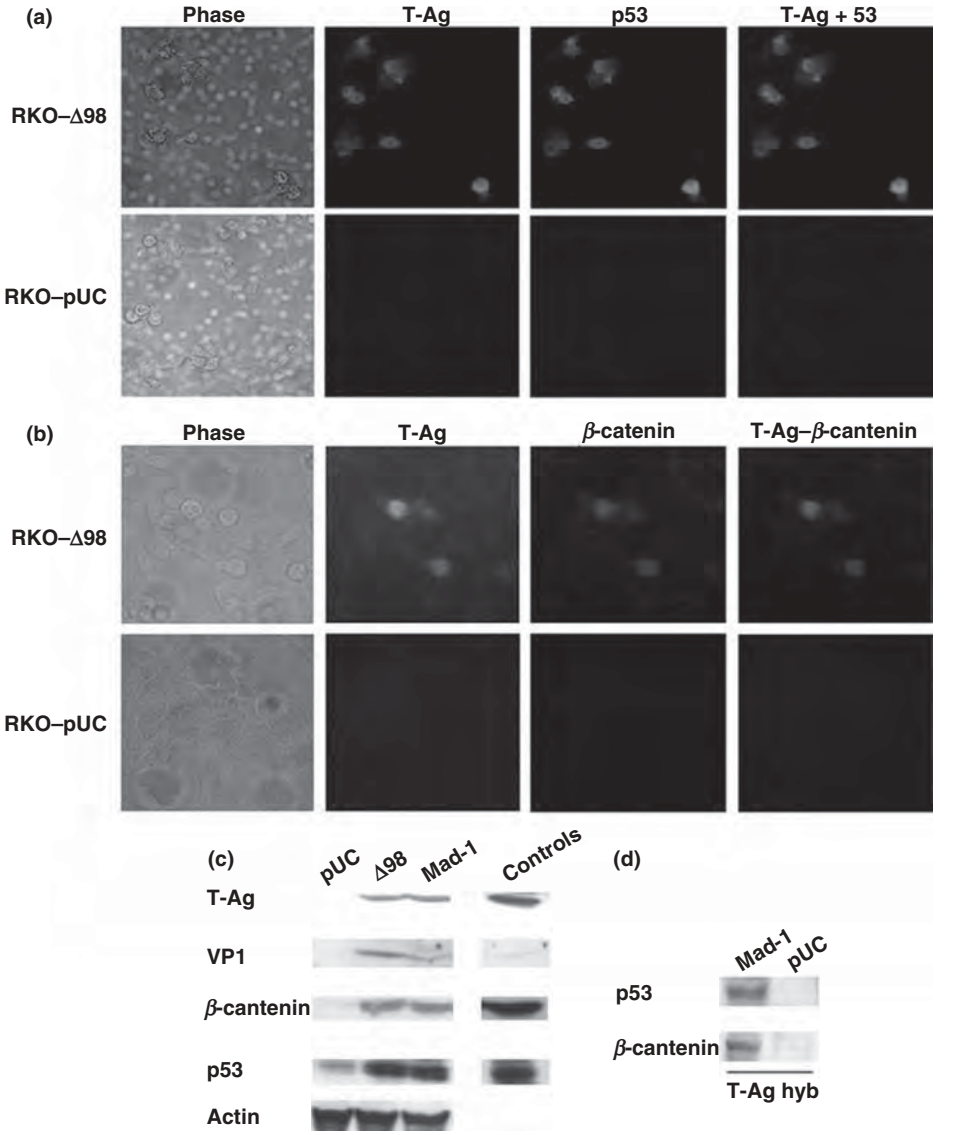

Figure 5.4. Interaction between JCV T-Ag, p53, and β-catenin. Dual immunofluorescence microscopy illustrates T-Ag–p53 (a) and T-Ag–β-catenin (b) interactions. T-Ag expression is observed in Δ98-transfected RKO cells and colocalizes with nuclear p53 and β-catenin, while negative controls (RKO–pUC) show no increase in T-Ag or β-catenin/p53. Western blot analysis (c) and coimmunoprecipitation (d) at day 7 posttransfection confirm what was seen at microscopy. Expression of T-Ag, VP1, β-catenin, and p53 increased in Δ98- and Mad-1-transfected RKO cells. The expression of these proteins decreased after >7 days posttransfection (data not shown). Coimmunoprecipitation experiments confirmed interactions between T-Ag, and β-catenin, as well as between T-Ag and p53 (Ricciardiello et al., 2003).

TABLE 5.4. Effects of JCV Transfection on RKO Cells

	7 days				14 days				21 days			
	WT	Mad-1	Δ98	pUC	WT	Mad-1	Δ98	pUC	WT	Mad-1	Δ98	pUC
PCR (T-Ag and TCR)	–	Y	Y	–	–	Y	Y	–	–	Y	Y	–
Southern blot	–	Y	Y	–	–	–	–	–	–	–	–	–
T-Ag expression	–	Y	Y	–	–	–	–	–	–	–	–	–
VP1 expression	–	Y	Y	–	–	–	–	–	–	–	–	–
p53 expression	–	Y	Y	–	–	–	–	–	–	–	–	–
Nuclear β-catenin	–	Y	Y	–	–	–	–	–	–	–	–	–
Chromosomal aberrations	–	+	+	–	–	++	++	–	–	+++	+++	–

JCV Mad-1 and Δ98 variants produce T-Ag in epithelial cells, but replicate inefficiently in RKO cells. In the early stages posttransfection, T-Ag is capable of binding to β-catenin and p53, leading to CIN, which increases between days 7 and 21, with concomitant progressive losses of viral genomes and proteins. Both Mad-1 and Δ98 strains of JCV were transfected. WT refers to the wild-type (unmanipulated) cells, and pUC refers to transfection of the plasmid without a JCV sequence (Ricciardiello et al., 2003).

in the suppression of viral gene expression and in the induction of latency (Mikovits et al., 1998; Robertson et al., 1999). More direct evidence for such roles of viruses has come from recent studies of polyomaviruses, wherein it has been suggested that the key protein mediating methylation events is the T-Ag. For instance, SV40 T-Ag is believed to mediate the cellular transformation of cultured epithelial cells by regulating the activities of a key *de novo* DNA methyltransferase, DNMT3b (Slack et al., 1999; Soejima et al., 2003). Clinical evidence supporting this was obtained when a significant correlation was observed between the presence of SV40 T-Ag and the methylation of multiple genes in non-Hodgkin's lymphomas (Shivapurkar et al., 2004) and malignant mesotheliomas in T-Ag-positive cases (Suzuki et al., 2005).

JCV infects the GI tract in early childhood without causing overt disease, and by virtue of encoding a gene for T-Ag, has the latent capability of causing the methylator phenotype in colorectal epithelial cells later in life. We hypothesized that T-Ag interacts with DNA methyltransferases to initiate the process of aberrant hypermethylation that may result in the establishment of CIMP in colorectal epithelium. Once the methylator phenotype has been established and sufficient genomic instability is established, JCV may be lost from a mature carcinoma, either by accidental loss (since it is no longer necessary) or by negative selection of infected cells.

More than 50% of sporadic CRCs demonstrate CIN, and the remainder are predominantly represented by CIMP (Goel et al., 2003). Recent work from our laboratory suggests that CIN and CIMP represent two independent and inversely related mechanisms of genetic and epigenetic instability in sporadic CRCs, and further confirm that MSI in these cancers is usually a consequence of CIMP, which leads to hypermethylation of the *hMLH1* gene (Goel et al., 2007).

JCV is present in the GI tract of most people and can induce CIN in cultured cells. The mechanistic basis of MSI cancers is relatively clear (i.e., due to the loss of DNA mismatch repair [MMR] activity) and is a consequence of either CIN or CIMP. The causes of CIN and CIMP in human CRCs are the principal unresolved issues. As discussed in the earlier sections, we already have presented *in vitro* data that JCV T-Ag can induce CIN. However, there are no direct data indicating whether T-Ag is involved in the genesis of CIMP. If JCV can be shown to induce CIMP, it would provide a complete explanation for the genesis of CRC.

To test this hypothesis, we analyzed 100 sporadic CRCs for JCV T-Ag protein expression and aberrant methylation of several tumor suppressor genes and sought associations between T-Ag expression and MSI, CIN, and CIMP (Goel et al., 2006). We observed that T-Ag DNA sequences were present in 77% of CRCs and that 56% of these expressed T-Ag protein, confirming the prior observations. We then found significant associations between the expression of T-Ag protein and CIN in these neoplasms, which supported the *in vitro* findings. Importantly, we also found that a significant majority of T-Ag-expressing CRCs had the CIMP mutational signature, based on the methylation of multiple genes. Therefore, we speculate a broad and multifaceted role for JCV and the viral oncogene it encodes, and suggest that JCV may be involved in colorectal carcinogenesis through multiple mechanisms of genetic and epigenetic instability. Interestingly, in recent *in vitro* studies, we have more direct evidence that JCV may induce CIMP. We selected a panel of colon cancer cell lines that have

low or absent basal levels of promoter methylation at several tumor suppressor genes. When these cell lines were transfected with a plasmid containing JCV T-Ag DNA, we observed *de novo* hypermethylation of several gene promoters, supporting a mechanistic role for JCV in CIMP (unpublished data).

SUMMARY AND CONCLUSION

In summary, we have found JCV DNA in most CRCs and in the normal colon of people with or without CRC. Substantially more copies (100-fold) of the virus can be found in the tumors compared with nonneoplastic tissue. T-Ag, the transforming oncogene of JCV, is expressed in CRC tumor cells, but never in nonneoplastic tissue. Only Mad-1 strains are found in the GI tract. Transfection of JCV into diploid CRC cells induces CIN, and interactions can be demonstrated between the T-Ag and p53 as well as β-catenin proteins. T-Ag expression is associated with CRCs that have either CIN or CIMP. Preliminary evidence suggests that JCV may also be involved in the induction of CIMP. There is strong evidence that JCV is mechanistically involved in the genesis of cancers throughout the GI tract.

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6

POSSIBLE ASSOCIATION OF BK VIRUS WITH PROSTATE CANCER

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INTRODUCTION

BK virus (BKV) is a member of the polyomavirus family that persistently infects nearly all humans by early childhood. The virus was initially isolated from the urine of a renal transplant patient in 1971 and derives its name from the initials of that patient (Gardner et al., 1971). It establishes a lifelong, persistent infection in the kidneys and can reactivate under immunosuppressive conditions (Reese et al., 1975; Padgett and Walker, 1976; Barbanti-Brodano et al., 1998). BKV causes significant morbidity in bone marrow and renal transplant recipients, and perhaps other solid organ transplant patients, in whom lytic viral replication occurs in the bladder and kidney, respectively. BKV reactivation leads to allograft dysfunction in up to 10% of renal transplant patients, ultimately leading to graft loss in at least half of these cases (Randhawa and Demetris, 2000; Hirsch, 2002). The incidence of BKV reactivation has been observed to be 26% in pediatric transplant patients, with high viral DNA titers in the blood directly correlating with impairment of renal allograft function and increased risk of developing BKV nephropathy (PVN) (Ginevri et al., 2003). As is the case for all polyomaviruses, BKV encodes at least two proteins that deregulate the ability of the cell to control its growth. In this chapter, we will discuss BKV infection in humans, its known transformation characteristics and

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oncogenicity *in vitro* and in animal models, and what is known about the potential role of BKV in human prostate cancer. The reader is referred to the chapter on polyomaviruses in *Fields Virology* for details on the basic biology of BKV (Imperiale and Major, 2007).

THE BKV LIFE CYCLE

BKV is a nonenveloped virus with an icosahedral capsid that surrounds a circular, double-stranded DNA genome that is a little over 5 kb in length (Imperiale, 2000, 2001; Tognon et al., 2003). BKV is very similar to JCV, SA12, and SV40, three other primate polyomaviruses, in DNA sequence and genome organization. The capsid is comprised of 72 pentamers of the late protein VP1 arranged in a $T = 7$ icosahedron (Rayment et al., 1982; Liddington et al., 1991; Li et al., 2003; Nilsson et al., 2005). Each VP1 pentamer is associated with one copy of either of the other two capsid proteins, VP2 or VP3. The BKV genome is divided into three functional regions: the early region, the late region, and the viral regulatory or noncoding control region (NCCR). The early genes are transcribed from one strand of the genome, and the late genes are transcribed from the complementary strand. Both transcriptional promoters are found in the NCCR, which also contains the origin of DNA replication. The BKV genome encodes at least six viral proteins. Two of these proteins, large tumor antigen (TAg) and small tumor antigen (tAg), are expressed from the early region, and four proteins, the capsid components VP1, VP2, and VP3, and another protein called the agnoprotein, are expressed from the late region. The tumor antigens are so-named based on the early discovery that they were the major antigens recognized by the immune systems of animals harboring virally induced tumors (Black et al., 1963).

The virus binds to the cell through an interaction with the gangliosides GD1b and GT1b (Low et al., 2006), although it has also been reported that sialic acid-containing glycoproteins can serve this function (Dugan et al., 2005). After internalization, the viral particle traffics to the nucleus, where the DNA genome is uncoated and transcription from the early promoter begins. The early genes are expressed from two alternatively spliced mRNAs that have the same 5′ and 3′ ends. Once TAg accumulates, it recruits the host DNA polymerase α/primase complex and other components of the host DNA replication machinery to the viral origin of DNA replication, thereby allowing viral DNA replication to initiate (Waga et al., 1994). TAg also represses early gene transcription and stimulates expression of the late genes. The capsid proteins are expressed from alternatively spliced mRNAs whose transcription initiation and polyadenylation sites are identical. Once the viral DNA has been replicated and new structural proteins have been produced, progeny virions are assembled and released from the cell concomitant with cell lysis.

Polyomaviruses have a dual lifestyle, depending upon the environment of the host cell. If the cell is permissive to viral DNA replication, the viral life cycle is completed and the cell is killed. If the cell is nonpermissive to viral DNA replication, however, the outcome is either an abortive infection or oncogenic transformation (Imperiale, 2000, 2001; Tognon et al., 2003). The latter outcome is dependent on continued expression of the two tumor antigens.

BKV INFECTION OF HUMANS

Numerous serological analyses indicate that primary infection with BKV occurs during early childhood (Shah et al., 1973; Knowles, 2001). Seropositivity among adults worldwide ranges from 46% to 94% (Knowles, 2001). Nearly all individuals are infected by 10 years of age except for small, segregated populations living in isolated regions of the world (Gardner, 1973; Mäntyjärvi et al., 1973; Shah et al., 1973; Portolani et al., 1974; Brown et al., 1975). While the natural route of transmission has not been established, the early age of seroconversion, along with evidence that the virus can be detected in the tonsils (Goudsmit et al., 1982), suggest that transmission is through aerosolized virus (Goudsmit et al., 1982; Brown et al., 1984). However, the virus has been detected in feces and urine, making it difficult to rule out fecal–oral or urine–oral routes (Bofill-Mas et al., 2001). Other reported routes of BKV transmission include semen, transfusion of blood products, and organ transplantation (Andrews et al., 1988; Dolei et al., 2000; Shah, 2000). The presence of BKV replication in lymphocytes suggests that infected lymphocytes may carry BKV in the bloodstream, allowing the virus to spread to different target organs (Portolani et al., 1985; Dörries et al., 1994; De Mattei et al., 1995; Chatterjee et al., 2000; Dolei et al., 2000). Following this initial viremia, renal tubular epithelial cells and urothelia become infected with BKV, where it persists for the lifetime of the individual.

BKV can be shed periodically in the urine of healthy individuals, but clinically relevant reactivation of BKV infection is only known to occur when the immune response is compromised. This is mainly due not only to immunosuppressive therapy in kidney, bone marrow, and other solid organ transplantation, but also to pregnancy, chemotherapy, HIV, diabetes, systemic lupus erythematosus, and use of cytotoxic drugs (Kahan et al., 1980; Goudsmit et al., 1982; Chesters et al., 1983; Brown et al., 1984; Knowles, 2001). Reactivation of BKV in renal transplant patients causes hemorrhagic cystitis, urethral stenosis, and interstitial nephritis (Colvin and Mauiyyedi, 2001; Suh et al., 2003). BKV has also been shown to be associated with hemorrhagic cystitis in recipients of bone marrow transplants (Azzi et al., 1994; Stoner et al., 2002). BKV-associated nephropathy (also called polyomavirus nephropathy), characterized by deterioration of the graft, is a serious and growing source of complications in kidney transplantation (Ginevri et al., 2003).

There are other reported clinical manifestations of BKV infection that have not been thoroughly investigated, including inflammatory syndromes and a variety of urinary tract diseases such as prostatic hypertrophy, renal calculi, bladder cancer, and urethral stricture, in which BKV DNA has been reported (Cobb et al., 1987; Bratt et al., 1999; Cubukcu-Dimopulo et al., 2000; Nickeleit et al., 2000a,b; Reploeg et al., 2001). Viral DNA and RNA have also been reported to be found in the brain, peripheral blood mononuclear cells, kidney, bladder, prostate, uterine cervix, sperm, lips, and tongue (Elsner and Dörries, 1992; De Mattei et al., 1994, 1995; Dörries et al., 1994; Monini et al., 1995a,b, 1996; Martini et al., 1996, 2004; Dolei et al., 2000; Zambrano et al., 2002; Das et al., 2004; Lau et al., 2007), although the validity of some of these findings is uncertain.

ONCOGENIC TRANSFORMATION BY BKV

BKV is recognized as a tumor virus because of its ability to cause changes in cell growth and tumor formation in cultured cells and animal models, respectively. BKV oncogenically transforms nonpermissive rodent cells in culture and immortalizes permissive human cells alone or in the presence of other oncogenes including c-*ras*, c-*myc*, and adenovirus E1A (Imperiale, 2001). Cells from the kidneys and brains of hamsters, mice, rats, rabbits, and monkeys can be transformed by either the early region alone or the entire viral genome (Major and Mayorca, 1973; Portolani et al., 1975; Takemoto and Martin, 1975; Tanaka et al., 1976; van der Noordaa, 1979; Costa et al., 1977; Mason and Takemoto, 1977; Seehafer et al., 1977, 1979; Bradley and Dougherty, 1978; Portolani and Borgatti, 1978; Grossi et al., 1982a; Watanabe et al., 1982). BKV TAg and an activated c-*ras* oncogene transform hamster embryo cells more efficiently than TAg alone (Pagnani et al., 1988). Stable expression of TAg is essential for the maintenance of the transformed phenotype in hamster and mouse cells (Nakashatri et al., 1988). Anchorage-independent growth can be reversed in transformed rodent cells through expression of TAg antisense RNA (Nakashatri et al., 1988).

Infection of newborn or young hamsters, mice, and rats with BKV results in tumor formation (Corallini et al., 2001; Tognon et al., 2003). Tumors induced by BKV contain integrated or episomal viral DNA sequences and also express TAg. In hamsters, the route of injection influences the frequency of tumorigenesis: BKV is weakly oncogenic when inoculated subcutaneously, but induces tumors in a large percentage of the animals when introduced either intravenously or intracerebrally (Corallini et al., 2001; Tognon et al., 2003). The spectrum of tumors in hamsters include ependymoma, neuroblastoma, fibrosarcoma, osteosarcoma, pineal gland tumors, and tumors of pancreatic islets (Corallini et al., 2001; Tognon et al., 2003). In mice and rats, BKV causes fibrosarcoma, liposarcoma, osteosarcoma, nephroblastoma, glioma, and choroid plexus papilloma (Corallini et al., 1977; Noss et al., 1981; Noss and Stauch, 1984). Transgenic mice expressing the BKV early region develop hepatocellular carcinoma, renal tumors, and lymphoproliferative disease (Small et al., 1986; Dalrymple and Beemon, 1990). Purified BKV DNA is not oncogenic after subcutaneous or intravenous administration and only induces tumors at a very low rate when inoculated intracerebrally into rodents, probably due to the inefficiency of transduction of cells by the naked DNA (Corallini et al., 1982). A recombinant plasmid that expresses TAg and an activated c-Ha-*ras* oncogene produces highly undifferentiated, rapidly growing, malignant sarcomas when delivered subcutaneously to newborn hamsters, and brain tumors when inoculated intracerebrally (Corallini et al., 1987, 1988). However, if either gene is inoculated separately or TAg is expressed with a wild type c-Ha-*ras* proto-oncogene, tumors are not induced.

BKV does not transform human cells efficiently in culture, often causing abortive transformation (Shah et al., 1976; Portolani and Borgatti, 1978). In addition, human cells transfected with BKV DNA or infected with virus tend to be only partially transformed, acquiring morphological changes and an increased life span but not immortality, anchorage independence, or tumorigenicity in nude mice (Purchio and Fareed, 1979; Grossi et al., 1982b). On the other hand, human embryonic kidney cells

cotransfected with recombinant plasmids expressing the BKV early region and the adenovirus type 12 *E1A* gene are fully transformed (Vasavada et al., 1986), and coexpression of the early region with c-*ras* or c-*myc* induces a transformed phenotype but not immortalization (Pater and Pater, 1986). These observations suggest that BKV can induce complete transformation in conjunction with other oncogenic stimuli.

TRANSFORMING ACTIVITY OF THE BKV TUMOR ANTIGENS

As discussed above, BKV encodes two oncoproteins in its early region, TAg and tAg. TAg is a nuclear, 695-amino-acid phosphoprotein that plays crucial roles in the life cycle of the virus. tAg, a 172-amino-acid, cysteine-rich protein, is also important for efficient viral replication (reviewed in Rundell and Parakati, 2001). It is the continued expression of these proteins in the absence of lytic replication that leads to disruption of normal cell growth control and contributes to oncogenic transformation. During BKV infection, the virus wishes to establish a host cell environment in which DNA replication and progeny virion production are maximized. To do this, it carries out two key functions. First, it induces the cell to enter the S phase, in which the host DNA synthetic machinery that is required by the virus for replication of its genome is expressed. Second, it delays the onset of apoptosis, which is a natural cellular response to the attempt of the virus to drive the cell through the replication cycle.

The ability of TAg to transform cells therefore maps to its interactions with crucial cellular tumor suppressor proteins that are involved in these cellular events: the retinoblastoma susceptibility protein (pRb) and its family members, p107 and p130; and p53. pRb, p107, and p130 regulate cell cycle progression through their interactions with cyclin/CDK complexes and the E2F transcription factor family (Nevins, 1992; Weinberg, 1995, 1996; Sherr, 1996, 2004; Sherr and McCormick, 2002; Deshpande et al., 2005). The hypophosphorylated form of pRb binds to E2F and represses transcription of a set of genes that are required for progression into the S phase and for DNA replication. In a normal cell, mitogenic signals activate cyclin/cyclin dependent kinase (CDK) complexes that phosphorylate pRb. The phosphorylated form of pRb can no longer bind E2F, allowing the E2F to activate its target genes and drive progression through the cell cycle (Nevins, 1992; Weinberg, 1995, 1996; Sherr, 1996, 2004; Sherr and McCormick, 2002; Deshpande et al., 2005). TAg binds pRb, p107, and p130, resulting in cyclin/CDK-independent increases in free E2F and cell cycle progression in the absence of external mitogenic stimuli, and leading to uncontrolled host cell proliferation (DeCaprio et al., 1988; Dyson et al., 1989, 1990; Ewen et al., 1989; Ludlow et al., 1989; Chellappan et al., 1991; Wolf et al., 1995; Harris et al., 1996; Krynska et al., 1997; Howard et al., 1998). Mutational analysis of BKV TAg indicates that the activation of E2F requires both pRb binding and an N-terminal domain of the molecule called the J domain. This domain derives its name based on the structural and functional homology to the DnaJ family of molecular chaperones, and mediates a number of important TAg functions related to transcription, replication, and transformation (Campbell et al., 1997; Kelley and Georgopoulos, 1997; Stubdal et al., 1997; Brodsky and Pipas, 1998; Zalvide et al., 1998; Harris et al., 1998b; Sock et al., 1999). The working model for the induction of

oncogenic transformation by the J domain is that TAg interacts with the pRb protein family, leading to the release of E2F and subsequent J domain-mediated degradation of the pRb family.

BKV tAg shares its amino-terminal 80 residues with TAg, and therefore, the only overlapping functional domain that is known to be involved in transformation is the J domain. BKV tAg function is not well characterized and studies are very limited, but SV40 tAg has been very well studied; most of what is known about BKV is based on the presumed functional similarity to SV40 tAg, with which it shares 80% sequence identity (reviewed in Imperiale, 2000, 2001; Rundell and Parakati, 2001). Like TAg, tAg is important in inducing a cellular environment that is conducive to efficient replication (Pallas et al., 1990; Sontag et al., 1993; Rundell and Parakati, 2001). The unique C-terminus of tAg allows it to bind to the cellular serine/threonine phosphatase, PP2A, which regulates signal transduction pathways in the cell involving mitogenic protein kinases such as MAPK (Yang et al., 1991; Sontag et al., 1993; Baysal et al., 1998; Beck et al., 1998). The association of tAg and PP2A was first discovered in coimmunoprecipitation experiments aimed at identifying cellular proteins that interacted with the tAg of SV40 and BKV (Yang and Wu, 1979; Rundell et al., 1981; Walter et al., 1988). Subsequent cDNA cloning and peptide sequencing of proteins revealed the identification of these proteins as the A and C subunits of PP2A (Walter et al., 1989; Pallas et al., 1990). Through this interaction with tAg, PP2A is inhibited, resulting in increased levels of signal transduction that results in the stimulation of cell cycle progression.

The negative regulation of PP2A by tAg has been shown to be important for anchorage-independent growth in transformed cells and for tumorigenesis in animal models (Porras et al., 1996; Yu et al., 2001). SV40 tAg also enhances transcription of E2F-activated promoters of early growth response genes and plays a role in transformation under certain conditions (Pallas et al., 1990; Loeken, 1992; Mungre et al., 1994; Beck et al., 1998). Additionally, a BKV strain lacking a C-terminal segment of tAg, BKV-IR, exhibits a low level of stable transformation and reduced incidence of tumors with a longer latency period (Caputo et al., 1983; Pagnani et al., 1986).

The cell responds to this assault on growth control by mounting a p53 response. p53 mediates cell cycle arrest and apoptosis in response to DNA damage, oncogenic stresses, and stimulation of unscheduled DNA replication (Kastan et al., 1991; Lowe et al., 1993). Upon infection with polyomaviruses, cellular p53 levels become elevated due to stabilization of the protein. However, the p53 is bound by TAg, and therefore, does not retain its normal activity (Crawford, 1983; Deppert et al., 1989; Collot-Teixeira et al., 2004). This interaction stabilizes the p53 protein and prevents it from responding to DNA damage (Harris et al., 1998a; Kang and Folk, 1992) by inhibiting its transcriptional activation of its target genes such as GADD45, MDM2, p21 (WAF1/CIP1), and Bax, which are involved in cell cycle arrest and apoptosis (Bollag et al., 1989; Haggerty et al., 1989; Zhan et al., 1994; Selivanova and Wiman, 1995; Harris et al., 1998a; Pipas and Levine, 2001; Harris and Levine, 2005). In this way, TAg-expressing cells escape p53-mediated cell cycle arrest or apoptosis. During a productive infection of permissive cells, this mechanism keeps the cell alive as long as possible to maximize the production of progeny virions. If the host cell is nonpermissive for viral infection, or

if rearrangements in the viral chromosome occur, which interfere with viral DNA replication, this can lead to oncogenesis since the T antigens are expressed but the viral life cycle is not completed. One report demonstrated that the binding of SV40 TAg to mouse p53 completely inhibits p53 transactivation, whereas interaction with human p53 permits the transcriptional activation of genes involved in apoptosis and growth arrest (Sheppard et al., 1999). Hence, one can speculate that the difficulty associated with inducing transformation of human cells by SV40 or, by analogy, the human polyomaviruses, could be due to the inefficiency of the TAgs to completely inhibit the tumor suppressor activities of p53 and pRb.

TAg plays a potential third role in oncogenesis by inducing chromosomal damage, although the exact mechanism is not very well understood (Ray et al., 1990; Theile and Grabowski, 1990; Tognon et al., 1996; Trabanelli et al., 1998). BKV induces mutations in cultured rodent cells and in human peripheral blood lymphocytes (Theile and Grabowski, 1990). Increased chromosomal damage in circulating lymphocytes in humans correlates with high titers of antibodies against the virus (Lazutka et al., 1996). Human fibroblasts expressing BKV TAg exhibit cytogenetic alterations such as dicentric chromosomes, deletions, duplications, and translocations (Trabanelli et al., 1998). The chromosomal damage in BKV TAg-expressing cells is detectable prior to the appearance of morphological transformation, suggesting that the chromosomal damage due to the effect of TAg expression contributed to transformation. Cytogenetic studies on two human glioblastoma multiforme cell lines expressing both BKV and SV40 TAg exhibit similar chromosomal alterations (Tognon et al., 1996). The mechanism for the clastogenic effect induced by BKV is not known, although it may be due to the ability of TAg, in conjunction with topoisomerase I, single-strand DNA-binding protein, and ATP, to stimulate chromosomal damage as they unwind the DNA double helix (Dean et al., 1987; Simmons et al., 1996). Additionally, binding of TAg to p53 may enhance the level of DNA damage in the cell because p53-induced arrest and apoptosis would be blocked and damage would not be repaired. The mutagenic effects of BKV may alter the expression of genes regulating cell growth. It is possible that during a lifetime of persistence in humans, there might be cumulative effects due to low levels of TAg expression.

DETECTION OF BKV IN TUMOR SPECIMENS

Various manuscripts describing the presence of BKV in human cancers have appeared in recent years (reviewed in Das and Imperiale, 2006). Technological advances in the field of PCR have made it possible to detect DNA and RNA in small biopsy samples. However, concerns have been raised about the possibility of false positives during PCR-based analysis due to the sensitivity of the technique. Therefore, additional analyses such as sequencing of PCR products, *in situ* PCR, *in situ* hybridization (ISH), immunohistochemistry (IHC), or Southern blotting provide stronger evidence for the presence of the virus. In addition, *in situ* methods allow the determination of the exact cellular location of BKV in tissue sections. Another disadvantage of PCR-based screening of samples is that biopsy samples are not homogeneous populations of cells: A small

number of virus-positive normal cells in a tumor sample, for example, can make the tumor look positive. This is a significant concern due to the ubiquity of BKV in the human population. Its prevalence in nearly 100% of the population also precludes epidemiological studies. At this time, there are several contradictory reports on the presence of BKV DNA in human tumor tissues, and while TAg expression has been demonstrated in some tumors, data establishing a truly causal role for BKV have not yet been reported.

While older reports using Southern blotting, the state of the art for detecting specific DNA sequences at that time, failed to detect BKV in prostate cancers (Wold et al., 1978), there are several more recent reports on the presence of BKV DNA in urinary tract tumors (Monini et al., 1995b; Volter et al., 1997; Zambrano et al., 2002; Das et al., 2004; Lau et al., 2007). The urinary tract would appear to be a logical site for BKV-induced carcinogenesis since the virus is known to persist in the kidneys. Monini et al. (1995b) detected BKV sequences by PCR in greater than 50% of both normal and tumor tissues isolated from the urinary tract and prostate. Southern blotting was also used to demonstrate the presence of viral DNA in six of the approximately 70 tumors examined. BKV was not detected in normal tissue with this technique, however. There are two likely explanations for this observation: amplification of the viral genome in the tumor cells or selection in the tumor for cells containing BKV. The evidence favors the latter possibility, as the viral sequences were integrated into the host chromosome in all six tumors analyzed, although one also contained episomal viral DNA. In four of the tumors, the viral copy number was less than one, which may be explained by contamination of virus-positive tumor cells with surrounding stromal cells that do not contain any virus. Unfortunately, the authors were not able to rescue virus from the samples by transfection of tumor DNA into permissive cells. They did, however, sequence the viral origin of replication of nine positive samples, including three prostate hyperplasias. All the sequences were identical and represented a new strain of BKV, which was designated URO1. This strain had rearrangements in the viral regulatory region that were predicted to affect viral DNA replication. Various studies have shown that mutations in this region can decrease DNA replication efficiency and increase the transformation potential of the virus in cell culture (Watanabe and Yoshiike, 1982, 1985; Rubinstein et al., 1991). A block to lytic replication could lead to transformation if the T antigens were to be expressed continually, similar to the transformation of otherwise permissive cells by SV40 variants containing mutations in their origin of replication (Gluzman, 1981; Butel, 1986). It has not yet been reported, however, if the specific mutations in URO1 affect replication or transformation.

Since that report, three groups have examined prostate tissue for the presence of BKV. Zambrano et al. (2002) examined paraffin-embedded and fresh frozen tissue using PCR. While they found the results from fixed tissue to be inconsistent, in frozen tissues they found the virus in 3 out of 12 patient specimens analyzed. Two of these were tumor tissue and one was normal. A subsequent report described a more detailed analysis of prostate tissues. Das et al. (2004) demonstrated the presence of BKV sequences in the epithelium of benign ducts and atrophic lesions of prostates obtained from radical prostatectomies. Proliferative inflammatory atrophy (PIA) is thought to be the initial stage in prostate carcinogenesis (De Marzo et al., 2007). This study observed

the presence of the BKV early region in 94% of the samples and the regulatory region in 81% of the samples. Sequence analysis of the regulatory region demonstrated the presence of at least three BKV strains and ruled out laboratory contamination. *In situ* hybridization with a viral early region probe detected BKV DNA in two-thirds of the samples. DNA was found in normal epithelial cells and in PIA, but not in high-grade prostatic intraepithelial neoplasia or invasive tumor cells, both later stages of prostate cancer progression. The virus was also not found in stromal cells. Immunohistochemical analysis detected TAg in slightly less than half of the samples, and only in atrophic cells. No TAg staining was observed in normal or tumor cells. BKV late protein expression was not observed in any of these samples (Das et al., 2008).

The TAg detected in this prostate study was cytoplasmic. Cytoplasmic TAg can still be tumorigenic: It transforms cells in culture and induces tumors in transgenic mice at a rate equivalent to wild-type virus (reviewed in Lanford et al., 1985; Butel and Jarvis, 1986; Pinkert et al., 1987). The expression of TAg in the cytoplasm correlates with the lack of late protein detection since the virus would be unable to replicate and express late proteins if TAg were not in the nucleus. Interestingly, the staining pattern for p53 in the TAg-expressing cells was also cytoplasmic, with the two proteins colocalizing. Localization of p53 to the cytoplasm implies that p53 could not carry out its transcriptional activation function. In cells that were TAg negative, when p53 was detectable it was nuclear. A model to explain these observations would be that cells containing cytoplasmic p53 would be unable to repair DNA damage during replication, therefore allowing them to accumulate mutations that could ultimately lead to complete loss of growth control and tumorigenesis. As the clonal tumor cells grow out, viral sequences could be lost due to dilution of the episomal DNA or selected against by the immune system.

It is also possible that continued TAg expression is incompatible with the additional oncogenic mutations. This behavior would be consistent with observations in the mouse model of prostate cancer, TRAMP (*tr*ansgenic *a*denocarcinoma of *m*ouse *p*rostate). In this model, SV40 TAg expression, under the control of the prostate-specific probasin promoter, drives tumor formation. When tumor cells are grown in culture they lose TAg expression (Foster et al., 1997). Additionally, TAg is known to have proapoptotic effects, perhaps mediated through interactions with the pRb family proteins or its chromosomal damaging ability, which could be incompatible with cell growth as cancer progresses (Theile and Grabowski, 1990; Tognon et al., 1996; Trabanelli et al., 1998). A similar loss of viral sequences has been demonstrated in tumors caused by bovine papillomavirus type 4 (BPV-4) (Campo et al., 1985). Although the virus is required for the induction of papillomas of the alimentary canal in cattle, its presence is not necessary for progression to, or maintenance of, the transformed state. The mechanisms by which papillomaviruses and polyomaviruses oncogenically transform cells are very similar.

Most recently, Lau et al. (2007) also used ISH and IHC to examine cancerous prostate tissue. While they reported no immunoreactivity in their samples, their criterion for a positive result was nuclear staining. Therefore, it is possible that they had samples with cytoplasmic staining as in the report discussed above but ignored them. Their ISH results were slightly more positive, with 2 out of 30 specimens giving a

positive signal. It is not clear why the latter two groups saw a fairly significant difference in the percentage of BKV DNA positive prostates.

CONCLUSIONS

In summary, it can be stated that although BKV has all the qualifications to be involved in prostate cancer, additional studies are required to confirm or refute its role in carcinogenesis. Several issues need to be addressed. Since BKV does not replicate in other animal hosts, one also cannot easily fulfill Koch's postulates. An attractive alternative set of criteria for assigning viruses to be the cause of human cancer has been proposed (Barbanti-Brodano et al., 1998; Lednicky and Butel, 1999; zur Hausen, 1999, 2001). These include epidemiological and clinical evidence that a viral infection puts one at risk for tumor development, presence of the nucleic acid and/or the expression of the virus in specific tumor cells, induction of cell transformation or immortalization upon transfection of either the viral genome or its individual subdomains; and demonstration that the phenotypic characteristics of the tumor and the changes caused in cultured cells from the tumor are dependent on the expression of the viral genome. The evidence to date indicates that BKV may fulfill at least the latter three criteria: It transforms cells in culture and induces the development of tumors in animals or transgenic mice, and viral DNA and protein expression have been detected in a small number of human tumor specimens. However, additional analyses are clearly required before a definitive statement can be made regarding the role of BKV in prostate cancer.

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7

ONCOGENIC TRANSFORMATION BY POLYOMAVIRUS LARGE T ANTIGEN

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The discovery of polyomavirus emerged in a surprising fashion during studies on murine leukemia virus (MLV). In 1953, Ludwig Gross discovered that newborn mice injected with cell-free extracts of leukemia tissues developed salivary gland carcinomas and a range of other solid tumors, rather than leukemia. Due to its ability to induce multiple tumors, this agent acquired the name *polyomavirus*, which is derived from the Greek word *poly*, meaning many, and *-oma*, denoting cancer. Polyomaviruses are a family of nonenveloped DNA viruses with icosahedral capsids containing a small, circular, double-stranded DNA genome. This chapter will discuss the primate polyomaviruses simian virus 40 (SV40), JCV, BKV, lymphotropic polyomavirus (LPV), simian agent 12 (SA12), Karolinska Institute polyomavirus (KIPyV), Washington University virus (WUV), and the newly discovered Merkel cell polyomavirus (MCPyV).

The early region of these viruses encodes nonstructural proteins, a large T antigen (TAg) and a small t antigen (st) (Table 7.1). Some of the primate polyomavirus encode additional proteins (such as 17kT antigen [17kT], T'_{135}, T'_{136}, and T'_{165}) due to alternate splicing. The large tumor antigens interfere with cell cycle regulators such as p53 and pRb and enhance the expression of genes associated with DNA synthesis. Consequently, cells are forced to enter the S phase. Shortly afterward, viral DNA replication begins. Simultaneously, with the initiation of viral DNA replication, the late promoter becomes active, resulting in the expression of the late genes, including viral coat proteins, VP1,

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TABLE 7.1. Comparison of the Primate Polyomavirus Genomes and Their Major Proteins

Virus	Natural Host	Genome Length (bp)	Virus-Encoded Proteins[a]					HR[b] Domain	Tissue Specificity
			LT	st	VP1	VP2	VP3		
SV40	Rhesus monkey	5,243	708	174	364	352	234	Yes	Kidney
JCV	Human	5,130	688	172	354	344	225	Yes	Kidney, central nervous system
BKV	Human	5,153	695	172	362	351	232	Yes	Kidney
SA12	Chacma baboon	5,230	699	172	364	352	234	Yes	Kidney
LPV	African green monkey	5,270	697	189	368	356	237	No	B lymphocyte
KIPyV[d]	Human	5,040	641	191	378	400	257	No	Lung
WUV	Human	5,229	648	194	369	415	272	No	Lung
MCPyV	Human	5,387	258[c]	186	423	241	196	No	Skin

[a]The number of amino acid residues in each viral protein is indicated.
[b]Host range (HR) domain lies at the C-terminus of TAg and determines the capability of the virus for productive infection in certain cell lines.
[c]Truncated T antigen.
[d]KI polyomavirus Stockholm 60.

VP2, and VP3. This results in the assembly of progeny virions, host cell lysis, and release of viral progeny.

Both polyomavirus infection in laboratory animals and large TAg expression in transgenic mice result in a wide range of tumor formation. Tumorigenesis by these viruses mostly depends upon the route of injection. For example, hamsters injected intravenously with SV40 develop osteosarcoma, lymphoma, and leukemia (Diamandopoulos, 1972). In contrast, hamsters injected intracranially develop choroid plexus tumors and ependymomas (Kirschstein and Gerber, 1962), while hamsters injected intrapleurally develop mesothelioma (Cicala et al., 1993). The consequences of TAg expression in different tissues have recently been reviewed emphasizing cell type-specific effects (Ahuja et al., 2005). For example, ectopic expression of large TAg in the mouse choroid plexus epithelium (CPE) results in renewed proliferation and tumor formation of this epithelial tissue (Chen and Van Dyke, 1991). On the other hand, TAg expression in enterocytes of the intestine results in hyperplasia and dysplasia, but no distinct tumors are observed (Hauft et al., 1992; Kim et al., 1993). Transgenic mice expressing BKV TAg develop a variety of tumors such as primary hepatocellular carcinomas, renal tumors, enlarged thymuses (ranging from hyperplasia to thymomas and lymphomas), and renal adenocarcinomas (Small et al., 1986). Transgenic mice expressing JCV TAg exhibit distinct phenotypes such as dysmyelination, adrenal neuroblastoma, peripheral neuroblastoma, medulloblastoma, and pituitary adenoma (Small

et al., 1986; Franks et al., 1996; Krynska et al., 1999). Tumor induction by both JCV and BKV shows some viral strain variation with the prototype isolate. Finally, transgenic mice expressing LPV early region developed choroid plexus tumors (Chen et al., 1989). The potential of the remaining primate polyomaviruses to develop tumors in laboratory animals has not been tested.

The polyomaviruses encode dominant acting oncoproteins that are capable of interfering with the functions of tumor suppressors and oncogenes. Therefore, they serve as important tools for exploring molecular events leading to transformation and tumorigenesis. SV40 has been intensively studied to provide a model system for the study of basic eukaryotic cell processes, including DNA replication, transcription, malignant transformation, and signal transduction. This chapter will provide an overview of the molecular mechanisms used by SV40 large TAg and TAgs of other primate polyomaviruses to transform cultured cells and induce tumors in animal models (Table 7.2).

LARGE TAg ACTION ON Rb PROTEINS

All TAgs from primate polyomaviruses contain a leucine-X-cysteme-X-glutamic acid (LXCXE) motif that is essential for their interaction with the Rb family of tumor suppressors. The importance of this sequence in mediating the interactions with Rb proteins, and in transformation, was first demonstrated for the adenovirus E1A protein (Whyte et al., 1988). The inactivation of Rb proteins by TAg appears to be a common feature of polyomaviruses as coimmunoprecipitation assays performed by Dyson et al. (1990) show a stable protein complex between the TAgs of either mouse, monkey, baboon, or human polyomaviruses, and pRb.

SV40 and Rb

SV40 TAg was shown to bind Rb proteins (pRb, p130, and p107) via the LXCXE motif (DeCaprio et al., 1988; Ewen et al., 1989). Mutants of TAg that are defective for Rb binding also fail to induce transformation (Kalderon and Smith, 1984; Rutila et al., 1986; Zhu et al., 1992; Srinivasan et al., 1997). TAg-transformed mouse embryo fibroblasts (MEFs) show reduced levels of p130 due to proteasome-dependent degradation of p130, increased levels of p107, and no change in pRb protein levels (Stubdal et al., 1997). The LXCXE motif is required for growth to a high density in low- and high-serum concentrations, and for anchorage-independent growth of MEFs both in a normal and Rb-null background (Zalvide and DeCaprio, 1995). Similarly, mutations in this sequence render TAg defective in these assays when tested in MEFs lacking both p130 and p107 (Stubdal et al., 1997), suggesting that inactivation of all three Rb family members is necessary for transformation.

Several reports suggest that cooperation between the LXCXE motif and the J domain is essential for the disruption of Rb–E2F complexes (Zalvide et al., 1998; Sullivan et al., 2000a). A model to explain this cooperation has been proposed (Brodsky and Pipas, 1998) (Fig. 7.1). According to this model, TAg associates with the Rb–E2F

TABLE 7.2. Interaction of Polyomavirus Large T Antigen with Cellular Proteins

Cellular Protein	Viral Protein	Biological Consequences	References
pRb/p130/p107	SV40, JCV, BKV, T′(JCV)	Promotes G1/S transition	Bollag et al. (2000); DeCaprio et al. (1988); Dyson et al. (1990); Harris et al. (1996)
p53	SV40, JCV, BKV	Promotes G1/S transition, blocks apoptosis	Bollag et al. (1989); Pipas and Levine (2001)
hsc70	SV40	Disruption of pRb–E2F complex	Sullivan et al. (2000a, 2001)
p300/CBP	SV40	Unknown	Poulin et al. (2004)
Cul7	SV40	Unknown	Ali et al. (2004); Kohrman and Imperiale (1992)
Bub1	SV40	Override spindle checkpoint	Cotsiki et al. (2004)
Nbs1	SV40	Genomic instability	Digweed et al. (2002); Wu et al. (2004)
Fbw7	SV40	Unknown	Welcker and Clurman (2005)
IRS-1	SV40, JCV	Signal transduction	Fei et al. (1995); Lassak et al. (2002)
β-catenin	JCV	Change in gene expression	Gan and Khalili (2004)
TEF-1	SV40	Change in gene expression	Dickmanns et al. (1994)
AP-2	SV40	Change in gene expression	Mercurio and Karin (1989); Mitchell et al. (1987)
Sp1	SV40	Change in gene expression	Johnston et al. (1996)
TBP	SV40	Change in gene expression	Damania and Alwine (1996); Gruda et al. (1993); Martin et al. (1993)
TAF1 ($TAF_{II}250$)	SV40	Change in gene expression	Damania and Alwine (1996); Johnston et al. (1996)
TAF4 ($TAF_{II}130$)	SV40	Change in gene expression	Damania and Alwine (1996); Johnston et al. (1996)
TAF5 ($TAF_{II}100$)	SV40	Change in gene expression	Damania and Alwine (1996); Johnston et al. (1996)
RNA polymerase II (140-kDa subunit)	SV40	Change in gene expression	Johnston et al. (1996)
c-Jun	SV40	Change in gene expression	Bharucha et al. (1994)

complex via the LXCXE motif, and to the chaperone hsc70 via the J domain. The J domain stimulates hsc70 ATP hydrolysis, and the resulting energy is used to release E2F from Rb. This release results in the up-regulation of E2F transactivation activity and subsequent progression of cells into the S phase. Studies in cell culture show that the TAg J domain plays a role in transformation. An in-frame deletion mutant within the J domain is defective for the induction of dense foci despite being able to bind Rb

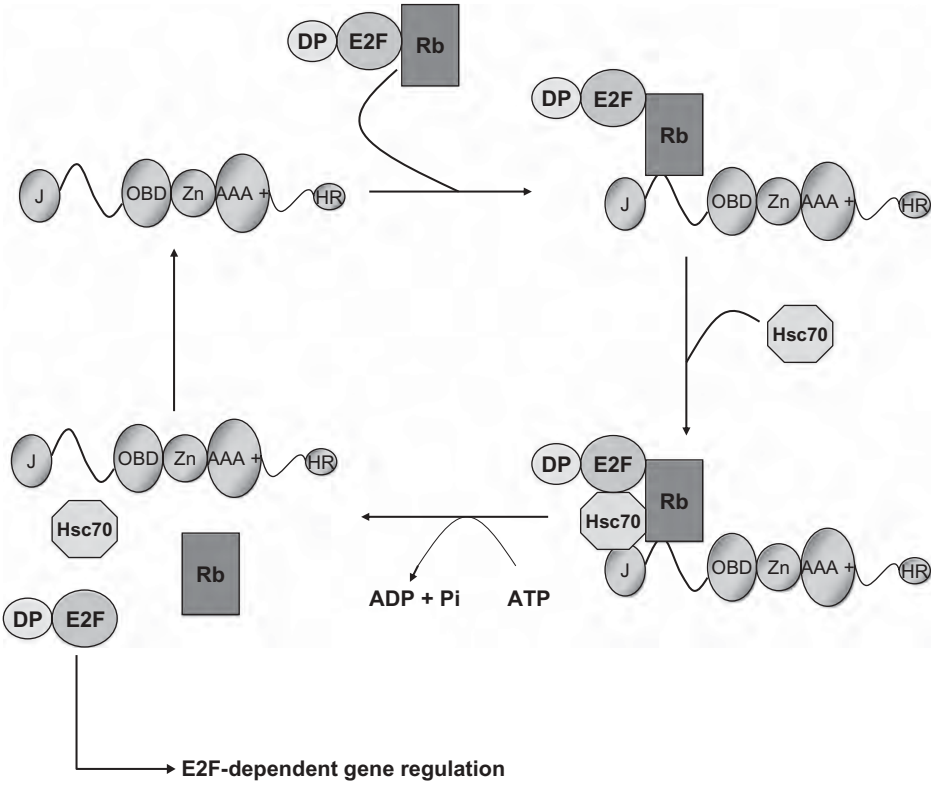

Figure 7.1. The chaperone model for disruption of Rb–E2F complexes. The large T antigen J domain recruits hsc70 and stimulates its ATPase activity, which in turn releases Rb from E2F. Free E2Fs are now able to transactivate genes required for cell cycle progression. See color insert.

proteins and p53 (Collins and Pipas, 1995). In contrast, single amino acid substitution mutants unable to bind hsc70 or to disrupt p130–E2F4 complexes induce focus formation and anchorage-independent growth, although at a somewhat reduced frequency compared with wild-type TAg. In general, J domain mutants are defective for altering p130 and p107 phosphorylation, and are unable to degrade p130; however, these mutants do not affect the phosphorylation state of pRb (Stubdal et al., 1997). Because different J domain mutants show different phenotypes in cell culture, the role of the J domain in transformation is confusing. The J domain is essential for transformation in at least one animal model; expression of full-length TAg in murine enterocytes results in intestinal hyperplasia, which progresses to dysplasia with age (Hauft et al., 1992; Kim et al., 1993). In contrast, similar transgenic mice expressing J domain mutant D44N do not develop hyperplasia (Rathi et al., 2007). Unlike mice expressing wild-type TAg, mice expressing D44N do not show reduced protein levels of p130, and the D44N protein is unable to dissociate p130–E2F DNA binding complexes. Furthermore, mice expressing D44N in a null p130 background are still unable to develop hyperplasia.

These studies demonstrate that the ectopic proliferation of enterocytes induced by TAg requires a functional J domain and suggest that the J domain is necessary to inactivate all three Rb family members.

BKV, JCV, and Rb

BKV and JCV are found widespread throughout the human population, with ~80% of adults exhibiting a persistent infection that is usually subclinical. However, BKV and JCV can reactivate in immunocompromised individuals and cause nephropathy (Comoli et al., 2006) and progressive multifocal leukoencephalopathy (PML), respectively (Khalili et al., 2003). Both BKV and JCV are tumorigenic in rodents and can transform cells in culture albeit with reduced efficiencies in comparison with SV40.

The TAgs from BKV and JCV are very closely related to SV40, sharing about 75% amino acid sequence identity (Frisque et al., 1984). The TAgs of both BKV and JCV contain a J domain and an LXCXE motif, and both bind to all Rb family members. BKV TAg–Rb complexes are detected only when large amounts of total protein are used. The levels of BKV TAg normally produced from viral promoter are too low to bind a significant amount of the Rb family proteins in the cell. However, these low levels of TAg are sufficient to down-regulate the expression and to induce phosphorylation of all three Rb family members and are also capable of inducing serum-independent growth (Harris et al., 1996). On the other hand, JCV-transformed cells show reduced levels of p130 and increased steady-state levels of hyperphosphorylated p107 and pRb (Bollag et al., 2000; Helt and Galloway, 2003). An interaction with hsc70 has not been reported for either BKV or JCV. Thus, the TAgs of SV40, BKV, and JCV alter levels of Rb family proteins differently. Whether this explains the variability in their transforming abilities in different cell types remains to be elucidated.

Using electrophoretic mobility shift assays, Harris et al. (1998) showed that free E2F (unbound to an Rb family member) was induced in cells transformed with BKV TAg. Despite the low levels of BKV TAg expression, the amount of free, transcriptionally active E2F is similar in cells expressing BKV TAg or SV40 TAg. Consistent with the chaperone model proposed for SV40, this increase in free E2F is dependent on both the J domain and LXCXE motif. By using a reporter assay, the authors showed that this increase in free E2F was paralleled by an increase in E2F-dependent transcription. However, the effect of BKV TAg on E2F target genes was not analyzed.

The mechanism by which JCV TAg targets Rb family members is not as well understood. JCV TAg contains a functional J domain, and therefore, like SV40, can be predicted to inactivate the Rb family proteins via a chaperone mechanism. In fact, a study by Sullivan et al. (2000b) showed that the J domain of JCV TAg could functionally replace the SV40 TAg J domain in disrupting p130–E2F complexes. In addition to the TAg and st, JCV encodes three additional splice variants of TAg termed as T'_{135}, T'_{136}, and T'_{165} (Trowbridge and Frisque, 1995). The T′ proteins also contain an LXCXE motif and a J domain, and bind to Rb family members with different efficiencies (Trowbridge and Frisque, 1995; Bollag et al., 2000). BKV also produces alternatively spliced, "truncated" versions of TAg, although the biological relevance of these proteins is not known.

Other Primate Polyomaviruses and Rb

The large TAgs encoded by SA12, LPV, KIPyV, WUV, and MCPyV have an LXCXE motif and a J domain approximately in the same positions found in SV40 large T-antigen (LTAg). The LPV LTAg was shown to bind pRb *in vitro* (Dyson et al., 1990). However, experiments determining a functional interaction of these TAgs with Rb–E2F complexes, or demonstrating that they have DnaJ molecular chaperone activity, have not been performed.

TAg INTERACTION WITH THE TUMOR SUPPRESSOR p53

SV40 and p53

p53 was first discovered as a cellular protein bound to TAg in SV40-infected or -transformed cells (Lane and Crawford, 1979; Linzer and Levine, 1979). TAg directly binds to the DNA-binding surface of p53, thus blocking its ability to bind promoters and to regulate gene expression (Bargonetti et al., 1993; Jiang et al., 1993). Complex formation between TAg and p53 interferes with the expression of p53-regulated genes, involved in several biological processes such as cell cycle (p21, cyclin G1), DNA repair (GADD45), apoptosis (Bax), and signal transduction (IGF-BP3). For example, TAg blocks the activation of p21, a universal Cdk inhibitor, and as a result the cells are unable to arrest in G1 and progress to S phase. Additionally, the binding of p53 by TAg prevents its degradation by Mdm2 and results in p53 stabilization. Therefore, high levels of p53, which are thought to be functionally inactive, are found in SV40-transformed cells (Oren et al., 1981). TAg can also block p53-dependent transcription and growth arrest through a mechanism that is independent of p53 binding but requires both the J domain and the LXCXE motif (Quartin et al., 1994; Rushton et al., 1997). Regardless of the mechanism, it is widely accepted that TAg creates a p53-null environment in the cell. Alternatively, TAg may manipulate p53 functions to benefit the virus and subsequently contribute to transformation.

BKV, JCV, and p53

TAgs from both viruses can form a complex with p53 (Bollag et al., 1989; Trowbridge and Frisque, 1993) but in comparison with SV40 and BKV, a smaller fraction of JCV TAg binds to p53. Like SV40, BKV and JCV also stabilize p53, and few studies suggest that this interaction blocks p53-dependent transcription of target genes (Shivakumar and Das, 1996; Krynska et al., 1997). More studies are needed to elucidate the role of TAgs–p53 interaction in BKV- and JCV-mediated transformation.

Other Polyomaviruses and p53

SA12, LPV, KIPyV, and WUV, but not MCPyV (due to a truncated TAg), have conserved sequences important for complex formation with p53. Nevertheless, an association of LPV TAg with p53 is not clear due to contradictory results by two research

groups (Symonds et al., 1991; Kang and Folk, 1992). Experiments testing the ability of LTAgs of SA12, KIPyV, and WUV to associate with p53 have not been reported.

TAg INTERACTION WITH THE CREB BINDING PROTEIN FAMILY

p300 and CBP are homologous transcriptional coactivators that possess both distinct and overlapping functions. These coactivator proteins are recruited by many different transcription factors such as cAMP response element binding (CREB), nuclear receptors, signal transducers and activators of transcription (STAT), NF-κB, and AP-1, and therefore play a broad role in multiple biological processes, including cell growth, cell cycle progression, and development. Not surprisingly, CBP/p300 have been linked with cancer (Goodman and Smolik, 2000; Iyer et al., 2004). The adenovirus E1A protein binds directly to p300/CBP through an N-terminal domain and CR1 region, and both regions are essential for its transforming activities. Genetic studies demonstrated that SV40 TAg can complement p300/CBP-binding-deficient E1A mutants to restore transformation (Yaciuk et al., 1991). In contrast to E1A, p300/CBP interacts with SV40 TAg indirectly, with p53 serving as a scaffold to bridge the interaction (Poulin et al., 2004). TAg induces the phosphorylation of p53 at S15, which in turn stabilizes the interaction between p53 and p300/CBP (Borger and DeCaprio, 2006). In turn, CBP acetylates TAg on K697 in a p53-dependent manner (Poulin et al., 2004). The biological consequences of this acetylation are not understood. This acetylation site is conserved on the TAgs from JCV, BKV, and SA12 (Poulin et al., 2004; Cantalupo et al., 2005), but not in those from LPV, KIPyV, WUV, and MCPyV. One report suggests that TAg helps deliver p300/CBP to E2F-dependent promoters, which may enhance the transcription of E2F target genes and progression to S phase (Ait-Si-Ali et al., 2000). This hypothesis is supported by the observation that activation of E2F-dependent promoters by the murine polyomavirus TAg requires both the inhibition of Rb proteins and an interaction with p300/CBP (Nemethova et al., 2004). Nevertheless, the functional consequences of large TAg binding to p300/CBP are unclear, and the role of this interaction in transformation has yet to be determined.

ADDITIONAL TAg TARGETS

Genetic studies suggest that in certain cases, inactivation of pRb and p53 is not sufficient to induce SV40 TAg-mediated transformation (Sachsenmeier and Pipas, 2001), thus indicating the presence of additional targets of TAg contributing to transformation. In the past few years, several additional potential targets of TAg have been discovered. While some of them appear to be important for transformation, others await more studies (Fig. 7.2). The following is a brief description of additional proteins that bind to SV40 TAg, and the possible relation to other TAgs.

Cul7 is a scaffold protein that forms the core subunit of an E3 ubiquitin ligase complex involved in degradation of proteins (Ali et al., 2004). SV40 TAg binds to Cul7 via residues 98–102, located between the J domain and the LXCXE motif. Mutants

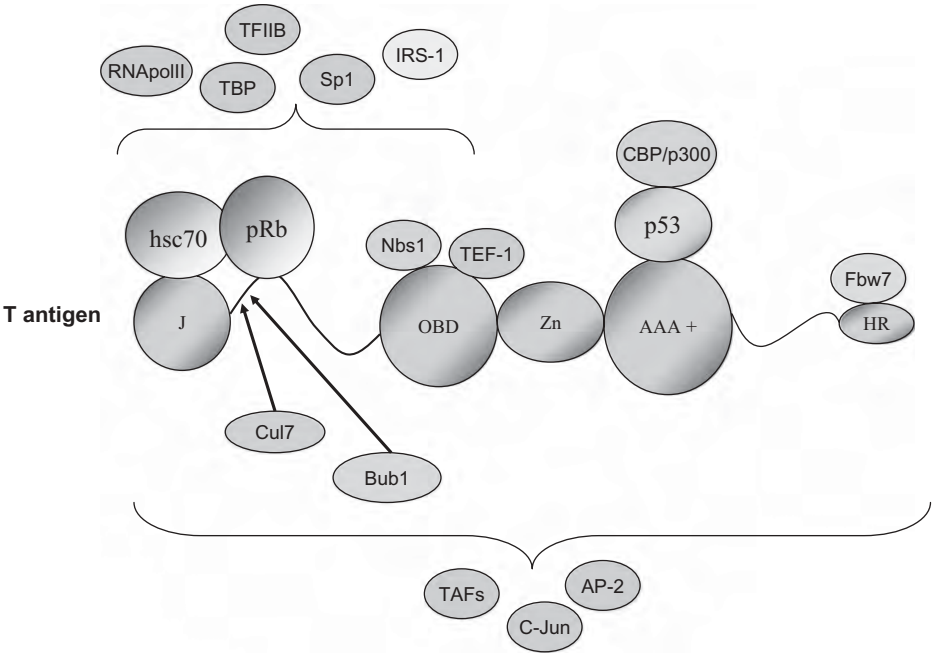

Figure 7.2. Interaction of cellular proteins with different domains/regions of SV40 large T antigen. T antigen binds to Rb-family proteins via an LXCXE motif, Hsc70 via the J domain, p53 via the p53 binding site, CBP/p300 via p53, Nbs1 and TEF-1 via the origin binding domain (OBD), and Fbw7 via the host range (HR) domain. In addition, the amino-terminus of T antigen interacts with TBP, TFIIB, RNApolII, Sp1, and IRS-1. T antigen was shown to be coimmunoprecipitated with AP-2, c-Jun, and TAFs. See color insert.

defective for Cul7-binding (F98A or deletion mutant 98–99) are unable to induce anchorage-independent growth of MEFs and fail to support growth in low serum (Ali et al., 2004). However, these mutants are capable of inducing cell division and growth to high densities when expressed in Cul7-deficient MEFs (Kasper and DeCaprio, 2005). The residue F98 is conserved in the TAgs of SA12, LPV, KIPyV, and WUV, but not MCV. These observations suggest that in addition to p53 and the Rb family of proteins, TAg must target Cul7 to fully transform cells.

Bub1, a mitotic spindle checkpoint protein, was found to interact with SV40 TAg in a yeast two-hybrid analysis (Cotsiki et al., 2004). TAg mutants defective in Bub1 binding (W94A or W95A) fail to induce focus formation in Rat-1 cells. However, these mutants do immortalize rat embryo fibroblasts (Cotsiki et al., 2004). The residue W98 is conserved in LTAgs of all primate polyomaviruses. The interaction between TAg and Bub1 results in perturbation of the spindle checkpoint and may induce aneuploidy and genomic instability (Woods et al., 1994; Chang et al., 1997; Cotsiki et al., 2004). It is yet to be determined whether genetic instability induced by TAg–Bub1 association contributes to transformation.

Nijmegen breakage syndrome protein 1 (Nbs1) is a component of the MRN (Mre11/Rad50/Nbs1) complex that functions in double-stranded DNA break repair. SV40 TAg can bind to Nbs1 via its origin binding domain (residues 147–259). TAg binding to Nbs1 induces endoreduplication and genomic instability. It has been also reported that TAg disturbs the accumulation of Mre11 at sites of DNA damage (Digweed et al., 2002). However, at present, there is no known role of this interaction in transformation.

Fbw7 is a human tumor suppressor and the substrate recognition component of the SCF^{Fbw7} ubiquitin ligase. SV40 TAg binds to Fbw7 via a phosphorylated epitope within the host range (HR) domain of TAg (Welcker and Clurman, 2005). The Fbw7 interaction site is conserved in the TAgs of JCV, BKV, and SA12, but not in LPV, KIPyV, WUV, and MCPyV, since these TAgs do not contain an HR domain. This interaction interferes with the Fbw7-driven turnover of physiological substrates such as cyclin E, thus causing stabilization of these substrates (Welcker and Clurman, 2005). This suggested a hypothesis where TAg, functioning as a competitive inhibitor, competes with cellular proteins for Fbw7 binding in a substrate-like fashion. Whether this interaction contributes to TAg-mediated transformation is not known.

Fei et al. (1995) have reported a direct association of SV40 TAg with insulin receptor substrate (IRS-1), a major substrate for both the insulin receptor and insulin-like growth factor type I receptor (IGF-IR). The amino-terminal 250 amino acids of TAg were shown to be required for this interaction; however, further studies with point mutants are needed to pinpoint the specific binding site. In at least one case, signaling through IGF-IR was reported to be required for SV40-mediated transformation (DeAngelis et al., 2006). JCV TAg has also been reported to bind IRS-1 and cause its translocation to the nucleus (Lassak et al., 2002). Additionally, competition for IRS-1–TAg binding by a dominant negative mutant of IRS-1 inhibited anchorage-independent growth of JCV TAg-transformed cells (Del Valle et al., 2002; Lassak et al., 2002).

SV40 TAg modulates the transcription of both cellular and viral genes by targeting various transcriptional regulatory proteins such as transcription-enhancing factor-1 (TEF-1), TBP, hTAF, TFIIB, RNApolII, Sp1, c-Jun, and AP-2 (Moens et al., 1997). How these interactions affect the transcription of cellular genes is not completely understood, but SV40 TAg binding to TEF-1 via the origin binding domain (OBD) (residues 133–249) inhibits the binding of TEF-1 to target DNA sequences. A TAg mutant deficient for TEF-1 binding (S189N) of TAg was shown to be seven- to eight-fold less efficient than wild-type TAg in a focus formation assay (Dickmanns et al., 1994). The residue S189 is conserved in TAgs of SA12 and MCPyV but not in the other primate polyomaviruses.

Finally, a physical interaction of JCV TAg with one of the components of the Wnt pathway, β-catenin, has been reported using a series of deletion mutants in a glutathione S-transferase (GST) pull-down assay (Gan and Khalili, 2004). Additionally, immunoprecipitation assays using a mouse medulloblastoma cell line expressing JCV TAg show association of β-catenin with TAg, but only a small percentage. More studies are warranted to investigate this interaction and its biological significance.

CONCLUDING REMARKS

Since their discovery, polyomaviruses have continuously provided insight into various cellular processes such as DNA replication, gene expression, posttranscriptional processing, tumor suppressor functions, and cell cycle regulation. Specifically, the ability of large TAgs to affect so many diverse biological functions is partly explained by the fact that they are multifunctional proteins that interact with both viral and cellular targets. TAgs are unique in their ability to induce transformation in multiple cell lines and to induce tumors in animal models, therefore serving as important tools to understand the mechanisms of oncogenic transformation in a cell type-specific context. The study of these viruses will continue to resolve the mysteries of fundamental cellular processes and help find treatments for immunocompromised and immunosuppressed individuals whose active polyomavirus infection becomes life threatening.

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8

SIMIAN VIRUS 40, HUMAN INFECTIONS, AND CANCER: EMERGING CONCEPTS AND CAUSALITY CONSIDERATIONS

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INTRODUCTION

Since its discovery in 1960, polyomavirus simian virus 40 (SV40) has been studied intensively as a model cancer virus and as a biological tool for investigating cell growth regulation pathways. Recently, it has received attention as a potential human pathogen. The finding of SV40 markers in human cancer has sparked new avenues of investigation that have confirmed human associations for the virus, but the biological significance of those findings remains under debate. Part of the reason for the lack of consensus is that discussants have focused on different requirements for proof of viral causality. The aims of this review are to identify the sets of criteria proposed to establish viral causation of human cancer, to evaluate the current status of evidence regarding a potential role for SV40 in human neoplastic disease, and to analyze the extent to which causality criteria are being met for different types of tumors.

CAUSALITY CRITERIA

The epidemiological definition of a causal factor in infectious disease is "either a factor that must exist for disease to occur (necessary) or always produces disease (sufficient)"

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(Franco et al., 2004). It is more difficult to define cancer causes, as cancer development is a multistep process that occurs over a long period of time and reflects the contribution of multiple factors and the accumulated effects of multiple events. Suggested categories of cancer causation include spontaneous, hereditary, environmental, and interactive (gene–environment) (Knudson, 1997). Viruses are environmental factors, but their role in cancer would likely be influenced by hereditary and interactive factors.

Among the many viruses that exist, a few are known to be cancer-causing agents in animals and/or humans. Estimates are that viruses account for up to 20% of all human cancers worldwide, reflecting, predominantly, the role of viruses in cervical and liver cancers (Butel, 2000; Pagano et al., 2004). Evidence accumulated from studies of virus-induced tumors in animals and virus-associated human malignancies led to the articulation of the general tenets of viral carcinogenesis (Table 8.1). These tenets highlight distinctions between viruses and other types of carcinogens. Because viruses are infectious agents, disease outcomes must be analyzed with an appreciation of the specific characteristics of the viral replicative cycle and of virus–host interactions. These biological imperatives, driven by the goal of viral survival in nature, have resulted in great diversity among viral agents and their replicative strategies. They also have made it inevitable that many hurdles are encountered in establishing an etiologic role for a virus in human cancer. Typically, experimental findings that do not fit the standard conceptual models are met with skepticism, and two or more decades elapse before the scientific and public health communities reach a general acceptance of the likelihood of an agent being associated with human cancer. Several sets of criteria have been proposed to help guide the process of determining if a given infectious agent is causally related to a malignancy (Franco et al., 2004); none is applicable to all human cancer virus systems.

Bradford Hill (1965) first proposed the general epidemiological criteria to evaluate causation between a disease and an environmental factor. These guidelines were formulated before the availability of molecular approaches. The criteria were strength of association, consistency, specificity of association, temporality, biological gradient,

TABLE 8.1. Tenets of Viral Carcinogenesis[a]

1. Viruses can cause cancer in animals and humans.
2. Tumor viruses frequently establish persistent infections in natural hosts.
3. Host factors are important determinants of virus-induced tumorigenesis.
4. Viruses are seldom complete carcinogens.
5. Virus infections are more common than virus-related tumor formation.
6. Long latent periods usually elapse between initial virus infection and tumor appearance.
7. Viral strains may differ in oncogenic potential.
8. Viruses may be either direct- or indirect-acting carcinogenic agents.
9. Oncogenic viruses modulate growth control pathways in cells.
10. Animal models may reveal mechanisms of viral carcinogenesis.
11. Viral markers are usually present in tumor cells.
12. One virus may be associated with more than one type of tumor.

[a]Butel (2000).

biological plausibility, coherence, experimental evidence, and analogy. Hill's criteria are more useful for environmental and occupational factors than for infectious agents, and specificity, plausibility, and analogy are not thought to be important considerations for relationships in cancer (Franco et al., 2004). In addition, biological gradient (dose response) needs to be redefined for replicating infectious agents. The most important criterion from Hill's list is that the correct temporal relationship must occur (i.e., the agent is present before the cancer occurs).

Evans and Mueller (1990) devised a set of epidemiological and virological guidelines that would support an etiologic role for a virus in a human cancer. Epidemiological guidelines included the following: geographic distribution of viral infection should coincide with that of the tumor, adjusting for the presence of known cofactors; presence of viral markers should be higher in cases than in controls; viral markers should precede the tumor; incidence of tumors should be higher in persons with the marker than in those without; and prevention of viral infection should decrease tumor incidence. The latter suggestion, while important in determining the long-term effect on tumor incidence, is impractical in the short term. Virological guidelines included the following: the virus should be able to transform human cells *in vitro*; the virus should be able to induce the tumor in an experimental animal; and the viral genome should be demonstrated in tumor cells.

Fredricks and Relman (1996) suggested causality criteria based on nucleic acid assays. These included the following: nucleic acid of the pathogen should be present in most cases and preferentially in diseased organs; few microbial copy numbers should occur in hosts or tissues without disease; detection of agent DNA should predate disease; the microbial agent identified should be consistent with the biological characteristics of that group of organisms; tissue–nucleic acid correlates should be sought at the cellular level; and evidence should be reproducible. The second point is not applicable in all situations; as human cancer viruses establish persistent infections (not all of which lead to cancer), control subjects and normal cells in cancer patients may be infected.

Carbone and Pass (2004) proposed criteria that could be used in the absence of epidemiological data to identify viruses that cause or contribute to human cancer development. These included the following: agent causes or contributes to tumors in animals of the same type as associated with the agent in humans; agent transforms or contributes to transformation of human cells in culture of the same cell type from which associated human malignancies arise; and molecular evidence shows that the agent interferes with key molecular pathways in human cells, which lead to the formation of human cancer.

Pagano and colleagues (2004) reviewed several virus–cancer relationships and summarized how criteria for causality were weighted differently when specific infectious agents were linked to particular human cancers. For human papillomavirus and cervical cancer, all causality criteria have been met, with molecular evidence preceding the acquisition of epidemiological support. Seroepidemiology was key to the acceptance of causality for Kaposi's sarcoma herpesvirus (KSHV) and Kaposi's sarcoma, for human T-cell leukemia virus type I (HTLV-I) and adult T-cell leukemia, and for both hepatitis B virus and hepatitis C virus and liver cancer. No animal models exist

for some of these virus–cancer relationships. In contrast, for Epstein–Barr virus (EBV) and lymphoproliferative disease, supporting evidence is consistency of association and the ability of the virus to immortalize B cells in culture, with epidemiological support lacking. Other EBV–human cancer links are not yet accepted as causal. For nasopharyngeal carcinoma, EBV genomes are consistently present, but host genetics and environmental cofactors appear to contribute to cancer development, and the relative roles of the various factors, including that of EBV, remain to be defined. With respect to Burkitt's lymphoma, EBV is not present in all cases and is currently considered to serve as a cofactor. Finally, the EBV association with Reed–Sternberg cells in Hodgkin's disease is inconsistent, with its mechanistic contributions to the tumor unclear. The authors concluded that the minimal criteria to establish causality included consistency of association between an agent and a malignancy, the presence of viral markers in tumor cells, and the production of tumors in experimental animals and transformation of cells in culture. These criteria would apply to direct-acting oncogenic agents. More difficult to define are criteria to identify viruses that contribute to cancer causation through indirect mechanisms.

Those same authors (Pagano et al., 2004) presented several important suggestions. In instances where a virus is not associated with 100% of a given type of tumor, subsets of tumors may be defined by the presence of virus, which could lead to the recognition of distinct sets of neoplastic properties and targeted therapies. To strengthen viral causality, polymerase chain reaction (PCR)-based data should be complemented with evidence obtained using other techniques (such as detection of viral mRNA and viral antigens in tumor cells), and epidemiological studies should be based on discriminative assays that can unambiguously identify virus infection.

As will be discussed in this review, gaps exist in our knowledge of the pathogenesis of human infections by SV40, and this complicates the marshalling of experimental data to meet causality criteria. Current deficiencies include the unknown prevalence of SV40 infections in humans, the unknown extent of geographic variation in prevalence rates, the lack of validated assays to identify infected individuals, and the unknown role of cofactors in SV40-associated cancers (with the exception of the established synergy between asbestos and SV40 in mesotheliomas). Also unknown is what effect, if any, viral strain variation may have on disease development. At this time, evidence is most persuasive of a causal link between SV40 and malignant mesotheliomas.

HISTORY AND CHARACTERISTICS OF SV40 AS A CANCER VIRUS

Contaminated Vaccines

The natural hosts for SV40 are Asian macaques, especially rhesus monkeys, in which the virus establishes persistent infections in the kidneys. The early polio and adenovirus vaccines were prepared using primary cultures of rhesus monkey kidneys, and SV40 became an unrecognized contaminant of many of those vaccines (Butel and Lednicky, 1999; Rollison and Shah, 2001; Stratton et al., 2003; Vilchez and Butel, 2004). Shortly after its discovery, SV40 was shown to be oncogenic in hamsters and able to transform cells in culture. These findings raised concerns, as millions of people worldwide had

been exposed to potentially contaminated polio vaccines starting in 1955. Vaccines used after 1963 were thought to be free from SV40, but a recent study found that the Russian oral polio vaccine was contaminated beyond that time, perhaps as late as 1978 (Cutrone et al., 2005). It is not known how many people were infected by SV40 following exposure to SV40-contaminated vaccines.

Basic Virology

SV40 is classified in the Polyomaviridae family. Members of this family are small, nonenveloped virions (~45 nm in diameter) with a circular, double-stranded DNA genome 5 kb in size. Polyomaviruses encode replication proteins, designated T antigens (T-ag), that are essential for virus replication. SV40 large T-ag is multifunctional, counting among its properties the ability to stimulate resting cells to enter the cell cycle and to initiate viral DNA replication by binding to the origin of replication within the viral regulatory region (Imperiale and Major, 2007; McNees and Butel, 2008). Many (but not all) laboratory-adapted strains of SV40 possess a complex regulatory region with duplications or rearrangements in the enhancer region. In contrast, most natural isolates from monkeys contain a simple regulatory region structure lacking duplications, although isolates with complex regulatory regions have been detected in animals with compromised immune systems (Lednicky and Butel, 2001). Viruses with complex regulatory regions tend to replicate better in tissue culture than those with simple regulatory regions, but whether that same effect occurs *in vivo* in infected hosts is not known. Rearrangements in the viral regulatory region cannot be used reliably for genotyping purposes as changes in that part of the genome can occur within a given host (Lednicky and Butel, 2001).

Other than the noncoding regulatory region, SV40 appears to be genetically stable based on sequence analysis of laboratory strains and natural isolates of virus (Forsman et al., 2004). However, spontaneous mutations can occur in the T-ag in transformed cells that result in epitope variants that allow for escape from T-ag-specific cytotoxic T-lymphocyte clones (Mylin et al., 2007). Different SV40 viral strains exist; these can be grouped into three clades or genogroups (with several isolates remaining ungrouped) based on stable variations in the coding sequence for the extreme C-terminal portion of the T-ag protein, some of which result in amino acid changes (Forsman et al., 2004). Recognition of SV40 strain variation raises the possibility that SV40 isolates may differ in biological properties that affect pathogenesis of infection and disease in animals or humans.

Oncogenicity

SV40 is a potent transforming virus in many types of cells in culture and is highly oncogenic in Syrian golden hamsters (Butel and Lednicky, 1999; Vilchez and Butel, 2004). Large T-ag is the major oncoprotein of the virus, reflecting growth stimulatory functions expressed during the virus replication cycle. Functions of Tag and its ability to modulate cellular regulatory pathways have been well described (White and Khalili, 2004; Ahuja et al., 2005; Imperiale and Major, 2007; Moens et al., 2007). Major

functions that drive oncogenic transformation are T-ag effects on two tumor suppressor proteins, p53 and pRb, that lead to dysregulated stimulation of the cell cycle, but other T-ag properties also contribute to transformation.

SV40 has long been recognized for its ability to induce tumors in Syrian golden hamsters (*Mesocricetus auratus*) (Butel et al., 1972; Butel and Lednicky, 1999; Butel, 2000; Vilchez and Butel, 2004). Depending on the route of inoculation and host age at the time of infection, the types of tumors that frequently develop include brain cancers, mesotheliomas, bone tumors, and lymphomas. The hamster model, therefore, predicts the types of human cancers repeatedly associated with SV40 (Gazdar et al., 2002; Vilchez and Butel, 2004) and has been used to show that asbestos and SV40 can cooperate in the induction of mesotheliomas (Kroczynska et al., 2006).

We demonstrated recently that SV40 isolates differ in oncogenic potential in weanling hamsters (Sroller et al., 2008; Vilchez et al., 2004). Surprisingly, the viral regulatory region was found to exert a statistically significant effect on tumor incidence, with viruses having a simple regulatory region structure being more oncogenic than those with a two-enhancer arrangement. There was no difference among strains in transforming frequency *in vitro*, indicating that the *in vivo* distinctions must reflect some aspect of the host response to infection. These findings in hamsters showed that SV40 strains differ in biological properties and suggest that the risk of disease may depend on the viral strain causing an infection. The most oncogenic strains were SVCPC, which has been detected in several human cancers (Krieg and Scherer, 1984; Lednicky et al., 1995; Vilchez et al., 2002a; Arrington et al., 2004), as well as in the Russian oral vaccine seed stock (Cutrone et al., 2005), and Baylor, recovered from a 1956 type 2 Sabin oral polio vaccine.

SV40 AND HUMAN INFECTIONS

There is clear and convincing evidence that SV40 is causing human infections today, but methodologies currently available do not allow a definitive determination of the prevalence or geographic range of those infections. It is likely that SV40 is distributed unevenly geographically and that infections occur at relatively low prevalence rates. That statement is based on the assumptions that SV40 was introduced into humans recently (about 50 years ago) through the use of contaminated polio vaccines, that contaminated vaccines were not used uniformly throughout the world, and that SV40 replicative capacity in humans and efficiency of human-to-human transmission may be low. This missing knowledge makes interpretation of some molecular surveys and epidemiological studies difficult.

Molecular Evidence

SV40 DNA has been detected in peripheral blood cells from healthy individuals in the United States (David et al., 2001; Li et al., 2002a), Japan (Yamamoto et al., 2000; Nakatsuka et al., 2003), Italy (Martini et al., 1996, 1998, 2002; Paracchini et al., 2005), Germany (Heinsohn et al., 2005), and Egypt (Zekri et al., 2007b), as well as in renal

biopsies from patients with kidney disease (Butel et al., 1999a; Li et al., 2002a,b; Milstone et al., 2004). Polyomaviruses JC virus (JCV) and BK virus (BKV) are known to be excreted in urine, and SV40 has been detected in urine samples from children (Vanchiere et al., 2005b) and, on rare occasions, in urine from adults (Li et al., 2002a,b; Milstone et al., 2004; Thomas et al., 2006). SV40 was found to be excreted in pediatric stool samples (Vanchiere et al., 2005a), recalling the report that some infants fed contaminated oral polio vaccine excreted infectious SV40 in their stools for up to 5 weeks (Melnick and Stinebaugh, 1962). This suggests that a fecal–oral route of transmission for SV40 is feasible. The natural history of human infections by SV40 is not characterized, so the most appropriate specimens for molecular screening to detect SV40 infections are not known. The molecular-based evidence shows that SV40 infections occur in humans without neoplastic disease, but the data are not sufficient to answer questions regarding prevalence, geographic distribution, or identification of susceptible populations.

Serology

Serological assays that detect serum antibodies are often used to detect markers of past or current viral infections. Serological studies have estimated SV40 prevalences between 2% and 20% in different human populations (Butel and Lednicky, 1999; Shah et al., 2004; Vilchez and Butel, 2004). SV40 neutralizing antibodies have been detected in children and adults in the United States (Shah et al., 1974; Strickler et al., 1996; Jafar et al., 1998; Butel et al., 1999b; Rollison et al., 2003), Europe (Butel et al., 2003; Knowles et al., 2003; Minor et al., 2003), and Central Asia (Nurgalieva et al., 2005). Although highly specific, SV40 neutralization tests are too labor intensive to utilize in large population surveys.

Enzyme immunoassays (EIA) based on viruslike particles (VLPs) have been developed. Advantages of such assays are that they can measure serological reactivity to all three polyomaviruses (JCV, BKV, and SV40) and can be scaled up to test large numbers of sera. However, these assays detect all antibodies that bind to virus particles, including nonneutralizing antibodies, and are complicated by cross-reactive antigens shared by the human polyomaviruses. Although the EIA using VLPs are able to discriminate high-titered SV40 antibodies in sera from infected rhesus monkeys, they have not been validated for the ability to detect specific low-titered SV40 antibodies in human sera in the presence of high-titered JCV and BKV antibodies. Using those assays, SV40-reactive antibodies that were detected in human sera by EIA were low titered and usually could be removed by adsorption with VLPs of BKV and/or JCV. The authors of those studies concluded that SV40 reactivity was due, almost entirely, to cross-reactivity with BKV and JCV antibodies and that there was no evidence of human infections by SV40 or of SV40 association with human cancer (Shah et al., 2004; Shah, 2006).

Caution is warranted at this time regarding interpretations of SV40 prevalence based on serological surveys, as the limitations of currently available assays hamper determination of conclusive data. In addition, very little is known about the human immune response to SV40 infections or what influence immunity to BKV or JCV may have on SV40-specific responses. It has been observed that SV40 antibodies wane over

time (Lundstig et al., 2005), and there are examples of individuals with proven SV40 infections not possessing detectable SV40 neutralizing antibodies (Melnick and Stinebaugh, 1962; Arrington et al., 2004). If developed further, and if specificity could be verified, future-generation serological assays would be valuable tools for identifying SV40-exposed individuals.

Exposure Sources and Impact on Cancer Causality

Accepted sources of human exposure to SV40 in the past were the early contaminated polio vaccines, including both the inactivated (Salk) and the prelicensure live oral (Sabin) forms. Higher levels of infectious SV40 were present in the oral vaccines than in the killed Salk vaccines, as the latter contained residual infectious SV40 that had survived the inactivation step in vaccine production. Among those of an age to possibly have been exposed to those contaminated vaccines, it is not known who was actually infected. There are no data identifying which individuals received contaminated vaccines, how much infectious SV40 was present in the contaminated lots, who among the exposed were successfully infected, and whether some individuals were exposed to SV40 from other sources. Evidence of SV40 infections occurs in individuals too young to have been exposed to potentially contaminated polio vaccines between 1955 and 1963, so possible sources of modern exposure to SV40 must be considered. One source would be the widely used Russian oral polio vaccine that appears to have been contaminated until the late 1970s (Cutrone et al., 2005); whether other vaccines remained contaminated longer than popularly believed is not known. Zoonotic infections among laboratory workers and animal handlers exposed to SV40-infected monkeys and/or tissues can occur at relatively high frequency (Horvath, 1972; Zimmermann et al., 1983; Engels et al., 2004), but those individuals would represent only a minor fraction of the human population. Detection of excretion of SV40 in stool and urine suggests that fecal–oral and/or urine–oral routes of human-to-human transmission might occur, but it has not been directly demonstrated. There is no evidence of insect or lower animal reservoirs for SV40. These ambiguities about virus exposure complicate epidemiological studies, as well as interpretations of molecular assays on cancer specimens.

SV40 AND HUMAN CANCER

A growing body of literature reports an association of SV40 with human cancer. A number of recent reviews have addressed the evidence that SV40 is a potential human pathogen with links to certain types of malignancies and have come to different conclusions regarding the weight of that evidence (Butel and Lednicky, 1999; Arrington and Butel, 2001; Jasani et al., 2001; Gazdar et al., 2002; Barbanti-Brodano et al., 2004; Pagano et al., 2004; Vilchez and Butel, 2004; Poulin and DeCaprio, 2006; Rollison, 2006; Shah, 2006). Different causality criteria tended to be emphasized in those assessments. The major issues fueling the lack of consensus are that variable findings have been reported regarding the presence of SV40 DNA in human cancers and that

epidemiological studies have generally failed to support a virus–cancer link. This review will focus on the three types of malignancies that have received the most attention—brain tumors, mesotheliomas, and lymphomas. Recent controlled studies that used modern molecular techniques are summarized in this section. For inclusion in the following tabulations, the studies met the criteria of investigating primary cancer samples (not cell lines), of using PCR-based assays to detect SV40 DNA sequences, and of analyzing control tissue specimens (not cell lines) in parallel with cancer specimens.

In PCR-based studies, it is important to be cognizant of the possibility of laboratory contamination with plasmids (López-Ríos et al., 2004) and to guard against it by using proper precautions, procedures, and dedicated facilities. In the controlled studies described below, contamination is ruled out, as it would be expected to occur randomly and be found equally in cancer and control specimens. Also, plasmid contamination would not result in T-ag expression within cancer cells or aberrant methylation of host genes, findings described below for SV40 DNA-positive cancers.

Brain Cancer

Brain tumors are not common, accounting for about 2% of all malignancies in adults and 20% of cancers in children. They were the first type of malignancy to be linked to SV40, with reports dating to the 1970s (Arrington and Butel, 2001; Vilchez and Butel, 2003; White et al., 2005). The early studies relied primarily on DNA hybridization techniques, whereas the more recent investigations were mostly PCR based (Bergsagel et al., 1992; Martini et al., 1996, 2002; Suzuki et al., 1997; Huang et al., 1999; Weggen et al., 2000; Malkin et al., 2001; Engels et al., 2002; Rollison et al., 2005) (Table 8.2). In fact, the first application of PCR technology to the science of polyomaviruses and human cancer detected SV40 sequences in pediatric brain tumors (Bergsagel et al., 1992). It was a tumor from that series that yielded a novel infectious isolate of SV40, designated SVCPC (Lednicky et al., 1995), that turned out to be identical to a virus cloned a decade earlier from an adult meningioma in Germany (Krieg and Scherer, 1984). Although polyomavirus JCV also has been linked with human brain tumors (White et al., 2005), molecular assays are able to identify with certainty the specific virus detected in clinical samples.

Many histological types of brain tumors have been included in viral screening surveys. The types most often found to be SV40-positive include astrocytomas, glioblastomas, ependymomas, choroid plexus tumors, and meningiomas (Croul et al., 2003). Glial cells, the origin of many of the tumors, retain the ability to proliferate throughout life, and this may facilitate transformation by SV40. SV40 possesses neurotropic properties among its pathogenic characteristics. It can induce brain tumors in hamsters (Girardi et al., 1962), transform human astrocytes in culture (Shein, 1967), and cause brain infections and disease in monkeys immunocompromised because of simian immunodeficiency virus infection (Ilyinskii et al., 1992; Lednicky et al., 1998; Newman et al., 1998).

In PCR-based controlled studies (Table 8.2), SV40 was detected significantly more often in brain cancers than in control samples ($P = 0.007$). However, there is

TABLE 8.2. Detection of SV40 DNA by PCR in Controlled Studies of Primary Human Brain Cancers

Study	Country	SV40 DNA by PCR [No. Positive/No. Tested (%)]	
		Brain Tumor	Control
Malkin et al. (2001)	Canada	2/2 (100)	0/2 (0)
Bergsagel et al. (1992)	United States	20/31 (64)	0/112 (0)
Martini et al. (2002)	Italy	10/25 (40)	14/55 (25)[a]
Martini et al. (1996)	Italy	32/83 (38)	33/133 (25)[a]
Huang et al. (1999)	Switzerland	71/199 (36)	1/8 (12)
Suzuki et al. (1997)	Japan	7/33 (21)	1/21 (5)
Weggen et al. (2000)	Germany	5/267 (2)	0/87 (0)
Engels et al. (2002)	India	1/47 (2)	0/18 (0)
Rollison et al. (2005)	United States	4/225 (2)	0/19 (0)
Total	—	152/912 (16.7)[b]	49/455 (10.8)[a, b]

[a]Among control specimens positive for SV40 were 38 peripheral blood samples from blood donors.
[b]$P = 0.007$ (Mantel–Haenszel test).

inconsistency in reported frequencies of viral positivity. Two studies reporting SV40-positive brain tumors provided confirmatory data by detection of T-ag in tumor cells by immunohistochemistry. In a separate study, Zhen et al. (1999) showed by immunoprecipitation and Western blot analysis that T-ag and p53 were complexed in many brain tumor specimens. The expression of SV40 T-ag in brain tumors supported a possible functional effect of SV40 on cell properties in those tumors.

It is noteworthy that 38 of the 49 SV40-positive control samples from the brain tumor studies were peripheral blood samples from individuals. Perhaps these findings indicate the presence of SV40 infections in blood cells in healthy individuals. Similar results have occurred in lymphoma studies (Table 8.4), in which 28 of 39 SV40-positive control samples were blood samples.

A number of factors may contribute to the variable detection of SV40 among brain cancer specimens. One consideration is that different brain tumor types are often included in such surveys. If SV40 were preferentially associated with certain subtypes of cancer and their representation varied in different studies, that variation would influence overall viral detection rates. The prevalence of SV40 infections in the study area is another important factor. It was reported that SV40 sequences could be detected in brain tumors from Switzerland (where SV40-contaminated vaccines had been used) but not in those from Finland (where contaminated vaccines had not been used) (Ohgaki et al., 2000). Cancers can have more than one cause, and etiology may vary geographically. Examples exist of a virus being etiologically involved in a given tumor type in one area of the world and seldomly in another (e.g., hepatitis B virus and liver cancer). The fact that a virus is found in tumors only from restricted geographic areas does not rule out the virus having a functional role in those tumors in which it is present. Subsets of tumors need to be considered in this regard.

A recent case study of an adult meningioma involved a scientist patient who had a risk of laboratory exposure to SV40 10–15 years earlier (Arrington et al., 2004). SV40 DNA was detected in the tumor, and both the viral regulatory region and T-ag variable domain sequences genetically matched those of the exposure source. These data address the causality criterion of temporality; they are strongly suggestive, but do not prove, that the scientist suffered a laboratory infection and subsequently developed a virus-associated cancer.

It is clear that SV40 markers can be found in human brain cancers and that the association in controlled studies is statistically significant. Although the evidence is strong in some individual cases for a causal link, the unexplained inconsistency among studies, the lack of focus on defined brain tumor types, and the lack of mechanistic studies in target cell types *in vitro* currently preclude a decision about the causality of brain tumors by SV40.

Mesotheliomas

The human malignancy most strongly linked with SV40 is malignant mesothelioma. These cancers are very aggressive, with median survival after diagnosis of about 1 year. Mesotheliomas are rare tumors, the incidence of which has increased significantly over the last 50 years. Asbestos has been identified as a cause of this cancer, but observations suggest that other factors in addition to asbestos may be involved. Exposure to asbestos is associated with about 80% of mesotheliomas, but cases occur in persons with no exposure history and less than 5% of heavily exposed workers develop the cancer (Carbone et al., 2002). It has now been shown that SV40 and asbestos can synergize, acting as cocarcinogens in the genesis of mesothelioma.

SV40 DNA was first detected in human mesotheliomas in 1994 (Table 8.3). Most, but not all, subsequent studies confirmed the association. In controlled studies, there is a significant association of SV40 with mesotheliomas compared with control specimens ($P < 0.001$).

More is known about the mechanistic role of SV40 in the development of mesotheliomas than for other human cancers. Many important insights have come from tissue culture studies of primary human mesothelial cells. The cells are highly susceptible to SV40 infection, compared with fibroblasts, emphasizing that specific cell types need to be studied to understand the pathogenesis of viral infection and disease. Following SV40 infection of mesothelial cells, viral DNA remains episomal, low numbers of virus particles are produced, most cells survive, and a high rate of cell transformation ensues (Bocchetta et al., 2000; Cacciotti et al., 2001; Gazdar et al., 2002). Changes observed in infected mesothelial cells include induction of telomerase activity, which promotes cell immortalization (Foddis et al., 2002); activation of the hepatocyte growth factor receptor (Met) pathway, which leads to S-phase entry (Cacciotti et al., 2001); increased release of vascular endothelial growth factor (Cacciotti et al., 2002); and progressive methylation and loss of expression of tumor suppressor gene *RASSF1A* (Toyooka et al., 2002).

Using the human mesothelial cell culture system, it was shown that SV40 and asbestos can act as cocarcinogens, leading to cell transformation (Bocchetta et al., 2000;

TABLE 8.3. Detection of SV40 DNA by PCR in Controlled Studies of Malignant Mesotheliomas

Study	Country	SV40 DNA by PCR [No. Positive/No. Tested (%)]	
		Mesothelioma	Control
Griffiths et al. (1998)	United Kingdom	26/26 (100)	0/30 (0)
Cristaudo et al. (1995)	Italy	8/11 (73)	0/7 (0)
Foddis et al. (2002)	United States	5/7 (71)	0/7 (0)
Carbone et al. (1994)	United States	29/48 (60)	3/51 (6)
Procopio et al. (2000)	Italy	50/83 (60)	0/7 (0)
Ramael et al. (1999)	Belgium	14/25 (56)	0/60 (0)
Galateau-Salle et al. (1998)	France	10/21 (48)	4/25 (16)
Shivapurkar et al. (2000)	United States	57/118 (48)	0/20 (0)
Toyooka et al. (2001)	United States	32/66 (48)	0/40 (0)
Dhaene et al. (1999)	Belgium	13/28 (46)	1/10 (10)
Pepper et al. (1996)	United Kingdom	4/9 (44)	0/12 (0)
Procopio et al. (1998)	Italy	7/18 (39)	5/100 (5)
Strizzi et al. (2000)	Italy	9/23 (39)	6/25 (24)
Priftakis et al. (2002)	Sweden	3/30 (10)	0/10 (0)
Aoe et al. (2006)	Japan	2/35 (6)	0/10 (0)
Emri et al. (2000)	Turkey	0/29 (0)	0/15 (0)
The International SV40 Working Group (2001)	United States	0/25 (0)	0/25 (0)
Manfredi et al. (2005)	Croatia, United Kingdom, South Africa, United States	0/69 (0)	0/20 (0)
Total	—	269/671 (40.1)[a]	19/474 (4.0)[a]

[a] $P < 0.001$ (Z test).

Cacciotti et al., 2005; Kroczynska et al., 2006). In addition, animal experiments proved that cocarcinogenic interactions occur between asbestos and SV40 *in vivo*. In hamsters, lower doses of asbestos were necessary to cause mesotheliomas in the presence of SV40 (Kroczynska et al., 2006). In a transgenic mouse system in which SV40 T-ag expression was directed to mesothelial cells, few animals developed spontaneous tumors, whereas many did after exposure to asbestos (Robinson et al., 2006). A direct relationship was observed between SV40 transgene copy number and survival after exposure to asbestos (e.g., lower copy numbers corresponded to fewer tumors and longer survival).

It has been reported that SV40 increases the risk that people exposed to asbestos will develop mesothelioma (Cristaudo et al., 2005). There also are indications that patients with SV40-positive tumors have less favorable clinical outcomes. One study found that SV40 represented a negative prognostic cofactor in Italian patients with mesotheliomas having biphasic or sarcomatous morphology (Procopio et al., 2000), and a recent study involving Egyptian mesothelioma patients showed that the presence

of SV40 sequences in tumor tissues was significantly associated with poorer overall survival (Zekri et al., 2007a). Furthermore, combined positivity for SV40 and asbestos exposure was found to be an independent, negative prognostic factor for patient survival.

Microdissection of mesothelioma tissue coupled with PCR revealed the presence of SV40 DNA in tumor cells, but not in an adjacent nonmalignant lung, showing that SV40 was linked to the tumor (Shivapurkar et al., 2000). In other studies of mesothelioma samples, the presence of SV40 was significantly correlated with methylation of four tumor suppressor genes, including *RASSF1A* and *Cyclin D2* (Suzuki et al., 2005). These findings indicated a functional effect of SV40 in virus-positive mesotheliomas.

The role of SV40 in malignant mesotheliomas has been the subject of much debate (Pass et al., 2004; Shah, 2004), but a large body of complementary data now supports the etiologic involvement of SV40, most probably as a cofactor. There is strong, although not complete, consistency among surveys of tumor tissues, perhaps reflecting the occurrence of SV40 infections (or lack thereof) in the populations being studied. Importantly, data from studies of animals, cultured human mesothelial cells, and malignant mesotheliomas consistently show that SV40 and asbestos synergize to cause transformation/tumor development. Molecular and biochemical studies of infected human mesothelial cells and virus-positive tumors have identified viral dysregulation of regulatory pathways that can lead to cancer. Currently lacking is epidemiological data establishing a virus–cancer link on a population-wide basis. However, the totality of molecular findings is persuasive that SV40 is contributing causally to the development of a subset of mesotheliomas.

Lymphomas

Lymphomas, especially non-Hodgkin's lymphoma (NHL), are the human cancers most recently associated with SV40 (Table 8.4). NHL is a relatively common disease, with approximately 55,000 new cases annually in the United States (Vilchez and Butel, 2003). NHL is a heterogeneous grouping of hematologic malignancies, with the two most common histological types from B cells being diffuse large B-cell lymphoma and follicular lymphoma. Risk factors for NHL are not known and no environmental cofactors have been identified. Herpesvirus EBV is present in many NHL that arise in immunocompromised individuals, including almost all primary central nervous system lymphomas.

SV40 sequences have been detected most frequently in systemic tumors of diffuse large B-cell and follicular lymphoma types. In addition, SV40 was associated more often with NHL of the germinal center B-cell-like (GCB) subgroup, whereas EBV was more commonly found in the non-GCB phenotype NHL (Meneses et al., 2005; Vilchez et al., 2005). SV40 has very seldomly been detected in T-cell lymphomas. These findings indicate a degree of specificity to the association of SV40 with NHL types. In recent studies, the expression of SV40 T-ag was detected by immunohistochemistry in many virus DNA-positive NHL (Meneses et al., 2005; Vilchez et al., 2005). In one patient with an AIDS-related lymphoma, T-ag was detected consistently in samples taken at different times (Vilchez et al., 2005). In those cancers in which

TABLE 8.4. Detection of SV40 DNA by PCR in Controlled Studies of Human Lymphomas

Study	Country	SV40 DNA by PCR [No. Positive/No. Tested (%)]	
		Lymphoma	Control
Amara et al. (2007)	Tunisia	63/108 (56)	4/60 (6)[a]
Zekri et al. (2007b)	Egypt	85/158 (54)	4/34 (12)[a]
Shivapurkar et al. (2002)	United States	29/68 (43)	5/120 (4.2)
Vilchez et al. (2002b)	United States	64/154 (42)	0/240 (0)
Shivapurkar et al. (2004)	United States	33/90 (36)	1/56 (1.2)
Meneses et al. (2005)	Costa Rica	30/125 (24)	0/91 (0)
Vilchez et al. (2005)	United States	12/55 (22)	0/19 (0)
David et al. (2001)	United States	15/79 (19)	19/187 (10)[a]
Martini et al. (1998)	Italy	11/79 (14)	3/50 (6)[a]
Chen et al. (2006)	Taiwan	13/91 (14)	0/106 (0)
Nakatsuka et al. (2003)	Japan	14/122 (11)	3/64 (4.7)[a]
Capello et al. (2003)	Spain, Italy	17/500 (3)	0/15 (0)
Daibata et al. (2003)	Japan	3/125 (2)	0/31 (0)
MacKenzie et al. (2003)	United Kingdom	0/152 (0)	0/45 (0)
Sui et al. (2005)	Tasmania	0/50 (0)	0/50 (0)
Schüler et al. (2006)	Germany	0/77 (0)	0/61 (0)
Total	—	389/2033 (19.1)[b]	39/1207 (3.2)[a,b]

[a]Among control samples positive for SV40 were 28 blood samples from noncancer patients or healthy individuals.
[b]$P < 0.001$ (Z test).

T-ag was detected, not all tumor cells appeared to be antigen positive, and the reaction was less intense than that typically found with hamster tumors or transformed cell lines. These results suggest that SV40 could be affecting the growth properties of a subset of NHL; it is hard to imagine that an oncoprotein as powerful as T-ag would be present and have no phenotypic influence on the target cells. In addition, changes in promoter methylation profiles of select tumor suppressor genes were detected in SV40-positive NHL in two independent studies (Shivapurkar et al., 2004; Amara et al., 2007), revealing a virus-specific alteration in a subset of lymphomas. However, epidemiological studies have failed to support an SV40 link to NHL at the population level (Engels, 2005).

Among controlled studies, there is a statistically significant association of SV40 with lymphomas ($P < 0.001$) (Table 8.4). Positive reports have originated from the United States, Central America, Europe, and Asia. Two recent reports (Amara et al., 2007; Zekri et al., 2007b) have added North Africa to the list of regions containing SV40-positive lymphomas. However, there is substantial variation among studies in the reported detection of SV40 and explanations remain to be elucidated for this apparent inconsistency. As noted above, differences in the prevalence of SV40 in the populations being surveyed could contribute to variable frequencies of positive findings. If SV40 infections are not present within a study group, it is unlikely that SV40-related

cancers would be detected. Technical details may also play a role in some of the observed inconsistent findings.

SV40 is able to induce lymphomas of B-cell origin in Syrian hamsters inoculated intravenously (Vilchez and Butel, 2004) and to immortalize primary human B lymphocytes *in vitro* (Kanki, 1994), showing that the virus has lymphomagenic properties. Approaches are needed to identify cellular control pathways involved in SV40 transformation of lymphocytes as mechanistic studies of transformation by SV40 to date have emphasized other cell types.

In summary, molecular studies have unambiguously established that SV40 is significantly associated with a subset of NHL. However, heterogeneity among studies and the absence of mechanistic insights regarding SV40 functions in lymphoid cells make viral causality assessments premature at this time.

INTERPRETATIONS AND CONCLUSIONS

The last decade has seen many advances regarding SV40 and human infections and cancer. Experimental studies have shown that SV40 meets all criteria for being a tumor virus. It encodes a potent oncoprotein that has been well characterized for multiple transformation-related functions, it can transform a wide variety of cells *in vitro*, and it can induce tumors in hamsters similar in type to those associated with the virus in humans. A large body of research has overturned the old conventional wisdom that SV40 cannot infect humans; it is now established that SV40 markers can be found in humans without cancer as well as in selected human malignancies. Questions have been defined that need to be addressed going forward.

Animal models are needed that reproduce the features of SV40 infections in humans so that carcinogenesis in the context of the pathogenesis of persistent viral infections can be studied. Much is known about the characteristics of SV40 tumors induced in hamsters following inoculation of a large dose of virus or in mice following transplantation of tumor cells. Mice are nonpermissive for SV40 replication and are not an appropriate model of persistent polyomavirus infections in humans. The dynamics of long-term interactions of the virus with host cells and with the host immune response in naturally infected individuals must be considered. Critical gaps in our understanding are concentrated in the area of events that occur following virus infection and eventually lead to cancer development in a fraction of infected hosts. As carcinogenesis is a multistep process, the role of the virus in tumor development in the context of natural infections will undoubtedly differ in some respects from current paradigms based on rodent studies; alternative mechanisms should be considered as SV40 interactions with human cancer are characterized. The hamster model appears to support low-level SV40 replication *in vivo* and may be able to serve as an infection model. Primates, especially rhesus monkeys, are highly permissive for SV40 replication and could be used to define interactions of viral infection with carcinogens or other potential cofactors. Finally, the use of transgenic mice, while not an infection model, can be exploited in studies of cofactor effects on SV40 carcinogenesis.

Animal models can address the biological basis of observations from human cancer studies that do not fit the current thinking of SV40 carcinogenesis. For example, there are observations suggesting that viral markers are not present in all cells in an SV40-positive human tumor. This could be a result of tumor progression over a long period of time, whereby the viral growth stimulatory functions are no longer needed in advanced tumors and viral genome copies are lost (an event especially apt to occur if the viral DNA were episomal rather than integrated in cancer cells). Alternatively, there is the possibility that the virus may infect tumor cells after the tumor has arisen, then either persisting in a quiescent state or acting in a promoter role, contributing to late tumor phenotypic changes.

There is a statistically significant association of SV40, in controlled studies, with the three types of tumors analyzed (Tables 8.2–8.4). However, there is inconsistency among reported studies with respect to detection of SV40 in human specimens. Technical issues and sample selection may be responsible for some of the observed variability, but a more likely explanation hinges on the geographic distribution of SV40 infections. It is predicted that the prevalence of infection varies geographically, depending on how much virus was seeded into a particular population by the use of contaminated vaccines and how well the virus has spread from person to person since that time. Negative reports may reflect studies carried out in populations in which pockets of infection do not exist.

The prevalence of SV40 infection in human populations cannot be easily determined at this time due to a lack of validated sensitive and specific assays to detect markers of infection. Neither is it known how immunity to BKV and JCV might influence host immune responses to infection by SV40. Furthermore, human specimens most appropriate to test in molecular assays to reliably detect viral infections are not known. Correction of these deficiencies awaits further technical advances, and efforts should be made to achieve their development without delay. It may be that perceived inconsistencies among studies of SV40 and human cancer are not inconsistent once optimized assays are available and the presence or absence of SV40 infection in target populations is known.

It is not to be expected that SV40 will be the only cause of the types of human cancers with which it has been associated. It is time to think in terms of subsets of tumors and to develop ways to recognize and characterize those that involve the virus, as this could lead to clinical and public health benefits. For example, SV40-positive tumors may behave differently from their virus-negative counterparts and could respond to targeted therapies. A future vaccine can even be envisioned to prevent those cancers having a viral component.

One of the novel findings regarding SV40 and human cancer is the cofactor role discovered for SV40 in malignant mesotheliomas, in which it synergizes with asbestos. Therefore, future studies should consider that SV40 may cooperate with carcinogens in other neoplasias linked to SV40. The challenge is that specific causes are not recognized for brain tumors and NHL (except for a subset of lymphomas linked to other viruses). It is important, then, to identify factors that contribute to the etiology of those types of cancer so that possible SV40 cofactor effects can be evaluated.

Epidemiological studies reported to date have not supported links between SV40 and human cancer. Because of the unique circumstances surrounding SV40 infections in humans, the studies have had significant limitations (Dang-Tan et al., 2004; Rollison, 2006). Future epidemiological studies must utilize more sensitive and specific biomarkers of infection to identify subpopulations who are infected and at risk of SV40 cancers. The possible involvement of cofactors in SV40-related cancers will need to be addressed. Whereas the described epidemiological studies do not rule out a role for SV40 in human cancer, they probably serve to reassure that the use of contaminated Salk polio vaccines did not precipitate a widespread cancer epidemic (Rollison, 2006).

In conclusion, evidence regarding a causal role for SV40 in human neoplastic disease has accumulated substantially in a few years' time, but the strength of the association depends on the malignancy under consideration. The collected evidence strongly favors a causal link for SV40 with malignant mesotheliomas, probably most often as a cofactor with asbestos. Supportive data are not as complete for the brain tumor and lymphoma systems as for mesotheliomas. Additional information is needed to provide mechanistic underpinnings for the virus in subsets of those cancers before causality can be decided.

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9

SIMIAN VIRUS 40 AND MESOTHELIOMA

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SIMIAN VIRUS 40 (SV40) STRUCTURE AND LIFE CYCLE

SV40 is an endogenous virus of rhesus macaque. It belongs to the family Polyomaviridae, which includes dsDNA viruses with circular genome of about 5 kb. Polyomaviruses have small (40–50 nm in diameter) icosahedral virions that lack a lipoprotein envelope. The capsid is built from proteins VP1, VP2, and VP3. Viral DNA is packed into the capsid in complex with four host-derived core histones, H2A, H2B, H3, and H4 (Mueller et al., 1978).

The genome of Polyomaviridae typically encodes three structural proteins (VP1, VP2, and VP3) and two proteins with diverse functions in virus replication, transcription, assembly and host cell cycle modifications, large T antigen (Tag) and small t antigen (tag). Genomes of individual members of the family may encode additional proteins. SV40 encodes three such proteins: agnoprotein, 17K T, and small leader protein (Sedman et al., 1989).

During the early stages of infection, the viral capsid protein VP1 of SV40 mediates interaction with gangliosides (Tsai et al., 2003), major histocompatibility complex class I antigens (Breau et al., 1992), and other unidentified cell surface receptors (Atwood and Norkin, 1989), leading to endocytosis of virions via caveolae

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(Pelkmans et al., 2001). Several hours after uptake, the virus is transported from caveolae to the endoplasmic reticulum. Subsequently, the virus crosses the endoplasmic reticulum membrane and exits into the cytoplasm. The VP2/3 nuclear localization signal and its interaction with importins assure nuclear entry of virion DNA (Nakanishi et al., 2002).

The SV40 genome, like the genomes of all Polyomaviridae, consists of three elements: early and late coding regions, which are separated by the noncoding regulatory region. Expression of the early coding region is carried out by the transcriptional machinery of the host and results in the production of the nonstructural proteins T, t, and 17K antigens, all three proteins being products of differential splicing of the same transcript. Tag is the master regulator of the infectious process. It orchestrates the production of early mRNA, initiation of viral replication, and activation of late gene transcription. Tag directly recruits the host cell DNA polymerase complex to the origin of viral replication in order to initiate a bidirectional viral DNA synthesis. Activation of the late viral promoter by Tag and associated cellular transcription factors leads to the transcription of the late coding region and the production of structural proteins VP1, VP2, VP3, and agnoprotein (Eash et al., 2006). All three capsid proteins are derived from a common precursor mRNA by alternative splicing.

VP3 and, possibly, VP2 have an inherent lytic property and participate in host membrane permeabilization, which facilitates virus exit (Daniels et al., 2006). Host necrosis is also mediated via stimulation of poly(ADP-ribose) polymerase (PARP) by VP3 (Gordon-Shaag et al., 2003; Daniels et al., 2006). PARP is an abundant nuclear enzyme that functions in the decision between cell survival, apoptosis, and necrosis. It modifies proteins by catalyzing the formation of extensive branched polymers of poly(ADP-ribose) by using NAD^+ as a substrate. When PARP is overactivated, depletion of cellular stores of NAD^+ and adenosine triphosphate (ATP) and necrotic cell death ensues. Necrotic pathway, unlike apoptosis, is characterized by the cellular swelling and loss of membrane integrity, and thus facilitates the release of mature SV40 virions from the cell.

SV40 natural host (rhesus monkey) cells are permissive for virus replication (Topp et al., 1981). After infection of these cells, the virus completes the cycle outlined above. Viral particles are assembled and the cells are lysed, releasing hundreds of infectious virions per cell (Ahuja et al., 2005). SV40 can infect other species too, but its fate in these species varies. Human fibroblasts and epithelial cells were found to be permissive for SV40 replication. Rodent and hamster cells, on the other hand, are capable of being infected by the virus, but for reasons that are not clear do not allow its replication or the transcription of the late region. These cells are said to be nonpermissive. Since SV40 does not replicate, the cells lose it within few cellular divisions. In about $1/10^8$ infected cells, SV40 may become stably integrated into the host genome.

A third, different, mode of viral cycle is supported by human mesothelial cells. These cells are semipermissive. After infection, the viral DNA is mostly recovered in episomal form (Fahrbach et al., 2008), cell lysis and viral production are limited (Bocchetta et al., 2000), and viral proteins are expressed in 100% of infected cells.

SV40 TRANSFORMING ACTIVITY

In rhesus macaque, its natural host, SV40 seems to be nonpathogenic, unless the animal is immunocompromised. It infects terminally differentiated, growth-arrested epithelial cells of the kidney. Since these cells are quiescent, they do not support DNA replication. SV40 Tag and tag drive infected cells into the S-phase, thus forcing them to express proteins involved in DNA replication, nucleotide metabolism, and chromatin assembly. The ability to stimulate cell cycling and fence off cellular defense mechanisms forms the basis of oncogenic properties of the virus in other hosts. Shortly after its discovery, SV40 was reported to induce tumor formation in newborn hamsters and immunocompromised mice (Eddy et al., 1962; Girardi et al., 1962). Depending on the route of infection, amount of virus, and immune status of the animal, SV40 was found to induce an array of tumors, including brain tumors, osteosarcomas, mesotheliomas, choroid plexus neoplasias, and lymphomas (Carbone et al., 1995). SV40 is also able to induce neoplastic transformation in rodent and human cell cultures (Ahuja et al., 2005).

SV40 promotes transformation and immortalization of the cells via a large number of pathways, many of which are only beginning to be investigated. Tag is responsible for both SV40 transforming activity and immortalization of the cells. In contrast, tag plays no known role in immortalization, but its role in transformation of the cells is essential, at least in human cell lines (Chang et al., 1985, de Ronde et al., 1989; Bocchetta et al., 2000; Yu et al., 2001). Tag targets a number of cellular proteins involved in cell cycling and apoptosis. Interactions with three of them—hsc70, retinoblastoma (Rb) family of tumor suppressors, and tumor suppressor p53—have been well characterized as necessary for transformation.

Rb family proteins regulate entry and exit from the cell cycle through their interactions with E2F transcription factors. E2F regulates the expression of multiple genes that encode proteins required for DNA replication, nucleotide metabolism, DNA repair, and cell cycle progression (reviewed in DeGregori, 2002; Dimova and Dyson, 2005). E2F acts as a transcriptional activator when it is associated with a dimerization partner, DP1 or DP2. Complex of E2F and Rb, on the contrary, represses transcription from E2F-dependent promoters. This repression is achieved through two mechanisms: Rb directly inhibits E2F's ability to activate transcription, and it can associate with various chromatin-modifying repressive complexes. Phosphorylation of pRb, or its binding to Tag, prevents its binding to E2F, leaving E2F free to interact with DP1 or DP2 and stimulate transcription. Consequently, the entry of the cell into the cell cycle is stimulated.

p53 is a cellular checkpoint protein. Normally, its levels in the cell are very low due to the rapid turnover. This balance is disturbed by several kinds of stress, including DNA damage, depletion of nucleotide pools, anoxia, and abnormal inhibition of pRb protein (Horn and Vousden, 2007). p53 is a potent transcriptional activator, capable of up-regulating the expression of a number of genes that mediate cell cycle arrest or apoptosis. Tag stabilizes p53 in the cell, but it also abrogates its ability to bind some cellular promoters through interaction with its DNA-binding surface (Bargonetti et al., 1992; Jiang et al., 1993); tag also participates in blocking p53 function (Pipas and Levine, 2001).

The first 70 amino acids of Tag have sequence identity with the J domain of the DNAJ class of molecular chaperones, through which they can bind to 70-kDa heat-shock proteins (Kelley and Landry, 1994). Tag, like other DNAJ chaperones, is capable of binding and working in concert with a partner chaperone of the DNAK class, hsc70 (Sullivan et al., 2001). This interaction is necessary for focus formation;, however, it is not clear why. In addition, Tag has been reported to interact with numerous other proteins, such as p300/CBP, Cul7, Bub1, TEF-1, Nbs1, and Fbw7 (reviewed in Ahuja et al., 2005). Finally, Tag has a DNA helicase activity (Stahl et al., 1986), which may contribute to chromosomal instability.

The only cellular protein known to interact with tag is PP2A. PP2A is a major protein serine/threonine phosphatase and is involved in many essential aspects of cellular physiology. The PP2A core enzyme consists of a catalytic subunit and a scaffolding subunit. To gain full activity toward specific substrates, the PP2A core enzyme interacts with a variable regulatory subunit. tag inhibits PP2A by directly binding to the core enzyme (Chen et al. 2007) and by modulating the assembly of the PP2A holoenzyme (Yang et al., 1991; Sontag et al., 1993). PP2A inhibition apparently stimulates multiple signaling cascades that converge in the activation of transcription of cyclins D1 and A (Porras et al., 1996; Watanabe et al., 1996; Skoczylas et al., 2004). By activating these cyclins, tag can drive both G1 and S-phase progression in the cell. 17K tag also appears to be able to stimulate the promoter of the cyclin A, independently of tag (Zerrahn et al., 1993; Boyapati et al., 2003).

Both Tag and tag are necessary for the induction of Notch-1 (Bocchetta et al., 2003). Notch-1 is a transmembrane receptor that plays an important role in the early development, but later, its expression is down-regulated to very low or undetectable levels. Deregulated expression of Notch-1 has been detected in several human malignancies.

Finally, SV40 infection induces telomerase activity in human mesothelial cells, but not in fibroblasts (Foddis et al., 2002). tag plays an important role in the induction of telomerase activity. This observation may be related to the immortal phenotype frequently exhibited by SV40-infected human mesothelial cells.

SV40 IN HUMAN POPULATION

Two members of the polyomavirus family naturally infect humans: BK virus (BKV) and JC virus (JCV). These viruses are widely distributed among the population worldwide; the infection lacks clinical manifestation except in immunosuppressed or otherwise predisposed individuals (Eash et al., 2006).

There is little doubt that SV40, despite being a simian virus, has by now crossed to humans. Personnel handling rhesus monkeys and rhesus monkey kidney cells have been reported to show a high incidence of SV40-neutralizing antibodies (Shah et al., 1971, 1972). However, a more dramatic route of infection might have involved vaccination. SV40 infected a significant segment of human population through the contamination of polio vaccines from 1954 through 1963. These vaccines were produced

by growing poliovirus in cell cultures derived from rhesus monkey kidney cells. The virus produces no cytopathic effects in these cells. Its presence in these cultures was not recognized until 1960, when high titers of virus were discovered in some lots of polio vaccines (Sweet and Hilleman, 1960). These vaccines were administered to millions of people both in the United States and worldwide (Shah and Nathanson, 1976). In the United States, vaccine lots approved in 1961 and later were required to be free of SV40, but lots approved earlier were not recalled, so SV40-containing vaccines might have been used until 1963 (Shah and Nathanson, 1976). Although there is no evidence of SV40 contamination in polio vaccines licensed in the United States and the United Kingdom after 1961, vaccines prepared in the former USSR through the late 1970s contained infectious SV40 (Cutrone et al., 2005). Another possible transmission of SV40 could have occurred between 1957 and 1960 through the administration of parenteral adenovirus vaccines contaminated with SV40 to military and, to a limited extent, civilian U.S. population. As recently as 1980, more than 150 newborn children were treated with a hepatitis A vaccine that contained SV40.

Limited data on the nature of SV40 infection in humans are available. Studies of stored specimens of experimental vaccines in which SV40 was an unrecognized contaminant were conducted (Shah and Nathanson, 1976). After intranasal administration, SV40 produced a low-grade infection, with virus shedding in the respiratory tract in a few participants and a low-level antibody response. After oral administration, there was no antibody response. After subcutaneous injection, there was a high-titered antibody response, although it is not known whether this response was produced by infectious or by inactivated SV40 present in the vaccine. Humans who were injected with SV40-contaminated polio vaccines developed neutralizing antibodies that persisted for at least 3 years (Gerber, 1967). No SV40-inactivating antibodies were detected in children who received contaminated oral live-attenuated polio vaccines, although some of the children excreted infectious virus in their stools for 3–5 weeks after ingestion of the vaccine (Melnick and Stinebaugh, 1962).

Very soon after its isolation from polio vaccines, SV40 was recognized as an oncogenic virus capable of inducing tumor formation in hamsters and the transformation of human cells in culture. The absence of an antibody response in children receiving contaminated oral polio vaccines, along with the lack of tumor development in hamsters given SV40 by mouth, suggested the hypothesis that only injectable vaccines were potentially oncogenic for humans. Nevertheless, this leaves a huge segment of human population that has been exposed to a potentially oncogenic virus. The notion that SV40 may contribute to the development of human cancers in exposed individuals (Fraumeni et al., 1963) thus became a subject of intense examination and much controversy (Shah, 2000; Gazdar et al., 2002; Garcea and Imperiale, 2003; Dang-Tan et al., 2004; Vilchez and Butel, 2004; Engels, 2005; Magnani, 2005; Barbanti-Brodano et al., 2006). Subsequently, a new problem presented itself. The recovery of SV40 DNA sequences from cancer tissues of children in the United States that were born long after the vaccines were required to be free of SV40. This implied that the virus had become established in human communities and was circulating by person-to-person transmission.

SV40 IN HUMAN CANCERS

There are three indirect ways to assess the role of SV40 in causing tumors in humans:

1. epidemiological studies in populations exposed to SV40-contaminated vaccines;
2. studies addressing the presence of SV40 DNA sequences or proteins in cancers; and
3. serological studies.

Flaws have been attributed to every technique utilized for virus detection. The validity of serological assessment has been confounded by the cross-reactivity of SV40 antibodies with two related human polyomaviruses, JCV and BKV (Carter et al., 2003; Viscidi et al., 2003). Cross-reactivity, contamination, and lack of reproducibility have been reported in the detection of SV40 nucleic acids and proteins by molecular techniques (Bergsagel et al., 1992; Testa et al., 1998; Strickler, 2001; Hubner and Van Marck, 2002; Vivaldi et al., 2003; Lopez-Rios et al., 2004).

SV40 viral sequences have been demonstrated in a variety of malignancies, including brain cancers, osteosarcomas, non-Hodgkin's lymphomas, and mesotheliomas (reviewed in Gazdar et al., 2002). The evidence for the association between SV40 and tumor development is considered the strongest for mesothelioma (Carbone et al., 2007).

SV40 AND MESOTHELIOMA

Mesotheliomas are uncommon neoplasms arising from the mesothelial cells lining the pleural, pericardial, and peritoneal cavities. Mesotheliomas are among the most aggressive human tumors, and median survival is about 3–6 months from diagnosis without therapy, and about 12 months with therapy (Ismail-Khan et al., 2006). Despite its rarity, there is great interest in this disease due to its connection to asbestos exposure. In fact, there were virtually no reported mesotheliomas before 1940. However, in the last 80 years, the incidence of mesothelioma has been continuously rising and now constitutes 2000–3000 newly diagnosed cases and approximately 2500 deaths per year in the United States alone (NIOSH Publication No. 2003-111. Section 7, 2002) and 5000 mortalities per year in Western Europe. The first convincing link between malignant mesothelioma and asbestos exposure was reported in 1960 based on evidence coming from South Africa (Cugell and Kamp, 2004; Robinson and Lake, 2005).

Despite a well-documented role of asbestos in the development of mesothelioma, only 2%–10% of individuals with heavy, prolonged asbestos exposure develop this disease (Ismail-Khan et al., 2006). Conversely, only about 80% of mesothelioma patients have a history of asbestos exposure. These facts prompted investigators to look for other etiologies or cofactors for this cancer. The role of SV40-contaminated vaccines in the development of mesothelioma is a subject of debate (Carbone et al., 2007).

In 2002, the Immunization Safety Review Committee of the Institute of Medicine (IOM) reviewed the possible link between SV40-contaminated polio vaccines and human cancer and reported that, "The evidence was inadequate to conclude whether or not the contaminated polio vaccine caused cancer" (Stratton et al., 2002).

SV40 Nucleic Acids

The IOM report listed 29 studies in which mesothelioma tissues were tested for SV40 DNA sequences, with the reported prevalence ranging from 0% to 100% (Stratton et al., 2002). Methods employed in these studies included mostly polymerase chain reaction (PCR), followed in some of the studies by sequencing or Southern blot of PCR products. One study (Carbone et al., 1997) involved *in situ* hybridization. This technique, although less sensitive than PCR, is considerably less liable to produce false negatives. In this study, 14 out of 34 mesothelioma samples were reported positive for SV40. In another study, SV40 DNA was not detected in any of the 25 specimens using Southern blot (Strickler, 2001). Also, one study, which reported the presence of SV40 RNA in seven out of seven samples, employed reverse transcription PCR (RT-PCR) (McLaren et al., 2000). Notably, at least one strain of the virus recovered from a human tumor specimen could be traced to a vaccine vial produced in 1954 (Rizzo et al., 1999). The point of contention for these studies might be the DNA input. SV40 DNA is typically found well below one copy per cell. It is, therefore, preferable to analyze at least 1 μg of DNA in each PCR reaction (Rizzo et al., 1998). The studies with negative findings used 100 ng of DNA from frozen samples and undetermined amounts from paraffin-embedded tissues. In particular, DNA obtained from paraffin-embedded tissues is of low quality.

Since the publication of the IOM report, 13 studies investigating the presence of SV40 DNA sequences in mesothelioma patients were published. Five of these studies (Hubner and Van Marck, 2002; Leithner et al., 2002; Mayall et al., 2003; Jin et al., 2004; Brousset et al., 2005) were negative (altogether, 194 tumor samples were tested). The other seven studies (De Rienzo et al., 2002; Gordon et al., 2002; Priftakis et al., 2002; Lopez-Rios et al., 2004; Cristaudo et al., 2005; Aoe et al., 2006; Ziegler et al., 2007) report on 685 mesotheliomas, with 40 (5.8%) being positive for SV40 sequences. The geographic origin of the tissues was similar for the positive and the negative studies, and tissues from the United States and Europe contributed positive as well as negative samples.

In a study by Ziegler et al. (2007), the authors examined PCR detection of the virus in detail and found persistent contamination of the samples with laboratory plasmids encoding SV40 sequences. In the effort to eliminate this contamination, routine decontamination of all laboratory equipment with DNA-destroying solution and strict compartmentalization of experiments were introduced. These measures led to a significant drop in SV40-positive samples; however, laboratory contamination could not be completely eliminated. After introducing measures to prevent and detect contamination, researchers were still able to show that 3 out of 18 mesothelioma tumor sections contained SV40 sequences, averaging a single viral copy per 200 cells (range 1 copy per 4.5–2000 cells).

Similarly, in the study by Lopez-Rios et al. (2004), researchers traced their positive results to contamination with laboratory plasmids. After eliminating this source of error, they reported significantly lower percentage of positive specimens (6%, or 4 out of 71, with the initial data being 56%–62% SV40-positive tumors). The SV40 DNA in the four positive samples was at the limit of the sensitivity of the assay, and the samples did not give consistently positive results. Moreover, none of the samples contained SV40 RNA as assayed by RT-PCR; the expression of SV40 Tag was also not detected by immunohistochemistry.

The wide discordance in results obtained by different laboratories has led to the evaluation of the PCR assays in two multilaboratory studies. The first study was sponsored by the National Institutes of Health and the Food and Drug Administration. In this study, nine laboratories participated in tests of masked mesothelioma (25 tissues) and control (25 normal lung) specimens and of masked panels of positive and negative controls. SV40 sequences were detected at similar low frequencies (2.3%–5.7%) in mesothelioma, normal lung, and negative control specimens. However, a major controversy among the coauthors, with several of them retracting their affiliation to this manuscript, stains the study (Ismail-Khan et al., 2006). The second study sponsored by the International Mesothelioma Interest Group, with four participating laboratories, confirmed the prevalence of SV40 in mesothelioma (Testa et al., 1998).

Detection of SV40 Proteins in Tumor Specimens

Seven studies in the IOM report have employed immunohistochemistry and immunoprecipitation methods to detect SV40 proteins in mesothelioma tissues. These studies generally reported positive results, with 88 out of 138 mesotheliomas positive for SV40 proteins. Two reports employing Tag immunostaining appeared since then (Brousset et al., 2005; Ziegler et al., 2007). In these studies 16/341 and 0/100 mesothelioma samples, respectively, were reported as positive. One additional study that assessed the prevalence of SV40 in mesotheliomas in Japan by PCR and by immunostaining was negative (Jin et al., 2004).

While the detection of viral proteins by Western blotting is not as likely to give false-positive results as PCR-based methods of detection of nucleic acids, the results are more liable to be false-negative. It has been shown that the sensitivity of the detection technique used determines the outcome of the experiment (Carbone et al., 2005; Elmishad et al., 2006). Using three techniques with different levels of sensitivity, the authors showed that the least sensitive procedure did not allow for the detection of SV40 Tag in any of the 20 mesothelioma samples, while the technique with intermediate sensitivity reported 7 of the 20 specimens as positive. Notably, the ultrasensitive procedure allowed the detection of SV40 in only two mesotheliomas because the sensitivity of the detection technique resulted in a very high background. Thus, the choice of technique with the appropriate level of sensitivity seems to be critical in the detection of viral proteins in tumor samples.

Seroprevalence

The presence of serum antibodies is a sensitive and specific marker of virus infection. Serological evidence of infection is expected to be more frequent in patients with cancer that is suspected to be associated with SV40 infection, as compared with cancer-free controls.

In contrast to the studies on the presence of SV40 nucleic acids in tumor tissues that gave widely contradictory data, the results of serological studies are comparable across the field. In these studies, typically, a low-level immunoreactivity of human sera to SV40 is shown. This is in agreement with earlier observations made in the 1960s (Shah, 1966).

The concern about these studies is whether the low-level immunoreactivity to SV40 that has been observed in the patients is indicative of SV40 infection or results from cross-reactivity with antibodies to the related human polyomaviruses BKV and JCV.

Enzyme immunoassays of the sera of rhesus macaque with viruslike particles of SV40, BKV, and JCV provide unambiguous evidence of immunologic cross-reactivity among these viruses (Viscidi et al., 2003). Competitive inhibition studies showed that the reactivity of the sera to BKV and JCV was completely eliminated by the prior incubation with SV40 viruslike particles (Shah, 2007). In another study, preincubation of SV40-reactive human sera with increasing concentrations of BKV or JCV viruslike particles completely inhibited their SV40 reactivity (Carter et al., 2003). While these results unequivocally demonstrate that BKV, JCV, and SV40 cross-react with antibodies present in these sera, they do not shed any light on the question of which viral agent elicited the immunologic response that resulted in the production of these antibodies. As observed for SV40, reactivity for either JC or BK viruses can also be eliminated by preincubation of the sera with either SV40 and BK or SV40 and JC viruses. Therefore, these experiments are biased toward the production of false-negative results.

In studies comparing SV40 seroreactivity in cancer patients and in controls, the prevalence of SV40-neutralizing antibodies was low (range 2.5%–15.2%). Close results with either cancer patients or controls exhibiting reactivity more often were reported, either in studies on malignant mesothelioma (Strickler et al., 1996) or on different types of cancers (Strickler et al., 1996; Carter et al., 2003; Rollison et al., 2003, 2005; de Sanjose et al., 2003; Engels et al., 2004, 2005). One study involving mesothelioma patients has specifically reported prevalence of SV40 seroreactivity as 8.8% (3/34) in cancer patients versus 2.9% (1/35) in control population (Strickler et al., 1996).

Epidemiological Studies

The risk of cancer in populations exposed to SV40-contaminated vaccines has been addressed in six epidemiological studies (Heinonen et al., 1973; Farwell et al., 1979; Engels et al., 2003; Strickler et al., 2003; Rollison et al., 2004; Thu et al., 2006), with

one of them (Strickler et al., 2003) concentrating on mesotheliomas. In this study, data from the Surveillance, Epidemiology, and End Results Program were used to estimate age- and sex-specific pleural mesothelioma incidence rates from 1975 to 1997. The prevalence, by birth cohort, of poliovirus vaccine exposure during the period of widespread contamination was determined from published survey data. The increase in the incidence of malignant mesothelioma in males was observed; this increase was mostly among males who were 75 years of age and older. This age group comprises the majority of mesotheliomas. Incidence rates among males and females in the age groups most heavily exposed to SV40-contaminated poliovirus vaccine remained stable or decreased from 1975 through 1997. The researchers concluded that age-specific trends in U.S. pleural mesothelioma incidence rates were not consistent with an effect of exposure to SV40-contaminated poliovirus vaccine. However, this study raised a number of objections (Puntoni et al., 2003; Vilchez and Butel, 2003). Malignant mesothelioma is a disease of the elderly. The latency period for pleural mesothelioma is longer than 40 years. In occupational studies of long-term asbestos exposure, time since first exposure ranges from 10 to 60 or more years. It can be hypothesized that, in case of SV40 infection, a similarly long latency period might be expected. If this is the case, the study in question would not have uncovered a higher incidence rate of mesothelioma among population exposed to SV40-contaminated vaccines due to the insufficient time that elapsed after exposure. Second, it is impossible to clearly separate exposed and nonexposed, and even less infected and noninfected, cohorts. Thus, in the United States, not every vaccine lot was contaminated with SV40, and the titer of live SV40 in the contaminated lots was expected to be reduced by formalin treatment. The successful infection rates by live SV40 are unknown. Third, the authors did not evaluate the possibility that the virus acts in combination with other agents, such as asbestos, and not as an ultimate carcinogen (Cristaudo et al., 2005). These facts confound epidemiological studies of cancer and SV40 exposure. In fact, IOM decided to "not recommend additional epidemiological studies of people potentially exposed to contaminated polio vaccine" until exposed and nonexposed cohorts can be reliably identified.

Hit-and-Run Hypothesis (Indirect Carcinogenesis)

It seems possible that, at least in some tumor samples, SV40 is present in very low amounts (the number of SV40 genomes/tumor cells cannot be estimated since mesothelioma biopsies often contain less than 5% of tumor cells, with the rest of cells representing reactive stroma and inflammatory cells). Moreover, viral transformation of human cells is not necessarily dose related: Often high viral doses are associated with cell lysis, while cells may have a higher chance to survive low-copy-number virus infections. In any event, what happens in the early stages of viral infection and cellular transformation may be independent of the maintenance of the transformed phenotype. In fact, the question of SV40 contributing to the process of carcinogenic transformation of human mesothelial cells is much more important than its presence

in tumor nodules. Given that SV40 is present in human cells as an episome, it is possible that it initiates the process of cellular transformation. Later on, when mutations accumulated by the infected human cells make its presence unnecessary, its loss from these cells may be facilitated by accelerated cellular proliferation and selective pressure by the immune system. This hypothesis is known under the name "hit-and-run" or indirect carcinogenesis: Scientists find it difficult to study the hit-and-run viral carcinogenesis because it is difficult to prove, but our difficulty in studying this phenomenon does not make this mechanism less important.

For the closely related JCV virus, strong evidence in support of hit-and-run mechanism has been obtained in the study of colonic cell transformation (Ricciardiello et al., 2003). In this study, cells transformed with JCV became chromosomally unstable. After beginning to accumulate genomic changes, cells started to lose viral DNA. This suggested that the virus, while involved early in carcinogenesis, was not required for tumor cell growth, and tumor cells containing the virus were selected against during tumor growth.

SV40 IN HEALTHY SUBJECTS

The prevalence of infection by SV40 in healthy subjects is indicative of how widespread the infection is, whether the virus is transmitted horizontally in the human population, and how many individuals are potentially at risk due to the oncogenic effects of the virus.

Despite the importance of the question, it has been addressed by a surprisingly small number of the studies (Heinsohn et al., 2005; Vanchiere et al., 2005). Many studies conducted to determine whether there is an association between SV40 and different types of cancer include information on the prevalence of genomic infection in healthy subjects. However, such subjects in these studies typically represent a convenience sample of controls, perhaps not representative of the general population. The data on healthy subjects in the studies addressing the prevalence of SV40 viral sequences detected by PCR has been reviewed recently (Paracchini et al., 2006). The total number of samples tested was 1103, with 88 subjects positive for SV40 sequences. Out of the 20 studies, 9 showed the presence of genomic infection, with an average prevalence from 1.3% to 25.6% (number of samples analyzed is 680). The other 11 studies showed no presence of infection. The authors found that results of the studies were statistically highly heterogeneous, which prevented them from calculating an overall prevalence of SV40 infection. The problems that plague PCR-based studies of the prevalence of SV40 sequences in tumor samples apply here as well.

A study of sera-neutralizing antibodies, conducted on a large sample of subjects from the United Kingdom, Africa, and Poland, found overlapping prevalence of infection rates among the three geographical areas (between 2.8% and 4.7%) (Paracchini et al., 2006). In another study, conducted to detect SV40 infection among healthy subjects in the United States utilizing serology, the prevalence was shown to be around 6% (Carter et al., 2003). Again, a concern about cross-reactivity of antibodies to BKV, JCV, and SV40 has been raised.

ASBESTOS AND SV40 COCARCINOGENESIS

The synergistic effects of SV40 and asbestos in causing mesothelioma are very well-documented. Bocchetta et al. (2000) reported that although focus formation was not observed in cultured human mesothelial cells treated with either asbestos fibers alone or transfected with plasmids encoding SV40 Tag, a high number of foci developed when the cells were exposed to both these agents. In a study by Kroczynska et al. (2006), the ability of SV40 and asbestos to cooperate in promoting mesothelioma formation was shown *in vivo* in hamsters. In this study, hamsters were injected with asbestos, SV40 dl883, or both. The dl883 mutant of SV40 does not express tag. Accordingly, hamsters injected with SV40 dl883 alone did not develop mesotheliomas, although a few animals developed lymphomas and sarcomas after a prolonged latency. Asbestos alone caused mesothelioma in 20% of the hamsters; in addition, it induced lymphomas, sarcomas, and liver carcinomas. Animals coinjected with SV40 dl883 and asbestos developed mesotheliomas at 90% incidence, with a significantly shorter tumor latency. This study unequivocally demonstrated that, at least in hamsters, SV40 and asbestos are cocarcinogens.

The observation that SV40 and asbestos act as cofactors in mesothelioma pathogenesis has been confirmed by two other studies. In one of these studies (Pietruska and Kane, 2007), the authors have demonstrated that the expression of Tag and tag in preneoplastic and neoplastic murine mesothelial cells enhances the formation of double-strand breaks induced by exposure to asbestos. Cells expressing these proteins also failed to senesce in response to asbestos fibers.

In another study (Robinson et al., 2006), it has been found that transgenic mice expressing high levels of Tag in the mesothelial compartment showed a relatively low level of spontaineous tumor development, but after exposure to asbestos, they rapidly developed fast-growing and invasive mesotheliomas.

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10

MOLECULAR IMMUNOBIOLOGY OF HEPATITIS B-ASSOCIATED VIRAL CANCER

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INTRODUCTION

Hepatitis B virus (HBV) is a small, enveloped virus with a 3.5-kb, partly double-stranded DNA semicircular genome that is the prototype member of the family Hepadnaviridae (Fig. 10.1; Seeger and Mason, 2000). HBV is an etiologic agent of liver diseases ranging from hepatitis to cirrhosis and liver cancer. There are several animal members of the Hepadnaviridae family (Table 10.1), and all are characterized by their ability to cause chronic infection, inflammation, and disease of the liver (i.e., hepatitis). However, despite similarities in genome composition, cross-species infections do not naturally occur and, unlike the other members of the family, duck hepatitis virus does not cause cancer. On the other hand, woodchuck hepatitis B virus (WHV) efficiently infects woodchucks and can cause liver disease and cancer in all infected woodchucks but does not establish infection in people (Tennant, 2001). Human hepatitis B only naturally infects and causes disease in people, although experimental infections of chimpanzees (Barker et al., 1975) and, more recently, the small vertebrate tupaia (Walter et al., 1996) are possible. This chapter will focus on human HBV as the clinically relevant and prototype member of the Hepadnaviridae family.

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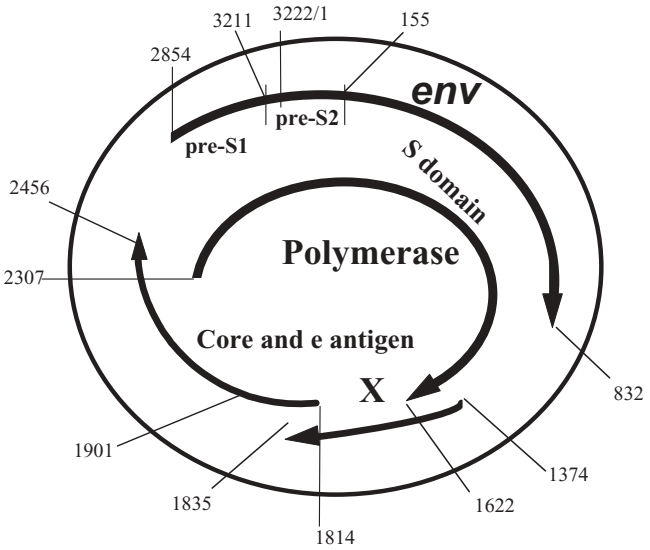

Figure 10.1. The HBV genome. HBV is a small DNA virus with a genome length of 3.2 kb. Nucleotide #1 is at the top in the pre-S2 domain. The approximate locations of the four open reading frames (ORFs) are shown: *env*, polymerase, X, and core. The four ORFs produce seven viral proteins. These are the L envelope protein, the M envelope protein, the S envelope protein (all three of these are produced from the *env* ORF), the viral polymerase (which also acts as a reverse transcriptase), the X protein, the core protein (which forms the nucleocapsid shell of the virus), and the e antigen. The start of each translational region is shown and numbered.

TABLE 10.1. Characteristics of the Hepadnaviruses

Family Member	Natural host	Experimental Host	Cancer in Natural Host	Gene Products
Human hepatitis B virus	Humans	Chimpanzees, tupia	Yes	Pol, *env* (S, L, M), core, X
Woodchuck hepatitis virus	Woodchucks	Woodchucks	Yes	Pol, *env* (S, L, M), core, X
Ground squirrel hepatitis virus	Ground squirrels	Beechey ground squirrels	Yes	Pol, *env* (S, L, M), core, X
Duck hepatitis virus	Peking ducks	Ducks	No	Pol, *env* (S, L), core

NATURAL HISTORY OF HUMAN HBV

Infection with hepatitis B results in acute, apparent or inapparent, infection ending in either resolution or in long-term "chronic" infection (Lok, 1992). There are multiple "genotypes" of the virus (A–E), and there is some evidence that pathogenicity correlates with genotype, but the likelihood of resolution depends mostly on the age at which a person is infected and, to a lesser extent, the amount of infectious virus in the inoculums (Yim and Lok, 2006). The majority of those who are infected as healthy adults resolve HBV usually without clinical evidence of infection. However, approximately 10% may experience chronic infections, with 1% developing life threatening hepatitis. On the other hand, the situation is reversed in the very young, with as many as between 60% and 90% of neonatal infections resulting in chronicity. People who are chronically infected with HBV have a between 10% and 30% lifetime risk of dying from liver disease.

EPIDEMIOLOGY

The numbers of people infected with HBV are staggering. Worldwide, more than 2 billion people have been infected, although as indicated above, most infections are resolved (Beasley, 1988). Nevertheless, as many as 350–400 million people worldwide remain chronically infected. It is this group of individuals who remains at high risk for serious illness due to HBV (El-Serag and Mason, 1999; Ganem and Prince, 2004; Gish and Locarnini, 2006; Parkin, 2006).

DISEASE ASSOCIATED WITH HBV

The most serious consequence of HBV is cirrhosis and hepatocellular carcinoma (HCC), which may not declare itself for many years, even after decades of inapparent infection that may or may not include hepatitis and liver fibrosis (Lok, 1992; Hoofnagle and Bisceglie, 1997; Lok and McMahon, 2001; Block et al., 2003). This chapter will discuss the current thinking about the molecular mechanisms involved in HBV disease, but it is worth noting that primary HCC is fifth in cancer incidence worldwide and the third leading cause of cancer death (Parkin et al., 1992; Marrero, 2006). The major etiology of HCC is hepatitis B, followed by hepatitis C (Di Bisceglie et al., 1998, 2003). Indeed, infection with hepatitis B and C viruses are responsible for at least 80% of all cases of HCC. The high mortality associated with HCC is primarily a result of poor diagnosis. That is, by the time the tumor is diagnosed, it is often unresponsive to treatment. The 5-year survival rate is less than 5%. With increasing hepatitis C virus (HCV) infection and immigration from HBV endemic populations, the number of deaths due to HCC in the United States is expected to rise over the next 20 years. Indeed, it is the fastest rising cancer in incidence in the United States.

IMMUNOLOGY OF HEPATITIS B INFECTION

Resolution of acute infection is associated with a vigorous polyclonal helper (Th) and cytotoxic T cell (CTL) response to multiple viral antigens in the infected livers (Milich and Chisari, 1982; Bertoletti et al., 1991, 1994; Rehermann et al., 1995, 1996; Rehermann, 1996; Wieland et al., 2000; Ganem and Prince, 2004). The first several months following infection may be critical. In this period of time, there is a growing antigenemia, with the cellular immune response lagging behind (Guidotti et al., 1999).

Although infection with HCV has been shown to induce numerous host innate response genes (Su et al., 2002), HBV is believed to induce few or no detectable host response genes, during at least the initial period of infection (Wieland et al., 2004; Wieland and Chisari, 2005). The reason why HBV can "fly under the radar screen" is not known.

There might be distinct mechanisms whereby HBV actively inhibits or avoids innate, cellular, and adaptive immune responses that contribute to tipping the balance between resolution and chronicity, and here we review one recently described (Block et al., 2006; Liu et al., 2007a,b).

MHC I presentation of viral epitopes depends on the degradation of viral proteins by cellular proteasomes. These peptides can come either from defective ribosome initiation products (Drips) or from the degradation of misfolded proteins. In the case of proteins that have entered the secretory pathway, this process is referred to as the endoplasmic reticulum (ER)-associated degradation (ERAD) pathway. Thus, processes that inhibit the degradation of viral proteins would be expected to inhibit the ability of the circulating immune system to recognize infected cells. Viral protein complexes that form within the ER, such as in the case of HBV envelope proteins, would be too large to be retrotranslocated from the ER to the cytosolic proteasomes. Such is the case with the HBsAg particles, which form immediately following protein synthesis, and at 22 nm, would certainly be too large to pass through translocon pores that may have limits of less than 100 Å. Moreover, since they are lipoproteins within vesicles, resistance to ERAD is all the greater. Thus, this may be a simple explanation for the relatively poor CTL response to the HBV envelope proteins, either in a natural setting (Rehermann et al., 1996; Webster et al., 2000) or with DNA vaccines (Geissler et al., 1999).

CANCER CAUSED BY HBV

HCC (liver cancer) is the primary cancer associated with HBV, although the virus has also been associated with pancreatic cancer (Hohenberger, 1984, 1985). The efficiency with which hepadnaviruses can cause liver cancer is quite striking, although the period of time between infection and clinical disease can be very long (years and decades). For example, all woodchucks chronically infected with WHV that do not die of other causes eventually develop HCC (Tennant et al., 2004). Thus, since hepadnaviruses have small genomes that can specify only a handful of gene products, it was assumed that identification of a virus specific "oncogene" would be fairly straightforward.

On the contrary, the molecular mechanisms of HBV oncogenesis appear to be quite complex and almost certainly involve host and viral factors, and no specific viral oncogene is sufficient.

Immune Destruction Theory of HBV-Associated HCC

Although it is well accepted that noncytolytic mechanisms exist by which the amount of HBV gene product can be negatively regulated by host immunological cells, and the virus has certainly evolved immune avoidance mechanisms, infection with HBV primarily causes a necroinflammatory disease (Ganem and Prince, 2004). The host immune system, led by CTL, recognizes epitopes derived from various viral gene products, such as pol, envelope, and core, presented by the MHC, and kills infected hepatocytes (Rehermann et al., 1996). The killing is incomplete, and surviving infected cells replicate to replace the killed hepatocytes, and the cycle of immunorecognition and killing repeats itself over days and years (Figs. 10.2 and 10.3; Nowak et al., 1996). Moreover, the regenerating hepatocytes are replicating in an environment of cytokines and, possibly, mutagen products left from the destroyed cells and immunological cells. It is reasoned that the continuous cycle of killing and regeneration results in opportunities for the regenerating hepatocytes to mutate themselves, and thus leads to transformation. In this model, specific viral oncogenes need not be implicated.

Having said that, HBV specifies at least five open reading frames, as shown in Figure 10.1, and each has been associated with oncogenesis. Cell proto-oncogenic pathways, and even the integration of viral genomic promoters near cellular proto-oncogenes, have all been suggested to play a role in HBV-mediated HCC. These categories are summarized below, topic by topic.

Integration of Viral DNA

Woodchuck viral genomes can be found integrated, adjacent to c-myc proto-oncogenes, in most HCC-derived cells in chronically infected animals (Moroy et al., 1986, 1989; Hsu et al., 1988). HBV DNA integration into host chromosomes is also common in malignant and nonmalignant hepatocytes (Koshy et al., 1981, 1983). However, unlike the situation with WHV-associated HCC, integration of human HBV DNA near specific proto-oncogenes in human HCC is a rare event and is not believed to be the major mechanism of oncogenesis.

Viral Gene Products

X. This is a 154-amino-acid polypeptide of unknown function that has been shown to enhance HBV replication in woodchucks and in culture (Zoulim et al., 1994; Bouchard and Schneider, 2004). It has been implicated in numerous activities that could have a role in oncogenesis, including calcium mobilization and activation of Pyk and FAK signaling pathways, c-fos, c-jun, c-myc, EGF, Wnt, Rb, p53, phosphoatidylinositol 3-kinase (PI3K)-Akt signaling pathways and MHC I (Zhou et al., 1990; Menzo et al., 1993; Feitelson, 1998, 1999; Yeh, 2000; Bouchard et al.,

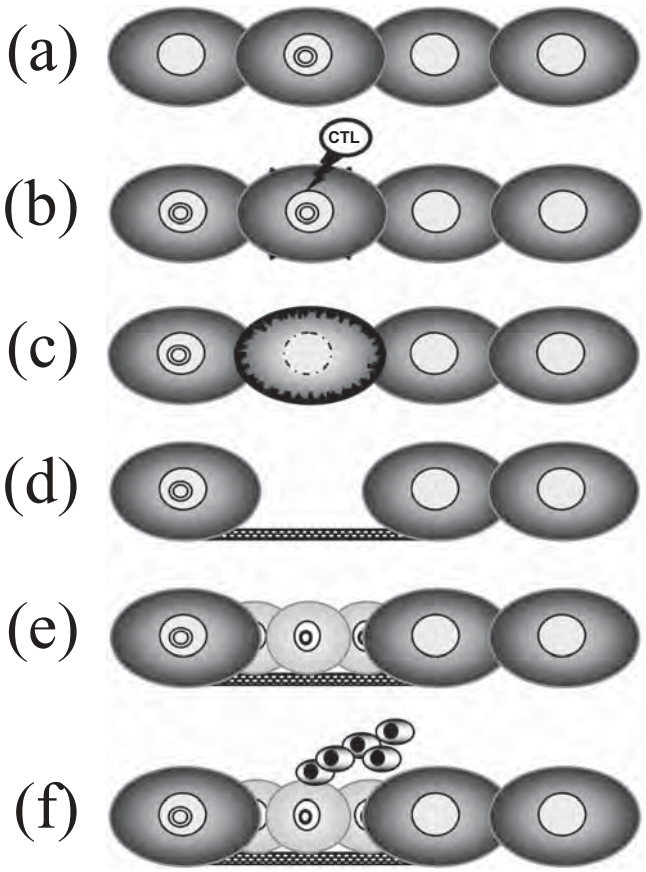

Figure 10.2. Immune-mediated destruction of infected hepatocytes leads to the development of HCC. (a) An infected cell is indicated by the presence of HBV CCC DNA in the infected cell nucleus. (b,c) The host immune system, led by cytolytic T cells (CTL), recognizes epitopes derived from various viral gene products, such as pol, envelope, and core, presented by the MHC, kills infected hepatocytes. (d) Collagen, fibrinogen, and other connective tissue is laid down at the site of damage. The killing is incomplete, and surviving infected cells replicate to replace the killed hepatocytes, and the cycle of immune recognition and killing repeats itself over days and years. (e,f) The regenerating hepatocytes are replicating in an environment of cytokines and, possibly, mutagen products left from the destroyed cells and immunological cells. It is reasoned that the continuous cycle of killing and regeneration results in opportunities for the regenerating hepatocytes to mutate themselves, and thus leads to transformation.

2001a,b, 2003, 2006; Wu et al., 2002; Pan et al., 2004) as well as p53 inactivation. Indeed, these are among the major oncogenic pathways that are activated in many types of human cancers, including HCC, and all have been reported to be influenced by HBV.

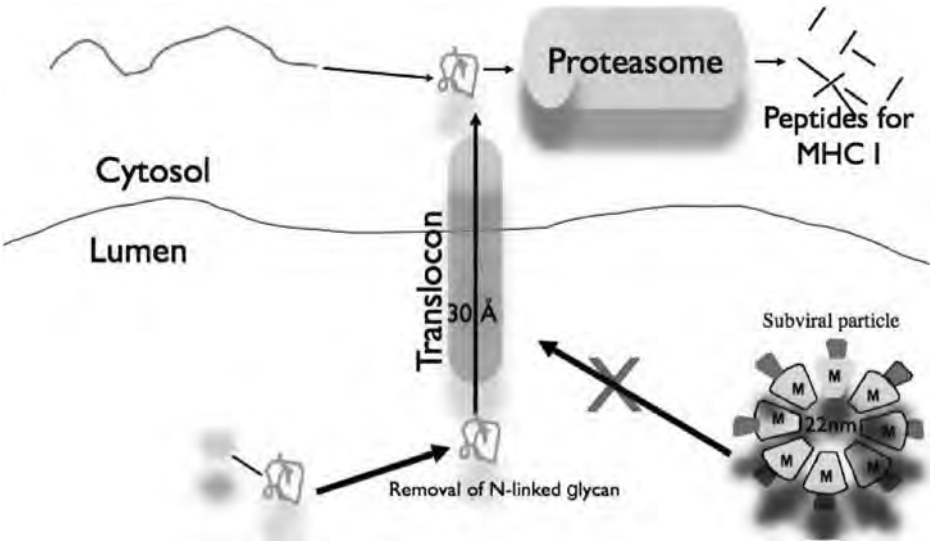

Figure 10.3. Peptides presented via MHC class I can arise from defective translation (defective ribosome initiation products [Drips]) or from the degradation of glycoproteins in the ER that fail quality control (ER-associated degradation [ERAD]). ERAD is the mechanism by which cells remove misfolded proteins from the ER. This is thought to involve the translocon pore complex, which is 10–30 Å in size. Protein aggregates, such as the HBsAg, theoretically, would not fit through this pore and would have to be degraded via a different mechanism.

However, the number of different functions and activities attributed to "X" make it very difficult to accept which, if any, function plays a role in malignancy. Nevertheless, based on the body of circumstantial evidence, there is a growing consensus, at the time of this writing, that "X" can contribute to HBV-mediated cancer.

Envelope Proteins. Accumulation of large envelope protein (LHBs) within the ER of hepatocytes has been shown to be associated with a predisposition to transformation in transgenic animal studies and with severe fulminant hepatitis in people (Chisari et al., 1987). HBV variants with mutations in pre-S regions were found to correlate with a diagnosis of HCC, strengthening the argument that aberrant metabolism of envelope proteins could lead to HCC (Santantonio et al., 1992; Tai et al., 2002). Indeed, recent reports have indicated that retained HBV envelope proteins are poorly degraded (Simsek et al., 2005; Liu et al., 2007b). The clinical consequences of this poor degradation could be severe, as reports have indicated that pre-S1 and pre-S2 mutations, which are often associated with the development of HCC, are retained in the ER for a prolonged period of time. This prolonged retention leads to an ER stress response and, possibly, to the eventual development of HCC (Xu et al., 1997; Hsieh et al., 2004; Hung et al., 2004; Shih and Tai, 2004; Wang et al., 2005).

Core and Precore. Core and precore mutations have also been associated with an increased risk of hepatitis (Baumert et al., 1998; Lin, 2005). Overall, HBV variants containing basal core mutations are detected in perhaps 50% of individuals with chronic HBV and a diagnosis of HCC. However, although there is a correlation, there is as yet no evidence of a causal relationship.

EARLY DETECTION OF LIVER DISEASE AND HCC

Regardless of the specific etiology, it is generally accepted that early detection of liver disease can result in improved outcomes. Liver fibrosis is an indication for therapeutic intervention with medication or other means, and early detection of cancer is associated with improved outcome (Sherman, 2005; Marrero, 2006).

The progression of liver disease in asymptomatic chronic carriers of HBV and HCV is monitored by serum liver function tests (LFTs) and ultrasound imaging for detection of small masses in the liver (Di Bisceglie et al., 1998). Many of the constituents of the LFT panel, for example, transaminase levels, vary throughout the course of chronic hepatitis and are of limited use in the early detection of HCC. Ultrasound detection requires at least a 3-cm tumor mass to be present, and often occurs at a stage at which the prognosis is very poor (Brechot, 1987; Hoofnagle, 1997). Since early surgical and chemotherapeutic intervention is the best hope for patient survival (Brechot, 1987; Di Bisceglie et al., 1998), early detection of HCC is necessary to identify the need for intervention. Thus, with a highly defined risk population, the potential for a significant impact on the disease is present.

The literature reports many biomolecular changes that occur during the development of HCC. These include, but are not limited to, increased serum activin-A (Pirisi et al., 2000), decreased serum retinol levels (Newsome et al., 2000), expression of cellular ets-1 (Baumann et al., 2000), repression of the cell surface marker CD81 (Inoue et al., 2001), and numerous changes that can be identified by comparative genomic hybridization (Zondervan et al., 2000) or, in hepatoma cell lines, by cDNA microarray analysis (Kawai et al., 2001; Patil et al., 2005). However, by far, the most useful change associated with HCC is the increase in serum alpha-feto protein (AFP) (Tatarinov, 1963). AFP is an oncofetal glycoprotein that is (hypothesized to be) produced by transformed liver cells after the development of HCC. However, AFP can be produced under many circumstances, including other liver diseases (Alpert et al., 1968; Ruoslahti et al., 1974; Di Bisceglie and Hoofnagle, 1989), and is not a definitive marker for the development of HCC. Analyses of the regulatory mechanisms of increased AFP synthesis in hepatic injury and in malignant transformations (Sawadaishi et al., 1988; Nakabayashi et al., 1989) have been unable to distinguish elevation of AFP between HCC and chronic liver disease. The detection of AFP is currently one of the methods by which the development of HCC is detected, although the poor sensitivity of this marker (30-60% sensitivity) has led people to question its true usefulness in the detection of HCC (Bruix and Sherman, 2005).

As a consequence of the poor performance of AFP as a biomarker of HCC, efforts have been made to discover more sensitive serum markers of early HCC. The principle

use of such a marker would be to select those patients for further analysis via imaging methodologies and following treatment. Hence, such a marker would not need to be 100% specific for HCC but should have the greatest level of sensitivity possible. Given these limited requirements, and the large amount of proteomic and genomic work, success has been limited (Santamaria et al., 2007; Wright et al., 2007).

Three of the most promising serum biomarkers of HCC are des-gamma carboxyprothrombin (DCP), glypican-3 (GPC3), and Golgi protein 73 (GP73) (Fujiyama et al., 1988; Weitz and Liebman, 1993; Kladney et al., 2000; Capurro et al., 2003; Block et al., 2005). DCP is a prothrombin molecule that is heterogeneous in gamma-carboxyglutamic acid (Gla) content with an average of five Gla residues/molecule compared with 10 Gla residues for native prothrombin and two Gla residues for DCP. This difference can be measured in serum using specific antibodies and has limited success in the detection of HCC. Performance characteristics are similar to AFP but are strongly linked to tumor size (Nakamura et al., 2006), hence DCP is thought to be a poor marker of early HCC. Efforts to improve the characteristics through the combination of markers are currently under way (Nomura et al., 1999; Marrero et al., 2003).

GPC3 is a proteoglycan that, like AFP, is highly up-regulated in cancer tissue with the development of HCC (Capurro et al., 2003; Patil et al., 2005). This marker can be found in the serum of 40%–60% of patients with HCC and was found in many patients who were AFP negative (Capurro et al., 2003). Hence, GPC3 may be useful, in combination with AFP, for the detection of early HCC.

GP73 was first identified as a Golgi-resident glycoprotein that was up-regulated in the livers of patients following viral or chemical insult (Kladney et al., 2000) and later was found in the serum of patients with HCC (Block et al., 2005; Marrero et al., 2005). The sensitivity and specificity of this protein was much greater than that observed with AFP and was detected in over 75% of AFP-negative patients (Marrero et al., 2005). In addition, the examination of specific glycoforms of GP73 has shown great promise and may increase the overall performance of GP73 in the detection of HCC (Drake et al., 2006).

MANAGEMENT OF HBV AND THE DISEASES WITH WHICH IT IS ASSOCIATED

Prevention

There are safe and effective vaccines to prophylactically prevent the establishment of HBV infection in people (Hoofnagle and Bisceglie, 1997). The most common are "subunits" of envelope antigen polypeptides derived from recombinant yeast. The currently used vaccines in the United States are approximately 90% effective in healthy individuals, although older age is associated with less effectiveness. A combination of vaccine and hepatitis B hyperimmune gammaglobulin given to newborns, within 24 h of birth from hepatitis B chronic carrier mothers, can prevent an almost certain establishment of chronic infection. Moreover, since immunity is humoral and directed against a single epitope, the "a" motif of sHBS, there is some concern about vaccine escape mutants (Gerlich, 2006).

Management of Chronic Infection

There are now four U.S. Food and Drug Administration (FDA)-approved oral medications, as well as parenterally administered interferon alphas, for the management of chronic HBV (Fung and Lok, 2004; Bartholomeusz and Locarnini, 2006; Hoofnagle et al., 2007; Lok et al., 2007). Since all of the current oral medications are polymerase inhibitors, resistant mutants may become a problem. Interferon alpha appears to be effective in less than 50% of those in whom it is used and its toxicity has limited its use against HBV.

Management of Fibrosis, Cirrhosis, and HCC

There are currently no U.S. FDA-approved antifibrotic medications. Bacteremia during cirrhosis is considered to be a later complication; a recent work by Mehta and Block (2008) suggests that bacteremia may be a factor much earlier than previously thought. Intervention with antibiotics in earlier fibrosis may be considered. Transplantation is reserved as an option for both decompensated cirrhosis and HCC. Depending on the stage, HCC is often treated with resection, chemoembolization, and ethanol ablation.

CONCLUSIONS

Although it is well understood that infection with HBV can lead to the development of HCC, much confusion still exists on how HBV causes cancer. There are, however, possible unifying themes, such as the constant immune-mediated destruction of infected hepatocytes, the accumulation of mutated HBV proteins that activate host proto-oncogenes, and host genetic alterations that reduce viral replication but lead to transformation. These all highlight the interplay between virus and host that often involves a roll of the dice of time and environment that determine the ultimate clinical outcome. The central question of which viral gene products are essential to these processes, and when in the process do they become unnecessary, remains the subject of study.

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11

HEPATITIS B VACCINE AND HEPATOCELLULAR CARCINOMA

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INTRODUCTION

Hepatitis B virus (HBV) infection and hepatocellular carcinoma (HCC) are examples of how research provides solid evidence to support the causal association of a virus and its related cancer. With the development of a vaccine, the virus infection is prevented, and thus the oncogenesis process by the virus is interrupted.

HCC is one of the five leading cancers in the world (Parkin et al., 2001). Persistent infection with HBV or hepatitis C virus (HCV) is associated with approximately 90% of HCC. Evidence from epidemiology, case control study, animal experiments, molecular biology, and so on supports the important oncogenic role, either directly or indirectly, of hepatitis B and C viruses in HCC. As evidenced by the large population infected with HBV in the developing world, HBV remains the most prevalent oncogenic virus for HCC in humans. HBV is estimated to cause around 55%–65% of HCC worldwide, while HCV accounts for around 25% of HCC (Bosch and Ribes, 2002).

There is a strong male predominance in HCC, with a male to female ratio of 4–7:1, even in children (Ni et al., 1991; Schafer and Sorrell, 1999). In most high prevalence areas

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of HCC, such as Southeast Asia and West Africa, the incidence increases after 30 years of age and peaks at 50–60. Familial clustering of HCC suggests the role of genetic predisposing factors in addition to the intrafamilial transmission of HBV infection (Chang et al., 1984). Liver cirrhosis is a common precancerous lesion, accounting for approximately 80% of patients with HCC, including children (Hsu et al., 1983, 1987). This sequela usually results from liver injury caused by chronic HBV or HCV infection.

Hepatitis B immunization provides the first evidence for the success of cancer prevention by vaccination in humans. In-depth research in epidemiology, transmission routes, clinical long-term follow-up of the consequence of persistent HBV infection, virology of HBV, and oncogenic mechanisms are most helpful in the development of effective strategies for the control of HBV infection and its related cancer. We review these data and present them in this chapter.

DISEASE BURDEN OF HCC

It has been estimated that approximately 564,000 new cases of HCC occurred worldwide in the year 2000 (Bosch and Ribes, 2002). Due to the high incidence as well as mortality rate of HCC, it remains as one of the three major causes of cancer death in man. According to the estimate of the World Health Organization (WHO), among a total of 58 million deaths worldwide in 2005, liver cancer accounts for 8.7% (662,000 deaths per year) of all cancer deaths (http://www.who.int/mediacentre/factsheets/fs297/en/). The occurrence of HCC ranges from 5 to 10 per 100,000 in low incidence areas, such as Northern Europe, among the Caucasian population in North and South America, Australia, and New Zealand, to more than 100 per 100,000 in high incidence areas, such as Southeast Asia and Africa. The annual incidence of HCC in Alaskan Eskimo males was 11.2 per 100,000, five times that of white males in the United States (Heyward et al., 1981).

The world geographic distribution of HCC overlaps well with that of the distribution for chronic HBV infection (Beasley, 1982). Regions with a high prevalence of HBV infection also have high rates of HCC. HBV causes 60%–80% of the world's primary liver cancer, which accounts for one of the three major cancer deaths in Asia, the Pacific Rim, and Africa. The southern parts of Eastern and Central Europe, the Amazon basin, the Middle East, and the Indian subcontinent are also areas with high prevalence of HBV infection and HCC (Lavanchy, 2004). In spite of the extensive HBV vaccination in children and adults, the number of cases of HCC is still increasing in adults, mainly due to the infection of HCV, which is another important oncogenic virus for HCC. HCV infection overrides the importance of HBV as an etiologic agent for HCC in adults, in low prevalence areas for HBV infection.

ONCOGENIC MECHANISMS IN HCC

The oncogenesis of HCC is a multistep process, which usually takes decades in a hepatitis virus chronically infected subject. Chronic hepatocellular injury and regeneration caused by persistent hepatitis virus infection may lead to hepatocarcinogenesis. Liver

TABLE 11.1. Oncogenic Mechanisms of Hepatocellular Carcinoma

1. Chronic liver injury caused by hepatitis virus or other agents, leading to hepatocyte transformation and finally HCC
2. Accumulation of genetic alterations with gain or loss of genes
3. Direct carcinogenic role of HBV or HCV
4. Interaction of HBV with other environmental factors

injury caused by chronic hepatitis B or C virus infection is the most important initiation event of hepatocarcinogenesis, which accounts for approximately 80% of HCC (Bruix et al., 2004). The role of HBV or HCV in tumor formation appears to be complex and may involve both direct and indirect mechanisms of carcinogenesis.

Worldwide, approximately 450 million people are infected by HBV and 200 million by HCV. Although the molecular hepatocarcinogenic mechanisms of HBV or HCV remain mostly unclear, the following mechanisms have been proposed (Table 11.1).

Severe Chronic Liver Injury, Regeneration, Preneoplastic Phase, and HCC

Persistent liver injury caused by chronic hepatitis virus (HBV or HCV) infection or other causes, such as hereditary tyrosinemia or hereditary hemochromatosis, can induce cell death and regeneration, liver fibrosis, and cirrhosis, which may then lead to hepatocyte transformation, low-grade and high-grade dysplastic lesions, and finally HCC. During this period of time, hepatic cycling is accelerated by up-regulation of mitogenic pathways (Thorgeirsson and Grisham, 2002). Alterations in the quantities of gene expression are mainly by epigenetic mechanisms in the pre-HCC lesions.

Following liver injury by hepatitis virus, hepatic compensatory proliferation can be driven by mitogens (e.g., tumor necrosis factor-α [TNF-α], interleukin-6 (IL-6), and hepatocyte growth factor) secreted by the surrounding nonparenchymal cells. The oncogenesis process can also be induced by hepatocyte proliferation, triggered by the secretion of growth factors (e.g., TGF-α) from the hepatocytes (Arsura and Cavin, 2005). The accumulation of genetic alterations during this multistep process accelerates the hepatocarcinogenesis.

Accumulation of Genetic Mutations

During the process of hepatocarcinogenesis, gains in chromosomes 1q, 8q, and 20q, and allelic loss (LOH) on chromosomes 1p, 4q, 6q, 8p, 9p, 13q, 16p, 16q, and 17q (Buendia, 2000; Villanueva et al., 2007) accumulate gradually. Some "hot spots" for breakage/LOH with known or putative tumor suppressor genes have been reported, such as E-cadherin, M6P/IGFIIR, TP 53, DLC-1, PRLTS, $p16^{INK/ARF}$, EXT1, Rb1, and p73 (Grisham et al., 2001). However, the relation of genetic mutation in the host to HBV or HCV infection remains to be further clarified.

Direct Carcinogenic Effect of HBV

Prolonged expression of the viral regulatory protein, hepatitis B x protein (HBx), and the large envelope proteins (LHBs) may contribute in deregulating the cellular transcription program and proliferation control, and sensitize liver cells to carcinogenic factors (Tang et al., 2006). HBx protein of HBV has a transactivator activity and upregulates growth-promoting genes during persistent viral infection. The presence of pre-S mutants carries a high risk of HCC in HBV carriers and was proposed to play a potential role in HBV-related hepatocarcinogensis (Wang et al., 2006). Subjects infected with HBV core promoter mutants were reported to have a higher risk of developing HCC. Furthermore, HBV mutants developed in many HCC patients during persistent HBV infection. Some HBV mutants result in loss of hepatitis B e antigen (HBeAg), hepatitis B core antigen (HBcAg), or hepatitis B surface antigen (HBsAg) expression of hepatocytes, which are the targets of immune-mediated cytotoxicity, giving those hepatocytes a distinct growth advantage.

Persistent HBV infection might promote host genomic instability, either through HBV DNA integration into the host genome or through the function of the HBx protein. Integration of HBV DNA into the host genome occurs mostly in random sites of the host genome at early steps of clonal tumor cell expansion. Even in childhood HCC, with shorter duration of chronic HBV infection and oncogenic process, HBV integration was present in the tumor and nontumor liver tissues (Chang et al., 1991). Occasionally, it may induce direct insertional mutagenesis of various cancer-related genes, which results in *cis*-activation of oncogenes or inactivation of tumor suppressor genes. The integration of woodchuck hepatitis virus near a cellular proto-oncogene, taking c-myc as a target, can contribute to the genesis of liver cancer (Hsu et al., 1988b). However, this does not seem to occur in man.

Interaction of HBV with Other Carcinogenic Factors

Aflatoxin B1 has been reported to promote the development of HCC in subjects with chronic HBV infection and P53 mutation (Bressac et al., 1991).

These data suggest that persistent HBV infection might trigger specific oncogenic pathways directly or indirectly, thus it plays a role beyond stimulation of host immune responses and chronic necroinflammatory liver disease (Laurent-Puig and Zucman-Rossi, 2006). HBV may share a number of basic strategies with other human oncogenic viruses. Most likely, collaborative mechanisms of the above-mentioned viral and host factors may contribute in the development of HCC.

HBV INFECTION AND HCC WITH EMPHASIS ON THE TRANSMISSION ROUTES AND PREVENTION

HBV infection is a worldwide health problem that may lead to fulminant, acute, or chronic hepatitis, liver cirrhosis, and HCC. It is estimated that more than 2 billion of the global population have been infected by HBV. Of these, approximately 360 million

are chronically infected and at risk of serious illness and death from cirrhosis and HCC, diseases that are estimated to cause 500,000–700,000 deaths each year worldwide (Hepatitis B vaccine, WHO position paper, 2004).

HBV infection is most prevalent in Asia, Africa, Southern Europe, and Latin America, where the HBsAg-positive rate in the general population ranged 2%–20%. In Asia and many other endemic areas, perinatal transmission through HBsAg carrier mothers accounts for 40%–50% of HBsAg carriers. Around 85%–90% of the infants of HBeAg seropositive carrier mothers became HBsAg carriers (Stevens et al., 1975), irrespective of the extent of HBsAg carrier rate in the population.

In endemic areas, HBV infection occurs mainly during infancy and early childhood. Mother-to-infant transmission accounts for approximately half of the transmission route of chronic HBV infections. In contrast to the infection in adults, HBV infection during early childhood results in a much higher rate of persistent infection and long-term serious complications, such as liver cirrhosis and HCC. Taking Taiwan as an example of endemic areas, the HBsAg carrier rate is approximately 10%–20% in its general population. Before the implementation of universal HBV vaccination program in Taipei City, the HBsAg seropositive rate was 5% in infants, increased to 10% at 2 years of age, and has remained at the same rate thereafter (Hsu et al., 1986). The data suggest that most chronic HBsAg carriers resulted from infections before 2 years of age in this population (Hsu et al., 1986).

Horizontal transmission, which accounts for another half of the transmission route, was through the use of unsterile needles or medical equipment, unsafe blood product or transfusions, unprotected sex, unsterile skin piercing, or other intrafamilial close contact. In Africa, where HBV infection is also endemic, horizontal infection during early childhood is the main route of transmission. In rural Senegal, by the age of 2 years, 25% of children are infected, while at age 15, the infection rate rises to 80% (Feret et al., 1987).

The age of HBV infection and the HBeAg status of the mother are two important factors affecting the outcome of HBV infection. The younger the age when infection occurs, the higher the rate of chronicity. Without immunoprophylaxis, more than 90% of infants who were infected by their HBeAg-positive HBsAg carrier mothers, will develop chronic HBV infection during follow-up (Stevens et al., 1975) (Table 11.2).

TABLE 11.2. Maternal Serum HBV Status and the Outcome of HBV Infection in Infants

Mothers	Infants	
	No Vaccination	With Immunoprophylaxis
HBeAg(+), HBsAg(+)	>90% chronic	Vaccine + HBIG→10%–15% chronic infection
HBeAg(–), HBsAg(+)	<5% chronic, with risk of FH, AH	<1% chronic infection and risk of FH, AH reduced
HBeAg(–), HBsAg(–)	Not infected	Not infected

FH, fulminant hepatitis; AH, acute hepatitis; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBIG, hepatitis B immunoglobulin.

This may be explained by the very high maternal viral load transmitting to the neonate with an immature immune system during perinatal period. An immune tolerance status to HBV is established during this early infection. For those who are infected at preschool age, the chronicity rate after HBV infection decreases to approximately one quarter (23%) (Beasley et al., 1982). Infection in young adults results in a much lower rate (2.7%) toward chronicity (Beasley et al., 1983b).

HBeAg is a soluble antigen produced by HBV. It can cross the placenta barrier from the mother to the infant. Transplacental HBeAg from the mother induces a specific unresponsiveness of helper T cells to HBeAg and HBcAg in neonates born to HBeAg-positive HBsAg carrier mothers (Hsu et al., 1992). This may help to explain why 85%–90% of the infants of HBeAg-positive carrier mothers became persistently infected (Beasley et al., 1977), while only approximately <5% of the infants of HBeAg-negative HBsAg carrier mothers became persistently infected. The immune tolerance state persists for years to decades after neonatal HBV infection.

Persistent HBV infection is closely related to the development of HCC. The peak age of HCC in adults is 40–60 years of age, in most countries. The seroprevalence rates of increased HBsAg in adult HCC patients vary in different countries and ethnic groups (Bosch and Ribes, 2002). In Asia, it ranges from as high as 79% in Taiwan, where HBV infection is endemic, to as low as 19% in Japan, where HCV infection is more prevalent than HBV. HCC can also develop in children in endemic areas, as demonstrated in Asia and Africa. No evidence of increased HCV infection has been found in children with HCC. Maternal transmission of HBV to their infants is the main route of transmission in children with HCC. HBsAg seropositive rate in the mothers of HCC children is 94%, which is significantly higher than that (50%) in the mothers of non-cancer HBsAg carrier control children (Chang et al., 1989). In one series of adults with HCC in Taiwan, HBsAg was positive in 74% of their mothers (Chen and Sung, 1980).

Chronic HBV infection in HCC patients begins mostly during childhood. Carcinogenesis takes years or decades after HBV infection. Nearly 100% of Taiwanese children with HCC are HBsAg seropositive, while 70%–80% of HCC adults are HBsAg seropositive. Although the role of HCV in HCC is increasing in many countries, the high rate and importance of HBV infection in childhood HCC has not changed. HCC patients are mostly (around 80%) anti-HBe seropositive at diagnosis (Chien et al., 1981). This implies that HCC occurs after long-term HBV infection and liver injury, and that the patients have seroconverted to anti-HBe. Consequently, at the diagnosis of HCC, they are already often in a low viral replication status. Subjects with prolonged high HBV replication levels or positive HBeAg after 30 years of age have a higher risk of developing HCC during follow-up (Beasley et al., 1981; Yang et al., 2002; Chen et al., 2006) (Table 11.3).

The prognosis of HCC is grave, unless it is detected early and complete resection or ablation is performed. Even in such cases, *de novo* recurrence of HCC is always a problem. Prevention is thus the best way toward the control of HCC (Chang, 2003). Strategies to prevent HCC include vaccination, chemoprevention of high-risk subjects (Jacobson et al., 1997; Chuang et al., 2000; Egner et al., 2001), antiviral therapy for hepatitis B or its related liver cirrhosis (Liaw et al., 2004; Lin et al., 2007), prevention of high risk behavior, changes in environment and/or diet, and liver transplantation for

TABLE 11.3. Incidence of HCC during Long-Term Follow-Up in HBsAg-Positive versus -Negative Adult Males

HBV Markers	No. of at Risk	No. with HCC	Incidence of HCC (10^5/Person-Year)	Risk Ratio	Authors
HBsAg(−)	19,253	9	5	1.0	Beasley et al.
HBsAg(+)	3,454	152	2,247	99	(1981)
HBsAg(−)/HBeAg(−)	9,532	29	39	1.0	Yang et al.
HBsAg(+)/HBeAg(−)	1,991	50	324	9.6	(2002)
HBsAg(+)/HBeAg(+)	370	32	1,169	60.2	
HBV DNA titer					
<300 copies/ml	—	—	108	1.0	Chen et al.
300–9999 copies/ml	—	—	111	1.0	(2006)
10^4–99,999 copies/ml	—	—	297	2.7	
10^5–999,999 copies/ml	—	—	962	8.9	
>10^6 copies/ml	—	—	1,152	10.7	

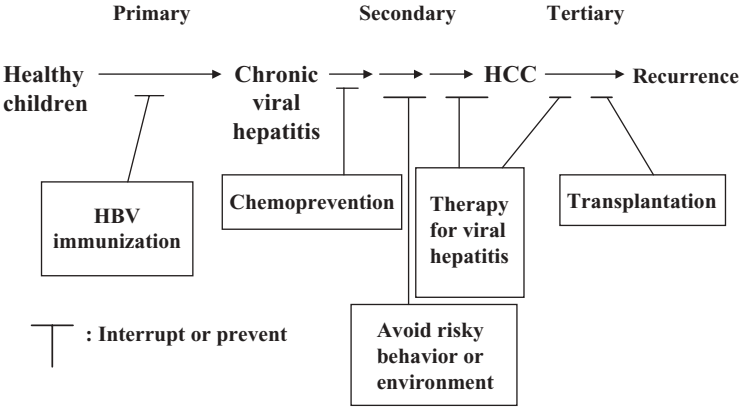

Figure 11.1. Strategies for primary, secondary, and tertiary prevention of liver cancer. HBV immunization is the most effective way. For persons who have been infected by hepatitis virus, antiviral therapy may delay or reduce the risk of developing HCC in a minor degree. The effect of other strategies such as chemoprevention and avoidance of risky behavior is still not confirmed and under investigation.

precancerous lesion, for example, liver cirrhosis (Fig. 11.1). However, the effects of chemoprevention against HBV-related HCC still remain to be seen.

Current antiviral therapies for hepatitis B, liver cirrhosis, and HCC are not satisfactory. Antiviral therapy for hepatitis B is aimed at the normalization of the liver enzymes, HBeAg clearance, and reduction of HBV DNA levels as well as inflammation and fibrosis in the liver. However, antiviral therapy and immune modulating agents did not reach a high sustained response rate. Even in those with sustained response, HBV cannot be eradicated from the hosts in the majority of treated cases

because it is difficult to eliminate cccDNA by these means. Viral resistance after prolonged use of antivirals is another problem. Antiviral therapy, therefore, may have borderline or limited beneficial effect in preventing HBV-related complications.

A prospective randomized controlled trial of antiviral therapy was conducted in 651 patients with HBV-related cirrhosis. HCC was diagnosed in 3.9% of lamivudine-treated patients and in 7.4% of placebo controls after a median follow-up of 32 months, with a borderline significant difference ($P = 0.047$) (Liaw et al., 2004). Another retrospective study revealed that the cumulative incidence of HCC at the end of 15 years (median 6.8 years) follow-up in the 233 interferon (IFN)-treated patients and 233 controls was 2.7% versus 12.5% ($P = 0.011$). Yet significant reduction of HCC was only observed in patients with preexisting cirrhosis and HBeAg seroconverters (Lin et al., 2007).

Furthermore, a large proportion of HCC patients are diagnosed late, are inoperable, or are not responsive to nonsurgical therapies. Recurrence of HCC after effective therapy is another common problem. Among all the preventive strategies, vaccination is still the only most effective way to prevent hepatocarcinogenesis.

DEVELOPMENT OF HBV VACCINE

Hepatitis B vaccine is a very successful protein-based subunit vaccine that contains HBsAg. Using three or four doses of the vaccine alone or in combination with hepatitis B immunoglobulin (HBIG) for infants of high-risk mothers can reach a high efficacy with long-term protection. In addition to active immunization with HBV vaccines, passive immunization with HBIG can neutralize HBV transmitted from the mother during perinatal period and provide temporary immunity. It must be given as soon as possible after birth, preferably within 24 h. After hepatitis B vaccine became available in 1982, active immunization with three or four doses of HBV vaccines without HBIG has proved to be immunogenic and efficacious in more than 90% of neonates of noncarrier mothers or HBeAg-negative carrier mothers.

In infants of HBeAg seropositive mothers, three or four doses of HBV vaccine reduced the HBV carrier rate from >90% to 24%. The injection of HBIG within 24 h after birth, followed by three doses of HBV vaccine, further reduces the carrier rate down to 3% in pilot studies (Beasley et al., 1983a), and to 14% in the study of high-risk infants of the general population in Taiwan's national mass vaccination program (Hsu et al., 1988a). According to the random sampling study of children in Taiwan 3 years after the universal HBV vaccination program, the protective efficacy was found to be 86% by HBIG plus HBV vaccine and 78% by three doses of HBV vaccine (Chen et al., 1987; Hsu et al., 1988). And thus, the optimal time for immunoprophylaxis against HBV infection and its sequelae is during the neonatal period or early infancy.

UNIVERSAL HEPATITIS B VACCINATION IN INFANTS

Comparing various strategies for HBV vaccination, a universal immunization program in infancy is better than immunization of the high-risk groups. Integration of HBV vaccination into the routine Expanded Program of Immunization (EPI) of infancy is

TABLE 11.4. Different Strategies of HBV Immunization and Their Cost and Efficacy

Immunization	Maternal Screening	Infants Receiving		Cost	Efficacy	Example
		Vaccine	HBIG			
Active only	No	Yes	No	Lowest	Modest	Thailand
Active + passive						
Type I	HBsAg	Yes	Infants of HBeAg(+)	Higher	High	Taiwan
	HBeAg		HBsAg(+) mothers			
Type II	HBsAg	Yes	Infants of HBsAg(+)	Highest	Highest (?)	United States
	Only	—	Mothers	—	—	—

Active immunization = hepatitis B vaccines.
Passive immunization = hepatitis B immunoglobulin (HBIG).

most plausible with highest compliance by the general population. The efficacy in preventing HBV infection is highest, and the requirement of the amount of HBV vaccine is the lowest. Three main types of strategies in universal immunoprophylaxis against HBV infection are used worldwide, depending on the prevalence of HBV infection and the resources of the countries (Table 11.4).

The first universal hepatitis B vaccination program in the world was launched in Taiwan since July 1984 (Chen et al., 1987). Pregnant women were screened for both serum HBsAg and HBeAg, or titers of HBsAg by reverse passive hemagglutination test (RPHA) at the beginning of the program. Infants of mothers with negative serum HBeAg, with reciprocal HBsAg titer lower than 2560, or with negative serum HBsAg, received four doses of hepatitis B vaccine at a schedule of 0, 1, 2, and 12 months in those born before July 1992, or three doses of recombinant hepatitis B vaccines at a schedule of 0, 1, and 6 months in those born after July 1992. In addition, newborns of high-risk carrier mothers with positive HBeAg received 0.5 ml HBIG within 24 h after birth. All of the costs of screening, vaccines, and HBIG were covered by the government. The coverage rate of hepatitis B vaccine for neonates is increased gradually from around 84% to >95%. The program was gradually extended to preschool children, older school children, and adults (Chen et al., 1987).

To save the cost of screening and HBIG, some countries with intermediate/low prevalence of chronic HBV infection or inadequate resources do not screen pregnant women and all infants receive three doses of HBV vaccines without HBIG. Using this strategy, the cost of maternal screening and HBIG can be saved. The efficacy of preventing the infants from chronic infection seems satisfactory (Poovorawan et al., 1989).

In countries with adequate resources, such as the United States, pregnant women are screened for HBsAg but not HBeAg. It is recommended that every infant receive three doses of HBV vaccine. In addition, infants of all HBsAg-positive mothers also receive HBIG within 24 h after birth (Shepard et al., 2006). This strategy saves the cost

and the procedure of maternal HBeAg screening but increases the cost of HBIG, which is the most expensive.

According to the WHO, hepatitis B vaccine was introduced throughout 164 WHO member states by the end of 2006 (WHO Department of Immunization, Vaccines and Biologicals data, September 2007). Global coverage of HBV vaccination was estimated to be 55%. It ranged from 86% in the Americas to 27% in the Southeast Asian region and 39% in the African region. Global coverage of infants in 1992 with three doses of hepatitis B vaccine was only 3% (http://www.who.int/immunization/documents/WHO_IVB_2008/en/index.html, WHO/UNICEF immunization coverage estimates, August 2006).

EFFECT OF HBV VACCINATION ON THE REDUCTION OF HBV INFECTION AND COMPLICATION

HBV Vaccination Can Prevent Acute and Chronic HBV Infection

In contrast to HCV vaccine, HBV infection has been prevented by hepatitis B immunization for more than two decades. After the universal vaccination program of HBV, the rate of chronic HBV infection was reduced to one-tenth of that before the vaccination program in the vaccinated infants worldwide. In Taiwan, the chronic infection rate (HBsAg carrier rate) decreased drastically from around 10% before to <1% in Taipei City after the vaccination program in children (Chen et al., 1996; Ni et al., 2001). The seroprevalence rates of HBsAg and hepatitis B core antibody (anti-HBc) in the children of Taiwan 20 years after the installation of the vaccination program were remarkably reduced (Ni et al., 2007). The HBV vaccination program has, indeed, reduced both the perinatal and horizontal transmission of HBV worldwide (Da Villa et al., 1995; Whittle et al., 2002). Right after the universal vaccination in the newborns, a drastic decrease of fulminant hepatitis in infants was noted (Kao et al., 2001).

Effect of HBV Vaccination on HCC Prevention

Since it usually takes 40 years or longer for HCC to develop after HBV infection in most occasions, we may expect to see the reduction of HCC in adults 40 years after the implementation of the universal HBV vaccination program after the year 2024. Studies of the changes of the incidence of HCC in children before and after HBV vaccination in a hyperendemic area may facilitate our understanding of the effect of HCC prevention by HBV immunization, if the HCC in children is similar to the HCC in adults.

The following lines of evidence support the similarity between childhood and adult HCC, and the close relationship between childhood HCC and HBV infection in endemic areas.

1. The histological features of HCC in children are the same as those in adults. As in adults, liver cirrhosis is present in 75%–80% of the children with HCC (Hsu et al., 1987).

2. Nearly 100% of children with HCC were HBsAg seropositive in Taiwan, a hyperendemic area of HBV infection (Hsu et al., 1987; Ni et al., 1991).
3. Very high seropositive rates (94%) of HBsAg in mothers, but not in fathers, of HCC children in Taiwan suggest that maternal transmission of HBV may play an important role in childhood HCC (Chang et al., 1989).
4. The integration of HBV DNA has also been demonstrated in the tumor tissues and the nontumor liver tissues in children with HCC (Chang et al., 1991).

After the implementation of the universal vaccination program of HBV in Taiwan in 1984, we demonstrated the decline of the occurrence of HCC in children. The incidence of HCC is reduced in children covered by the mass HBV immunization in Taiwan. The average annual incidence of HCC in children of 6–14 years declined from 0.52–0.54 per 100,000 children born before 1984 to 0.13–0.20 per 100,000 children born after 1984 (Chang et al., 1997, 2005). Further follow-up studies are anticipated to also reveal a decline of the incidence of HCC in young adults. In contrast, the incidence of hepatoblastoma in children and the incidence of HCC in adults during the same study period were not reduced.

The incidence of HCC in boys born after 1984 was significantly reduced in comparison with those born before 1978, with a relative risk of 0.72 (Chang et al., 2000). However, the reduction in HCC was not clearly demonstrated in girls born during the same period of time. The reasons for this gender difference were unclear and may be due to the limited number of HCC cases in girls. Further studies are being undertaken.

PROBLEMS REMAINING IN HCC PREVENTION

To eradicate HBV infection and its related diseases, we have to overcome the difficulties that hinder the success of universal HBV vaccination.

Low Coverage Rate

Inadequate Resources in Developing Countries. The relatively high price of HBV vaccine in comparison with the traditional EPI vaccines in use is the main cause of low coverage rate in the developing world in the past two decades. The failure to attract national government funding delays the integration of HBV vaccination into the EPI program in some countries. Even with integration into the EPI program, the coverage rate is still inadequate, although the price has fallen substantially in developing countries. One main reason is that the HBV vaccine is not totally free; the parents still have to pay for the HBV vaccines in these countries.

Since 1991, WHO has called for all countries to add hepatitis B vaccine to their national immunization programs. However, many low-income countries do not use the vaccine. In 1992, WHO further recommended that all countries with a high burden of HBV-related diseases should introduce hepatitis B vaccine in their routine infant immunization programs by 1995 and that all countries do so by 1997 (Kane, 1996). In 1996,

an additional target was added, that is, an 80% reduction in the incidence of new hepatitis B carriers among children worldwide by 2001 (Kane and Brooks, 2002). This is particularly urgent in areas where HBV infection and HCC are prevalent. With the integration of the hepatitis B vaccination program into EPI in most countries of the world, chronic HBV infection and its complications will be further controlled in the twenty-first century.

Unfortunately, the above-mentioned targets have not been met. How to reduce the cost of the vaccine and to increase funds for HBV vaccination to help children of endemic areas with poor economic conditions are important issues to solve for the eradication of HBV infection and its related liver cancer. The Global Alliance for Vaccines and Immunization (GAVI) was established in 1999. WHO has taken a leading role in GAVI. The main mission of GAVI is to vaccinate as many children as possible against vaccine-preventable diseases. The GAVI Alliance is a public–private partnership, which includes the GAVI Fund, national governments, the United Nations Children's Fund (UNICEF), WHO, the World Bank, the Bill and Melinda Gates Foundation, the vaccine industry, public health institutions, and nongovernmental organizations (NGOs). GAVI has introduced a new approach to international health funding: the Global Fund for Children's vaccines (GFCV). This fund will help 74 low-income countries to reinforce their national vaccine programs and introduce hepatitis B, yellow fever, and *Haemophilus influenzae* type b vaccines into their national immunization programs (http://www.who.int/mediacentre/factsheets/fs204/en/).

Since 2000, the efforts of the GAVI Alliance have ensured that 90 million children in the world's poorest countries were immunized against hepatitis B. WHO and UNICEF have supported the development and implementation of the country–GAVI project, through the national committees and/or groups.

Anxiety Regarding the Side Effects of Vaccination or Ignorance. In countries with adequate resources, the ignorance of the parents/guardians or an antivaccine mentality drives some of the people to refuse vaccination. Opposition to vaccination may be reduced by clarification of the vaccine-related side effects. For instance, although there is a lack of supporting evidence, the correlation of central nervous system demyelinating diseases and hepatitis B vaccine has been suspected. Clarification of this question may help to eliminate opposition to vaccination, which hampers the effort of HBV vaccination and, hence, the goal of eradication of HBV and its related liver diseases (Halsey et al., 1999). Education and propagation of the benefits of HBV vaccination will enhance the motivation of the public and the governments to accept HBV vaccination, even in low prevalence areas.

Vaccine Failure or Nonresponders

Further investigation of the mechanism of HBV vaccine failure or nonresponders is needed. Intervention to prevent intrauterine infection and development of a better HBV vaccine covering the pre-S components of HBsAg, the surface gene mutants, and for

immunocompromised hosts are possible ways to reduce the rate of vaccine failure or nonresponders.

Hepatitis B Surface Gene Mutants. The rate of HBsAg gene mutants in HBsAg carriers born after the vaccination program is increasing. The rate of HBV surface gene mutation was 7.8%, 17.8%, 28.1%, and 23.8%, respectively, at before, 5 years, 10 years, and 15 years after the launch of the HBV vaccination program (Hsu et al., 1999, 2004). However, as most carriage is prevented by immunization, the total number of HBsAg carriers with the vaccine escape mutants is negligible.

Intrauterine Infection. Although immunoprophylaxis for HBV infection is very successful, still around 2.4% of infants of HBeAg-positive mothers already had detectable HBsAg in the serum at birth or shortly after birth (Tang et al., 1998). They become HBsAg carriers despite complete immunoprophylaxis. Intrauterine HBV infection, though infrequent, is the possible reason. Risk factors of immunoprophylaxis failure include a high level of maternal HBV DNA, uterine contraction and placental leakage during the process of delivery, and low level of maternal anti-HBc (Lin et al., 1991; Chang et al., 1996).

Immunocompromised Host, Hyporesponders, or Nonresponders. Before receiving immunosuppressant or organ transplantation, hepatitis B markers and hepatitis B surface antibody (anti-HBs) need to be monitored routinely. Hepatitis B vaccination should be given to those with inadequate anti-HB levels. A double dose of HBV vaccine can be given to hyporesponders to enhance the vaccine response. Further development of a better vaccine is needed for nonresponders to conventional HBV vaccine.

Hepatitis B Surface Antibody Decay and Booster

A large-scale prospective community-based study enrolling 1200 children with complete HBV immunization received in infancy was conducted until the children were 14 years of age. Eleven children had new HBV infections with anti-HBc seropositivity during follow-up. None of them became positive for HBsAg or HBV DNA by polymerase chain reaction (PCR). The proportion of children with protective anti-HBs gradually decreased from 71% at age 7 years to 37% at age 12 years in 951 children who did not receive booster doses of HBV vaccine during follow-up. Among the 458 children with anti-HBs lower than the protective level at age 7 years, only 1 of the 200 children in the group who received booster doses and 2 of the 258 children in the group who did not developed a new HBV infection with anti-HBc positivity during long-term follow-up (Lin et al., 2003).

Sequential epidemiological studies in Taiwan revealed that universal HBV vaccination provides long-term protection for up to 20 years. In spite of the gradual reduction of anti-HB levels with age, the absence of an increase in HBsAg seropositive subjects at different ages in the same birth cohorts born after the vaccination program implied no increased risk of persistent HBV infection with age. Furthermore, surveillance in the adolescents and teenagers who received HBV vaccination in infancy did not show any increase of acute hepatitis B (Chen, 2005; Kao and Chen, 2005), and

thus, a universal booster is not indicated for the primary HBV vaccinees before adulthood (Chen, 2005; Kao and Chen, 2005; Ni et al., 2007). A booster is recommended for high-risk subjects, such as medical personnel, drug abusers, those who receive frequent blood products, family members, or sexual partners of HBsAg carriers.

IMPLICATION FOR CANCER PREVENTION IN HUMANS

It is most cost-effective to prevent liver cancer through successful control of HBV infection. Continuous monitoring of the reduction in incidence of HCC in children and adults is mandatory. A decline in the incidence of HCC in young adults and then all adults is anticipated in the near future. Since the peak age of HCC in adults is around 40–60 years, theoretically, we may expect a reduction of HCC in adults 40 years after the universal vaccination programs. However, based on the data on children, which demonstrated a herd immunity in older children born before the implementation of the universal vaccination program, the reduction of HCC in adults may come earlier than we expected.

Furthermore, the impact of HBV vaccination on the control of hepatitis B and its related diseases can be extrapolated to other infectious agent-related cancers, such as human papillomavirus and cervical cancer, Epstein–Barr virus (EBV), and endemic Burkitt's lymphoma, as well as nasopharyngeal carcinoma, human T-cell virus-type 1 (HTLV-1) and adult T-cell leukemia/lymphoma (ATL), and *Helicobacter pylori* and mucosa-associated lymphoid tissue lymphoma (MALTOMA) or gastric cancer.

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12

PATHOGENESIS OF ACUTE AND CHRONIC HEPATITIS C VIRUS INFECTION

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INTRODUCTION

Hepatitis C virus (HCV) is a hepatotropic virus that infects an estimated 200 million people worldwide. Roughly 170 million of these acute exposures persist and become chronic infections (Shepard et al., 2005). Over the past decades, there have been two major HCV epidemics: one in the 1960s, which was associated with the transmission of genotype 1 through medical procedures, and the second in the 1980s, which was associated with needle sharing among drug addicts (predominantly genotype 3) (Ebeling, 1998). Although the pathogenesis of chronic HCV is often asymptomatic for 15–20 years, underlying liver disease develops in the majority of infected patients (Racanelli and Rehermann, 2003), although only a minority of chronically infected patients develop severe liver disease (Rodger et al., 2000; Wiese et al., 2000). Among the features that characterize chronic HCV-associated liver disease are steatosis, diabetes mellitus, chronic hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). While the

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pathogenesis of chronic HCV infection is immune mediated, as shown by the development of a transient hepatitis in transgenic mice conditionally expressing HCV E1, E2, and core (Wakita et al., 1998), there is also considerable evidence suggesting the contribution of virus gene products to this process. The natural history of HCV infection is variable and dependent upon the interaction of various host (e.g., age, gender, human leukocyte antigen [HLA], transforming growth factor-beta 1 [TGF-β1] gene polymorphisms, tumor necrosis factor-alpha [TNF-α] production, body mass index [BMI]) and viral factors (e.g., HCV genotype, virus load, quasispecies diversity). This chapter will attempt to correlate various aspects in the pathogenesis of chronic infection with putative underlying mechanisms that provide novel diagnostic and prognostic markers, as well as therapeutic targets for understanding and treating chronic hepatitis C.

PATHOGENESIS OF ACUTE HCV AND PROGRESSION TO CHRONICITY

Natural History of Acute HCV Infection

In about 10%–15% of acutely exposed patients, HCV infection is resolved. Given that HCV has been virtually eliminated from the blood supply by detection of anti-HCV (Alter and Bradley, 1995), the major routes of transmission include intravenous and intranasal drug abuse. Clinical diagnosis of acute HCV infection is imprecise but consists of frequently high titer HCV RNA within 1 week of infection, followed by an acute increase in the levels of alanine aminotransferase (ALT) to more than 10 times the upper limit of normal in individuals with a history of likely exposure to HCV within the preceding 2–12 weeks (Thimme et al., 2001; Chung, 2005) (Figs. 12.1a and 12.2). Seroconversion to anti-HCV usually occurs during acute infection. Significantly, HCV RNA titers peak in the blood and are already falling sharply by the time elevated ALT, anti-HCV, and HCV-specific T cells appear (usually 1 month after the appearance of HCV RNA in the blood), suggesting that the virus "outpaces" the adaptive immune response.

RISK FACTORS IN ACUTE INFECTION ASSOCIATED WITH THE DEVELOPMENT OF CHRONICITY

Experimental infection of susceptible chimpanzees with HCV has shown that acute HCV infection triggers the intrahepatic production of interferon-alpha (IFN-α) and -beta (IFN-β), the latter of which stimulates the production of chemokines that attract mononuclear inflammatory cells to the liver (Chung, 2005), which in turn trigger hepatocellular death by apoptosis (Racanelli and Rehermann, 2003; Schinoni et al., 2006). Importantly, the process of apoptosis is a form of programmed cell death that normally does not result in the release of intracellular contents to the blood, since cell remnants remain enclosed within cell membranes that are engulfed by macrophages within the liver. When this occurs during acute infection, ALT values often remain normal or are mildly elevated, amplification of intrahepatic inflammatory cells is limited, and clinical disease never develops. Under these circumstances, the virus and liver disease often persist, and there is a high probability for the development of chronic infection

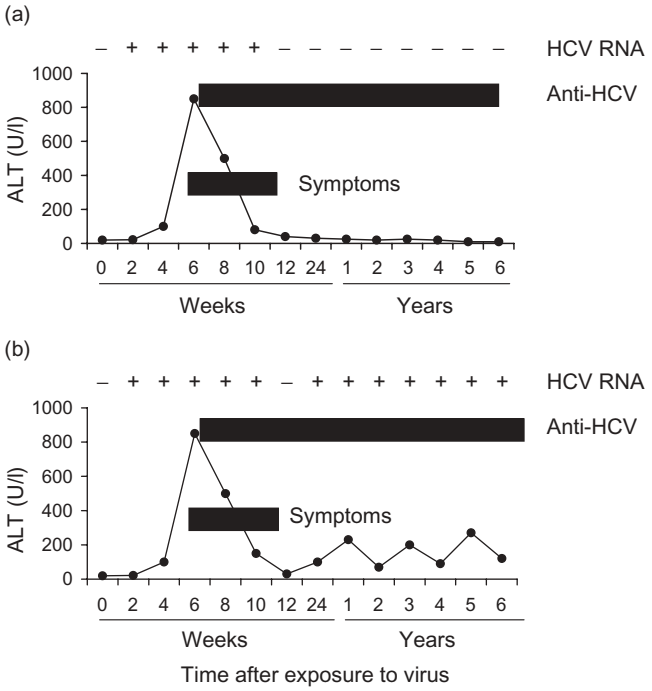

Figure 12.1. Outline of natural history of acute (a) and chronic (b) hepatitis C infections (modified from Hoofnagle, 1997).

(Figs. 12.1b and 12.2). However, when an acute infection is associated with vigorous HCV-specific $CD4^+$ and $CD8^+$ T-cell responses to multiple HCV antigens, acute hepatitis is resolved (Gerlach et al., 1999) (Fig. 12.2). In this context, when experimentally infected chimpanzees were treated with antibody to deplete $CD4^+$ T cells *in vivo*, rechallenge was associated with the establishment of chronic infection, underscoring the importance of $CD4^+$ T cells to the clearance of acute infection (Grakoui et al., 2003). The fact that acute HCV infection becomes chronic and progressive among immunosuppressed patients with human immunodeficiency virus coinfection (Ockenga et al., 1997), with immunoglobulin deficiencies (Bjoro et al., 1994), undergoing cancer chemotherapy (Castellino et al., 2004), and in an organ transplant setting (Somsouk et al., 2003) is also consistent with a major role of T-cell-mediated immunity in the outcome of acute HCV infection. Despite the fact that liver disease progresses more rapidly in the immunosuppressed setting, HCV is not directly cytopathic, since there is no correlation between HCV RNA titer in serum and the severity of liver disease (Pawlotsky, 2004).

In addition to immune status, the spontaneous resolution of acute HCV infection is more often seen among patients under 40 years old, among female patients, and, as mentioned above, among those with an asymptomatic acute phase infection (Gerlach et al., 2003). In addition, there is some evidence that individual homozygous for a polymorphism in the inhibitory natural killer (NK) cell receptor KIR2DL3 and its

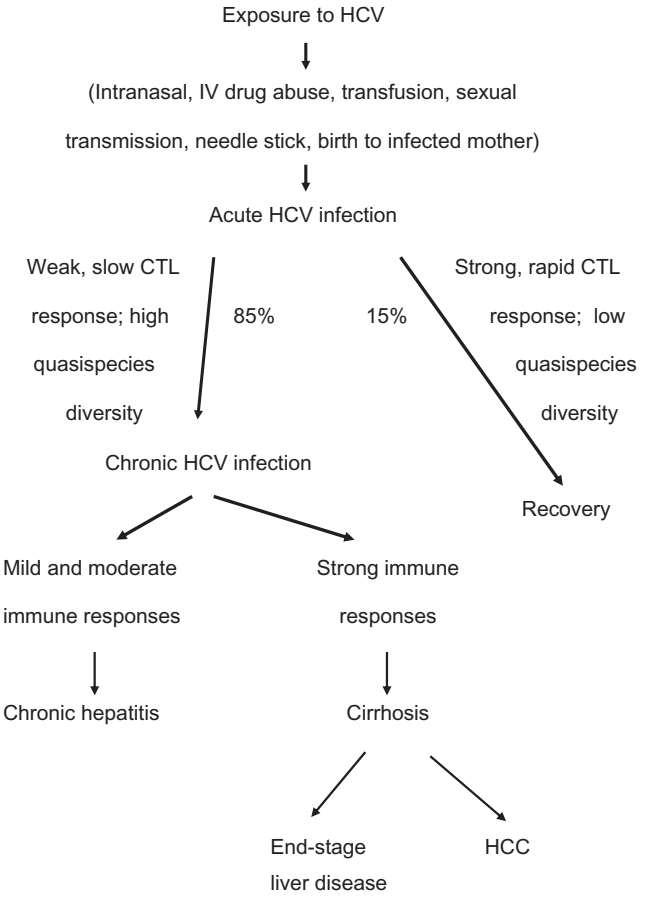

Figure 12.2. Hepatitis C: spectrum of disease and kinetics of immune response (modified from Hoofnagle, 1997).

HLA-C1 ligand are more likely to resolve acute infection, suggesting that immunogenetics may also be important in the host–virus relationship that develops (Khakoo et al., 2004). Further, there is a correlation between the genetic diversity of HCV in a given infection and the outcome of that infection. HCV encodes an RNA-dependent RNA polymerase (RdRp) that is responsible for virus replication but lacks proofreading capabilities. This results in the development of a lot of genetic microheterogeneity in the genome of the virus during infections, referred to as "quasispecies" (Holland et al., 1992). Acute infections are often characterized by infections with limited quasispecies diversity (and few amino acid substitutions within T- and B-cell epitopes of the virus), while infections that become chronic are associated with broad quasispecies diversity. Apparently, this diversity tends to increase the longer a person is infected, which suggests that strong and rapid immune responses to an infection with limited genetic diversity will favor resolution, while infections characterized by broad virus genetic

diversity are much more challenging to overcome (Farci et al., 2000). The ability of virus to "outpace" the development of adaptive immune responses, as mentioned above, may be due, in part, to the rapid generation of quasispecies, which remain infectious in the presence of neutralizing immunity against quasispecies that were dominant at the earlier stages of infection (Figs. 12.1b and 12.2).

PATHOGENESIS OF CHRONIC INFECTIONS: RISK FACTORS AND RAMIFICATIONS

Natural History of Chronic HCV Infection

Roughly 85% of individuals who are acutely infected with HCV develop chronic infections (Fig. 12.2). Clinical symptoms, such as jaundice, which correlate with T-cell-mediated liver injury, are sometimes observed in acute HCV infections but are commonly associated with the development of chronic infections. The latter is characterized by the persistence of HCV RNA in the blood, usually at titers lower than at the peak of acute infection and, in some patients, with persistently elevated ALT (Alter et al., 1989; Farci et al., 1991). The amplitude, specificity, and timing of the innate and adaptive antiviral immune responses are likely to play a major role in the outcome of infection, with slow, narrow (to only a few virus epitopes), and weak immune responses, permitting the virus to establish a chronic infection without being eliminated (Fig. 12.2). Chronic liver disease (CLD) often progresses and becomes more severe with the passage of time, although most patients remain asymptomatic for 10–30 years, even though they have persistently elevated ALT levels and moderate or severe CLD (Hoofnagle, 1997; Sharara, 1997). This is why chronic HCV is referred to as the "silent epidemic" (Lee, 1993). Interestingly, up to one-third of chronically infected patients with persistent viremia have consistently normal ALT levels, while ALT levels in others are intermittently elevated (Barrera et al., 1995). The apparent discordance between ALT levels and liver injury may be related to the proportion of liver cell damage and death due to apoptosis (which limits the inflammatory response) and necrosis (which promotes the inflammatory response). Among chronically infected patients with elevated ALT who become symptomatic, up to 35% develop cirrhosis by year 20 after infection, while almost a quarter of chronically infected patients develop HCC by year 30 (Kiyosawa et al., 1990; Sharara, 1997). Cirrhosis develops in 10%–20% of chronically infected patients over a period of 20–30 years, while HCC appears in 1%–5% of patients with cirrhosis per year (Di Bisceglie et al., 1991; Seeff et al., 1992) (Fig. 12.2).

Factors that Contribute to CLD

Factors that predict the severity of CLD have been established (Table 12.1). For example, when infection occurs in men over the age of 40, the development and progression of fibrosis is usually much faster than among patients infected under the age of 40 (Poynard et al., 1997). In addition, the age of infection determines the lag period between the time

TABLE 12.1. Putative Factors that Contribute to the Progression of CLD and Development of HCC

Male gender
Age of infection >40 years
Degree of fibrosis
Chronic alcoholism
Hyperinsulinemia/insulin resistance
Steatosis (fatty liver)
Non-insulin-dependent diabetes mellitus (NIDDM)
Infection with genotype 3
Body mass index (BMI)/obesity
Oxidative stress (elevated CYP2E1)
Stellate cell activation
Elevated TNF-α, TGF-β1 and β-catenin
Telomere shortening

of infection and the appearance of cirrhosis. The progression of CLD is also related to the underlying degree of fibrosis, with slow progression in patients with little fibrosis and rapid progression among people with advanced cirrhosis (Poynard et al., 1997). In other words, fibrosis is a risk factor for fibrosis progression. While the underlying mechanisms to explain this are not clear, they are probably associated with age-related changes in the liver. For example, recovery from a bout of CLD is often associated with liver regeneration. Hepatocytes, like other cell types, are capable of replicating a finite number of times before they undergo replicative senescence. With each round of regeneration, sequences at the ends of the chromosomes of replicating cells, known as telomeres, do not fully replicate. Over time, these telomeres become so short that the cell no longer undergoes further rounds of regeneration, thereby entering replicative senescence. The net effect is that regeneration slows with age. Intrahepatic inflammatory cells and cytokines also activate hepatic stellate cells, which then synthesize and secrete collagens and other extracellular matrix proteins that promote fibrogenesis (Tillmann et al., 2005). Independently, in the setting of liver transplantation for HCV, the age of the donor liver was found to significantly correlate with patient morbidity and mortality (Berenguer et al., 2002; Velidedeoglu et al., 2004), again suggesting that reinfection of the graft and the development and progression of CLD are age dependent.

Alcoholism in Chronic HCV Infection

There is now a strong evidence that even moderate alcohol intake (>10 g/day) promotes the progression of fibrosis and the development of HCC (Koff and Dienstag, 1995; Hezode et al., 2003), in part, by stimulating HCV replication (Sawada et al., 1993; Oshita et al., 1994) (Table 12.1). In addition, chronic alcoholism, in the setting of liver steatosis (fatty liver), promotes the development of fibrosis (Serfaty et al., 2002). In fact, alcohol exacerbates CLD so much that the National Institutes of Health (NIH)

consensus statement strongly discourages alcohol consumption among any chronically infected patients (National Institutes of Health, 1997). The underlying mechanisms for this are not clear, but it is possible that the environment created by ethanol oxidation results in altered signaling pathways that promote HCV replication. If so, then inhibitors that attenuate the effects of ethanol on the liver may also demonstrate an antiviral effect as well.

Steatosis, Fibrosis, and Diabetes

There is an increasing evidence that a disturbance in lipid and insulin metabolism might also contribute importantly to the development of fibrosis during chronic HCV infection. Hepatic steatosis is observed in 30%–70% of patients with chronic hepatitis C, which is two- to threefold higher than that found in other forms of CLD (Goodman and Ishak, 1995; Hwang et al., 2001; Lonardo et al., 2006). Steatosis is strongly associated with an increased severity of fibrosis in chronic HCV infection (Hourigan et al., 1999; Hwang et al., 2001; Hu et al., 2004), with higher grades of steatosis associated with higher rates of fibrosis progression (Adinolfi et al., 2001; Lonardo et al., 2006) (Table 12.1). In clinical samples, HCV core protein expression has been linked to steatosis (Fujie et al., 1999), suggesting that HCV directly contributes to these lesions. Further analysis showed that moderate-to-severe steatosis is often associated with infection by HCV genotype 3 (Hezode et al., 2004) (Table 12.1), that steatosis is more prevalent in genotype 3 (74%) compared with nongenotype 3 infections (48%) (Lonardo et al., 2006), and that steatosis resolves only among genotype 3 patients who have a sustained viral response following antiviral treatment (Kumar et al., 2002; Lonardo et al., 2006). The latter implies that genotype 3 polyprotein may trigger cytopathic effects, perhaps by blocking lipoprotein secretion that result in the development of steatosis (Rubbia-Brandt et al., 2000; Serfaty et al., 2001; Kumar et al., 2002), but this has not been directly demonstrated. Among nongenotype 3-infected patients, steatosis is associated with metabolic factors, such as BMI and obesity (Table 12.1), and as expected, a sustained antiviral response does not impact upon the severity of preexisting steatosis (Kumar et al., 2002; Poynard et al., 2003). Although the underlying mechanisms responsible for the development of steatosis and those connecting steatosis to fibrosis are poorly understood, steatosis is partially characterized by an increase in the proinflammatory cytokine, TNF-α, with increased production of collagen 1, and with the activation of cytochrome p450 2E1 (CYP2E1) (Gochee et al., 2003) (Table 12.1). Given that CYP2E1 activation is associated with oxidative stress (which results in lipid peroxidation) (Lonardo et al., 2004), that HCV infection triggers oxidative stress (Gong et al., 2001; Moriya et al., 2001), and that CYP2E1 also metabolizes ethanol (Salmela et al., 1998), perhaps provides a common denominator whereby ethanol or HCV promotes the development and progression of fibrosis (Patel et al., 2005) (Table 12.1). This mechanism might also help to explain, at least in part, how chronically infected alcoholics develop a rapidly progressive CLD. In addition, CYP2E1 is one of the targets of activated β-catenin (Loeppen et al., 2005), the latter of which is also up-regulated by HCV core and NS5A (Levrero, 2006). The combined action of elevated CYP2E1 and TGF-β1 results in increased oxidative stress, followed

by liver cell damage and death (Zhuge and Cederbaum, 2006), which would further exacerbate the intensity of CLD (Table 12.1). Finally, the transcriptional cooperation between TGF-β1 and β-catenin (Wnt) pathways has been shown to promote tumorigenesis in several tumor types (Labbe et al., 2007), and since both of these signaling pathways are activated in chronically infected livers, these may also promote the development of HCC.

Disturbances in lipid metabolism may also have an impact upon virus replication, since HCV replication is dependent upon cell membrane constituents for assembly and secretion of the HCV replication complex (Patel et al., 2005). For example, in HCV core transgenic mice that develop steatosis, there is impairment in very low-density lipoprotein assembly and secretion (Perlemuter et al., 2002). In an infected chimpanzee, up-regulated serum response element-binding protein (SREBP) signaling turns on in genes responsible for fatty acid and cholesterol metabolism, while in cultured cells supporting HCV replication (Table 12.2), activation in SREBP signaling is associated with a modest increase in virus replication (Su et al., 2002). Clinical studies have shown an inverse relationship between serum cholesterol levels and liver steatosis in HCV infection, which is corrected only among patients with a sustained virological response to treatment both in genotype 3 and nongenotype 3 (Poynard et al., 2003). Depletion of membrane sterols with lovastatin markedly suppresses HCV RNA levels in replicon cell lines, suggesting an important clinical association between lipid metabolism and HCV replication (Serfaty et al., 2001).

TABLE 12.2. Putative Factors that Contribute to the Persistence of HCV

In the liver:
HCV core inhibits intrahepatic IL-12, IL-2, and IFN-γ production
Resistance to type 1 IFNs (NS3/4A cleaves MAVS)
NS3/4A may block activities of IFN regulatory factors 3 and 7
NS5A inhibition of PKR
Inhibition of innate immunity to HCV
Impaired or late onset of T-cell effector functions
Intrahepatic toleragenic environment
Elimination of effector T cells by Fas killing
Alterations in fatty acid and cholesterol metabolism
Altered sensitivity of infected hepatocytes to immune-mediated apoptosis
In the blood:
Delayed trafficking of immune cells to liver
Virus quasispecies diversity (antibody and T-cell escape mutants)
Variable and low titer antibody production
Restricted (narrow) antibody specificities
Lymph nodes:
Generation of CD25 regulatory T cells
Reduced priming or proliferation of T cells
Reduced B-cell activation

Adapted from Rehermann and Nascimbeni, 2005.

The appearance of insulin resistance early on during chronic HCV infection is also associated with the development of fibrosis (Hui et al., 2003; Muzzi et al., 2005). High levels of blood glucose trigger hyperinsulinemia, which stimulates stellate cell proliferation, increased secretion of extracellular matrix proteins, and up-regulation of other factors, such as TGF-β1, that strongly promote fibrogenesis (Paradis et al., 2001; Levrero, 2006) (Table 12.1). In addition, insulin resistance contributes importantly to the development of steatosis by increasing lypolysis and fatty acid delivery to the liver (Kahn and Flier, 2000), resulting in the appearance of steatosis and steatohepatitis. Interestingly, HCV core overexpressing transgenic mice develop insulin resistance, then steatosis, and eventually HCC, suggesting a tight relationship between chronic HCV infection and the appearance of these lesions in the liver (Moriya et al., 1997, 1998; Shintani et al., 2004). Independent observations have shown the appearance of steatosis and eventually HCC in transgenic mice overexpressing the HCV polyprotein (Lerat et al., 2002). Moreover, in cells replicating HCV, there is colocalization between HCV core and HCV NS5A proteins with intracytoplasmic triglyceride-rich lipid droplets, suggesting that these HCV-encoded proteins are associated with altered lipid metabolism (Barba et al., 1997; Shi et al., 2002) (Table 12.2).

Non-insulin-dependent diabetes mellitus (NIDDM) has been documented in up to 30% of patients with chronic HCV infection, especially in those 40 years or older (Mehta et al., 2000; Ratziu et al., 2004). This is a much higher prevalence than NIDDM in patients with other CLDs and in the general population. HCV infection significantly increases the risk for developing NIDDM. Given that NIDDM and chronic HCV infection are linked to steatosis, the latter may be a mediator of insulin resistance. Together with steatosis, NIDDM and chronic HCV infection have been shown to be independent risk factors for the development of HCC (Ohata et al., 2003; El-Serag et al., 2004), suggesting that alterations in glucose metabolism and insulin signaling play important roles in hepatocarcinogenesis. Although little is known about the mechanisms that underlie these interactions, HCV core may promote cellular damage via prolonged oxidative stress and/or the binding of HCV core to the retinoid x receptor α (RXRα), the latter of which is a transcriptional regulator that controls aspects of cell growth and differentiation and regulates lipid metabolism (Zhu and Chung, 2003).

Apoptosis

Part of the mechanism, whereby steatosis causes fibrosis, may be through the induction of apoptosis (Walsh et al., 2004) resulting from increased oxidative stress and hepatic stellate cell activation (Clouston et al., 2001) (Table 12.1). In tissue culture experiments, however, HCV core protein bound to tumor necrosis factor receptor (TNFR1) potentiated TNF-α signaling, which either promoted or inhibited apoptosis (Ray et al., 1996; Zhu et al., 1998; Bantel et al., 2001) under different experimental conditions. HCV core also bound to other members of the TNF family of receptors, including the lymphotoxin β receptor (LTβR; Chen et al., 1997; Matsumoto et al., 1997) and Fas (Lai and Ware, 2000). The latter promoted or inhibited Fas-mediated apoptosis in cell culture (Marusawa et al., 1999). The effect of the core may depend upon which TNF family member it is binding to and the state (growing or quiescent) of the infected cell at the time of

signaling (Lai and Ware, 2000). However, it is not clear whether these events are happening in natural infections. Among patients with chronic HCV, there is an up-regulation in Fas expression on hepatocytes, especially in periportal regions near areas of piecemeal necrosis (Jin et al., 1997). The latter may promote the survival of virus-infected hepatocytes by trigger apoptosis in inflammatory T cells (Table 12.2). However, apoptotic bodies are also found in the livers of patients with viral hepatitis (Yoon and Gores, 2002), demonstrating that apoptosis is occurring *in vivo*. Perhaps conditions, in addition to the presence of HCV, determine the fate of infected hepatocytes *in vivo.* One of these "conditions" may involve the extent to which hepatic stellate cells are activated, not only because they synthesize and secrete extracellular matrix components, but also because they produce the profibrogenic cytokine, TGF-β1 (Fadok et al., 2001).

The ability of HCV to modulate the sensitivity of immune-mediated apoptosis of virus-infected hepatocytes is also likely to contribute importantly to the persistence of chronic infection. For example, when transgenic mice, expressing the HCV nonstructural proteins, are infected with recombinant adenovirus expressing β-galactosidase, adenovirus-infected hepatocytes (β-galactosidase positive cells) are resistant to Fas-mediated elimination, despite an apparently normal T-cell response (Disson et al., 2004). HCV core may play a major role in modulating apoptosis, which on one hand may promote the survival of infected cells (which eventually become neoplastic) and on the other, promote the apoptosis of infected cells, thereby releasing virus progeny and accelerating the development and progression of CLD (Table 12.2).

During CLD, apoptotic bodies taken up by Kupffer cells trigger activation and secretion of proinflammatory cytokines that activate stellate cells (Promrat et al., 2003). Stellate cells are also activated by uptake of apoptotic bodies, by oxidative stress, and by reactive oxygen intermediates (ROIs) (Akuta et al., 2001; Promrat et al., 2003). Activated Kupffer cells, stellate cells, and endothelial cells produce considerable amounts of TGF-β1, which stimulates the development of fibrosis. The finding that the grade of necroinflammation is a predictive factor for fibrogenesis in CLD (Poynard et al., 1997) is consistent with this mechanism being operative *in vivo*. The long-term production of proinflammatory cytokines and ROI is also associated with telomere shortening independent of hepatocyte turnover. Importantly, cirrhotic liver is characterized by the presence of shorter telomeres (Harley et al., 1990) and by the activation of senescence signaling pathways (Dimri and Campisi, 1994). As noted above, these features promote fibrogenesis in place of hepatocellular regeneration, thereby accelerating the progression of CLD (Table 12.1). Further, the enzyme telomerase is central to telomere reproduction. When telomerase-deficient mice were subjected to acute or chronic liver damage, they developed premature steatosis and fibrosis associated with elevated TGF-β1 production and stellate cell activation (Livni et al., 1995; Wiemann et al., 2002). Moreover, their ability to undergo liver regeneration was attenuated (Livni et al., 1995; Albrecht et al., 1997), which was characterized by both slower cell cycle progression and elevated levels of hepatocellular apoptosis, strongly suggesting that telomere integrity within hepatocytes is an important element in the response of the liver to the types of liver cell damage in chronic hepatitis C. In this context, telomere dysfunction induces cytochrome p450 activity, which in turn is important for the generation of ROI, the latter of which is involved in stellate cell activation (Kaita

et al., 1997; Powell et al., 2000). This would seemingly establish an inverse relationship between hepatocellular proliferation and stellate cell activation in the development of cirrhosis in the chronically infected liver.

ADAPTIVE IMMUNITY AND IMMUNOLOGIC ESCAPE MECHANISMS USED BY HCV

The timing and strength of innate immune responses against HCV contribute importantly to the adaptive immune responses that follow. Acute, resolving HCV, vigorous, multi-epitope $CD4^+$ and $CD8^+$ T-cell responses are readily detectable in blood, while in patients developing chronic hepatitis C, adaptive T-cell responses are late, transient, and/or narrow focused (Diepolder et al., 1995; Thimme et al., 2001; Wedemeyer et al., 2002) (Table 12.2). The centrality of the T-cell response to HCV infection was proven in infected chimpanzees, where transient depletion of either $CD4^+$ or $CD8^+$ T cells prevented HCV clearance and clinical recovery (Grakoui et al., 2003; Shoukry et al., 2003). There is also a correlation between the appearance of T-cell responses, the induction of IFN-γ expression, and decreased levels of HCV RNA in the blood (Su et al., 2002; Thimme et al., 2002), although it is not known whether this correlation reflects a cause and effect relationship. In addition, although there is an early onset of vigorous HCV infection following acute exposure, HCV-specific T cells appear in the blood 5–9 weeks later, and in the liver, some 6–12 months after infection (Thimme et al. 2001, 2002). The underlying reasons for this are unknown, but one contributing factor may be the fact that from an immunologic viewpoint, the liver is largely toleragenic, so that even immune responses arising from peripheral sites (e.g., extrahepatic lymph nodes) may not be able to carry out their effector functions effectively in the liver (Table 12.2). Even within the liver, which is rich in Kupffer cells and T cells, priming and/or effector functions may be attenuated. Given that the liver is exposed to the partial breakdown products of a multitude of foreign food-based antigens throughout life, and that most of these antigens do not trigger corresponding immune responses, suggests that the intrahepatic toleragenic environment is physiologically important but may also partially "protect" hepatotropic viruses from clearance. Alternatively, or in addition, HCV may simply not trigger the correct types or magnitude of immune responses needed for virus clearance. Hence, T-cell responses are only partially effective against virus-infected hepatocytes (Table 12.2).

Humoral immune responses against HCV are variable in that among patients with acute, resolving infection, anti-HCV titers often decrease and become undetectable over 10–20 years, while in chronically infected patients, antibody titers are often high and sustained (Takaki et al., 2000; Bartosch et al., 2003). Anti-HCV is also restricted in their isotype profile and is usually present at low titers compared with antibodies against other virus infections (Chen et al., 1999). However, antibodies against the envelope glycoproteins of HCV (E1 and E2) to a single HCV isolate neutralize infectivity of that same isolate in chimpanzees (Farci et al., 1994). In addition, envelope antibodies modulate HCV RNA levels in vaccinated and rechallenged chimpanzees (Forns et al., 2000), suggesting that they are partially neutralizing and can modify the outcome of

infection. Partial neutralization is due to the presence of infectious HCV quasispecies that escape neutralization (Table 12.2), which is one of the reasons why it will be so difficult to develop a prophylactic vaccine for HCV.

Unlike anti-HCV, virus-specific T-cell responses remain detectable in both patients and chimpanzees recovering from acute infection (Takaki et al., 2000; Shoukry et al., 2003). This may indicate that HCV is controlled but not completely eliminated after the resolution of acute infection. This, however, does not seem to confer lasting immunity, since chimpanzees that had previously recovered from HCV infection could be reinfected with homologous or heterologous batches of virus (Farci et al., 1992), although these animals usually develop an attenuated course of infection characterized by low virus titer and no liver disease (Shimizu et al., 1994). In both human and chimpanzee infections, there is evidence for the development of HCV escape mutants selected by antibodies (Shimizu et al., 1994) and by T cells (Chang et al., 1997) (Table 12.2). T-cell escape mutants affect epitope processing, binding of an, otherwise, immunogenic virus peptide to the major histocompatibility complex (MHC), and the ability of HCV antigens to stimulate the T-cell receptor (Chang et al., 1997; Frasca et al., 1999; Timm et al., 2004). There is also evidence that HCV core antigen binding to the C1q receptor (for complement) inhibits interleukin (IL)-12 production from receptor-bearing macrophages (Eisen-Vandervelde et al., 2004) and blocks both IL-2 and IFN-γ production by T cells, thereby attenuating T-cell proliferation (Kittelsen et al., 2000) (Table 12.2). Decreased IL-2 production results in incomplete maturation and differentiation of T cells (Francavilla et al., 2004). HCV may also impair dendritic cell function (Auffemann-Gretzinger et al., 2001). Further, there is some evidence that genetic polymorphisms in the gene promoters of chemokine receptor genes (Hellier et al., 2003) might partially modulate HCV immune responses, which may result in the production of IL-10-producing CD8 T cells in chronically infected liver (Accapezzato et al., 2004). Recent evidence has also demonstrated the presence of $CD4^+CD25^+$ regulatory T cells in the blood of chronically infected patients (Cabrera et al., 2004), suggesting that HCV may trigger T-suppressor cells during chronic infection (Table 12.2). Hence, the host–virus relationship that develops promotes the persistence of a chronic infection that does not resolve, although the relative importance of these various mechanisms, singly or in combination, at different times during chronic infection, remains to be elucidated.

MECHANISMS OF HCV RESISTANCE TO IMMUNE-MEDIATED CLEARANCE

Microarray analysis of serial liver biopsies from chimpanzees acutely infected by HCV showed strong up-regulated expression of genes involved in the type 1 IFN response within the first week of infection (Bigger et al., 2001; Su et al., 2002). However, HCV seems to be resistant to this IFN response *in vivo* (Table 12.2), even though HCV replicons are sensitive to IFNs *in vitro* (Frese et al., 2001). This resistance may explain

the high percentage of acute HCV infections that become chronic. This could occur by several putative mechanisms. First, the HCV serine protease (NS3-NS4A) may block signaling through the interferon regulatory factors 3 and 7 (IRF3/7) (Table 12.2), as demonstrated in cells replicating an HCV replicon (Foy et al., 2003). Second, HCV NS5A and the unglycosylated form of envelope protein 2 (E2) may inhibit the double-stranded RNA-induced protein kinase (PKR) (Table 12.2) by direct binding, which has also been shown to occur in replicon-bearing cells (Gale et al., 1997; Taylor et al., 1999). PKR would otherwise phosphorylate the eukaryotic translation initiation factor 2α (eIF2α), thereby inhibiting translation in virus-infected cells. In addition, PKR is a tumor suppressor, and its inactivation by NS5A may contribute importantly to the development of HCC, although this is yet to be shown. NS5A also blocks p53-mediated apoptosis by cytoplasmic sequestration (where p53 is not active), by modulation of intracellular Ca^{+2}, by binding to growth factor receptor bound protein 2 (Grb2) in signaling pathways, and by inducing the anti-inflammatory cytokine, interleukin-8 (IL-8) (Tan et al., 1999; Gong et al., 2001; Majumder et al., 2001; Polyak et al., 2001), all of which may partially block innate immunity to HCV (Table 12.2). Interestingly, sequence variation within the PKR-binding region of NS5A (known as the interferon sensitivity determining region or ISDR) differs among HCV subtypes, and these differences alter binding to PKR and corresponding IFN sensitivity (Simmonds, 2004). Third, HCV proteins may interfere with NK cell activation early after HCV infection, which is known to be associated with the clearance of HCV and clinical recovery (Khakoo et al., 2004). Although high concentrations of HCV E2 bind to and cross-link the HCV receptor CD81 on the surface of NK cells *in vitro*, blocking NK-mediated cytotoxicity and cytokine production, as well as blocking their ability to activate dendritic cells, it is not clear whether this occurs among infected people (Jinushi et al., 2004). Fourth, signal transducer and activator of transcription 1 (STAT1) signaling, which is how IFN mediates its antiviral and antiproliferative effects, has been found to be impaired in liver biopsies from patients with chronic HCV infection, resulting in reduced transcriptional activation of IFN-α-stimulated genes (Duong et al., 2004) (Table 12.2). This is accompanied by the up-regulated expression of protein phosphatase-2A (Duong et al., 2004). At this time, it is not known which of these pathways, singly or in combination, accurately reflect HCV resistance to immune-mediated clearance, nor is it known which of these mechanisms are rate limiting at different points in the pathogenesis of chronic infection.

HCV AND MITOCHONDRIA

Independent work has shown that mitochondria also play important roles in innate immunity (Piccoli et al., 2006). Recently, a protein referred to as mitochondrial antiviral signaling (MAVS), which is associated with the outer membrane of the mitochondria, stimulates the activity of the signaling molecules IRF3/7 and NF-κB, which in turn activate transcription from the IFN-β gene enhancer (Seth et al., 2005; Piccoli et al., 2006). Double-stranded HCV RNA, which is generated during virus replication, binds

to a complex of proteins, which include MAVS, and this triggers an innate IFN response. The HCV NS3/4A protease, however, inactivates MAVS by proteolytic cleavage, thereby providing resistance to the innate immunity provided by IFN-β (Li et al., 2005) (Table 12.2).

The effects of HCV infection upon mitochondria are likely to go beyond that of blocking innate immunity. Many labs have shown that HCV proteins partially colocalize with mitochondria (Li et al., 2005; Suzuki et al., 2005) and with the endoplasmic reticulum (ER) (Mottola et al., 2002). It is hypothesized that the accumulation of HCV proteins in the ER membranes causes a release of calcium (Brookes et al., 2004; Rizzuto and Pozzan, 2006) that may be taken up by nearby mitochondria, thereby precipitating a drop-off in mitochondrial respiratory rate and depolarization in mitochondrial membrane potential (Deryabina et al., 2004; Korenaga et al., 2005). Levels of nitric oxide synthase (NOS) and nitric oxide (NO) then accumulate within the mitochondria, which exhaust their antioxidant buffering capacity, triggering mitochondrial damage, cytochrome c release, and then apoptosis (Alderton et al., 2001; Ghafourifar and Cadenas, 2005). This outcome would favor the release of virus progeny and infection of neighboring liver cells. If apoptosis is not triggered and elevated NO favors cell growth (along with other signaling pathways activated by HCV proteins), the net effect may contribute to the development of HCC.

CONCLUSIONS

HCV is highly successful as a pathogen that establishes and maintains chronic infections that do not resolve. It does this by blocking pathways of innate immunity, thereby preventing the timely development of adaptive immune responses that would otherwise be needed to clear the virus. Once adaptive immune responses are established, they are not robust, long lasting, or sterilizing. This may partially be associated with the development of T-cell and B-cell escape variants that result from immunologic selection. HCV may also alter the susceptibility of hepatocytes to apoptosis, leading to intrahepatic Kupffer and stellate cell activation, with the development of steatosis and then fibrosis. There is also accumulating evidence that the HCV alterations in lipid metabolism contributes importantly to the development of diabetes in many chronically infected patients. Although it is clear that multiple mechanisms of HCV persistence exist, their relative contributions to the pathogenesis of chronic infection probably differ at different points during pathogenesis, and more work needs to be done to understand this better. However, once the molecular mechanisms underlying the pathological lesions are elucidated, they will provide opportunities to develop more specific therapies that target both the virus functions and host pathways that are operative in the pathogenesis of chronic hepatitis C.

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13

MOLECULAR MECHANISMS OF HEPATITIS C VIRUS-INDUCED CELLULAR TRANSFORMATION

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BACKGROUND

Hepatitis C virus (HCV) is a major health problem. There are approximately 170 million people worldwide that are chronically infected by HCV, with an estimated 3–4 million new HCV infections very year. Most patients infected by HCV fail to clear viral infection, and many of these chronic HCV patients will develop blood disorders such as mixed cryoglobulinemia, B-cell lymphoma (BCL), and severe liver diseases such as liver cirrhosis and hepatocellular carcinoma (HCC). Many of HCV patients will require liver transplantation for survival. There is no vaccine available for HCV. The current therapy for HCV patients uses interferon-α and the nucleoside analogue ribavirin. These two drugs have severe side effects, and their combined usage generates sustained response in roughly only 50% of the HCV patients.

HCV is a positive, single-stranded RNA virus that belongs to the Flaviviridae family. This virus has a high mutation rate. Based on their nucleotide sequences, HCV isolates have been grouped into six major genotypes and many more subtypes. The HCV genomic RNA is 9.6 kb in length and codes for a polyprotein, which is cleaved by both cellular and viral proteases, to generate 10 mature protein products. The structural proteins are located at the N-terminus of this polyprotein sequence. These

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structural proteins include the core protein, which forms the viral capsid, and E1 and E2 envelope proteins. Other HCV proteins are p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. The p7 protein is an ion channel protein that may play an important role in viral entry or release from infected cells. NS2-NS5B are nonstructural proteins involved in viral replication (Kolykhalov et al., 2000). In addition to these proteins, HCV also encodes a protein named F protein or alternative reading frame protein (ARFP) using a reading frame that overlaps with the core protein coding sequence (Branch et al., 2005). Many of the HCV proteins including the F/ARFP protein have regulatory functions.

HCV and hepatitis B virus (HBV) are two major causes of HCC, which is the fifth most common cancer in the world. HCV is the primary cause of HCC in many countries where HBV is not endemic (Vainio et al., 1994). A clinical study conducted in Japan on 161 chronic HCV patients revealed that cumulative HCC incidence rates among this group of patients were 24%, 51%, and 63% over the time periods of 5, 10, and 15 years, respectively (Ohata et al., 2003). Chronic HCV patients also have a twofold increased risk for non-Hodgkin's B-cell lymphoma (B-NHL) and a greater than threefold risk for diffuse large B-cell lymphoma (DLBCL) (Nieters et al., 2006).

HCV was first isolated in 1989 (Choo et al., 1989). During the past two decades, the research on this virus has generated a significant amount of information for understanding its replication and pathogenesis. HCV is an oncogenic virus and can apparently induce HCC indirectly through the induction of chronic liver inflammation and also directly through the activities of its gene functions. The latter was demonstrated by studies using transgenic mice that express either the HCV core protein or the full-length polyprotein. These transgenic mice were found to develop hepatic steatosis, a common histopathologic feature found in chronic hepatitis C patients (Ohata et al., 2003), as well as iron overload, which then progressed to HCC without chronic inflammation (Moriya et al., 1998; Lerat et al., 2002; Furutani et al., 2006). HCV can apparently induce cancer via multiple pathways that induce chronic inflammation, the production of reactive oxygen species (ROS), DNA damage, endoplasmic reticulum (ER) stress, autophagic response, and apoptosis. In this chapter, we will review these different pathways in HCV oncogenesis.

CHRONIC LIVER INFLAMMATION

Similar to HBV, the infection by HCV can induce chronic liver inflammation. During inflammation, immune cells, including cytotoxic T lymphocytes (CTL), are recruited to the liver to induce hepatocellular death. For many infections, this inflammatory response is self-limited and will cease when infected cells are removed. However, for chronic liver inflammation, this immune response is sustained, which leads to repeated liver injuries and regenerations. This, in combination with DNA damage that may be caused by the ROS produced by the immune cells including phagocytes and macrophages (see below), may lead to fixation of mutations in proliferating progeny cells and the eventual transformation of hepatocytes into tumor cells. The most direct evidence to support a role of chronic liver inflammation in the development of HCC comes from

the studies on transgenic mice that expressed a noncytopathic amount of HBV envelope protein known as the hepatitis B surface antigen (HBsAg). Due to the expression of HBsAg from the transgene, there was immunotolerance to this viral protein in the mice. Immune response against HBsAg was reintroduced into thymectomized and lethally irradiated transgenic mice by the adoptive transfer of bone marrow and spleen cells from syngeneic nontransgenic mice that had been previously immunized with HBsAg. This reconstitution of the immune system in transgenic mice induced chronic liver inflammation and the development of HCC (Nakamoto et al., 1998). Chronic liver inflammation together with its associated liver fibrosis remains the most important cause of HCV-induced HCC.

ROS

ROS are by-products of normal metabolism. They include oxygen ions, free radicals, and peroxides. Elevated levels of ROS can also increase the activity of carcinogenic xenobiotics by facilitating their activation to reactive compounds (Adelman et al., 1988). One of the most important consequences of ROS production is DNA damage. Normal cells control ROS with reductants, such as glutathione, and with the expression of enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase. However, in response to various factors, ROS levels may dramatically increase to cause oxidative stress, leading to DNA damage and cell death.

HCV infection can induce oxidative stress, which is evidenced by decreased levels of hepatic and plasmatic glutathione, increased levels of 4′-hydroxynonenal (HNE) and 8-hydroxyguanosine, markers for oxidative DNA damage (Choi and Ou, 2006), and elevated levels of circulating ROS in patients (Farinati et al., 1995; Houglum et al., 1997; Mutlu-Turkoglu et al., 1997; Kageyama et al., 2000). This induction of oxidative stress by HCV is apparently the combined effects of chronic inflammation and HCV gene products. HCV core protein can bind to the outer membrane of the mitochondria, can enhance mitochondrial Ca^{2+} uptake, can reduce electron transport complex I activity, and can increase mitochondrial ROS production (Li et al., 2007). HCV NS3 and NS5A can also be associated with mitochondria and ER and alter calcium homeostasis in cells to induce oxidative stress (Bureau et al., 2001; Gong et al., 2001). NS3 also activates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2, which generates ROS (Bureau et al., 2001). In addition, HCV infection can also induce oxidative stress by inducing the production of tumor necrosis factor-α (TNF-α), a cytokine known to cause oxidative stress by stimulating the release of ROS (Kizaki et al., 1993).

DNA DAMAGE

ROS such as hydroxyl radicals (·OH), peroxynitrite ($ONOO^-$), and nitric oxide (NO) can damage DNA directly to cause single- or double-stranded DNA breaks, nucleotide modifications, DNA adducts, and DNA–protein cross-links. Normal cells respond to these damages by arresting the cell cycle for DNA repair to complete or by eliminating

themselves through apoptosis. However, if mutations occur in critical genes, such as tumor suppressor genes or proto-oncogenes, DNA repair or apoptosis may be suppressed, and neoplastic transformation of cells may follow.

HCV infection can lead to an increase in DNA damage. This is supported by studies showing that HCV-infected patients have elevated levels of 8-hydroxydeoxyguanosine (8-OHdG), a well-known marker of DNA damage (Kato et al., 2001). In addition to the induction of DNA damage via the induction of ROS, HCV can also affect DNA damage–repair. For example, HCV NS3 and NS4A proteins have been shown to impair DNA repair by interacting with ataxia-telangiectasia mutated (ATM), an important protein kinase that responds to DNA double-strand breaks (Lai et al., 2008). HCV NS3 can also bind to the tumor suppressor p53 to affect the DNA repair process (Deng et al., 2006). HCV infection can also induce error-prone DNA polymerase ζ, polymerase ι, and activation-induced cytidine deaminase, which together enhance mutations of many somatic genes (Machida et al., 2004). These mutations may cause deleterious mutations in proto-oncogenes or tumor suppressor genes and result in the eventual oncogenic transformation of cells.

ER STRESS

Another contributing factor to HCV oncogenesis is the induction of ER stress. The ER is very sensitive to a variety of cellular stresses, such as alteration of calcium homeostasis, inhibition of protein glycosylation, and viral infection (Kaufman, 1999). These stresses lead to the inhibition of protein folding in the ER, resulting in the accumulation of unfolded or misfolded proteins in the organelle. This in turn leads to the activation of the unfolded protein response (UPR). The UPR is the cellular stress response of the ER and contains three different sensors: pancreatic ER kinase (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF 6), and inositol-requiring enzyme 1 (IRE1) (Ron and Walter, 2007) (Fig. 13.1), which work to alleviate ER stress by halting protein translation and activating the signaling pathways that lead to the increase in molecular chaperones involved in protein folding. In conditions of prolonged stress, the UPR changes from promoting cell survival to promoting apoptosis (Lin et al., 2007).

ER stress has been linked to cancer. Activated UPR has been found in a variety of tumors from both patients and animal studies (Ranganathan et al., 2006). ER stress has also been shown to play a critical role in tumor development (Fels and Koumenis, 2006; Koong et al., 2006). For example, UPR, in particular, the activated PERK signaling pathway, has been shown to confer a survival advantage of tumor cells under hypoxia (Fels and Koumenis, 2006).

HCV infection can cause ER stress. A number of HCV proteins, as well as the subgenomic RNA replicon that contains the HCV NS3-NS5B sequence and can replicate, have been shown to activate the UPR signaling pathways (Liberman et al., 1999; Tardif et al., 2004; Benali-Furet et al., 2005; Chan and Egan, 2005; Ciccaglione et al., 2005; Pekow et al., 2007). HCV infection seems to cause a continuous and prolonged state of ER stress in the cell. This ER stress may lead to repetitive cycles of cell death and regeneration and increase the likelihood of cancer-causing errors in cellular genes. ER stress has also been shown to induce autophagy, which has been linked to cancer.

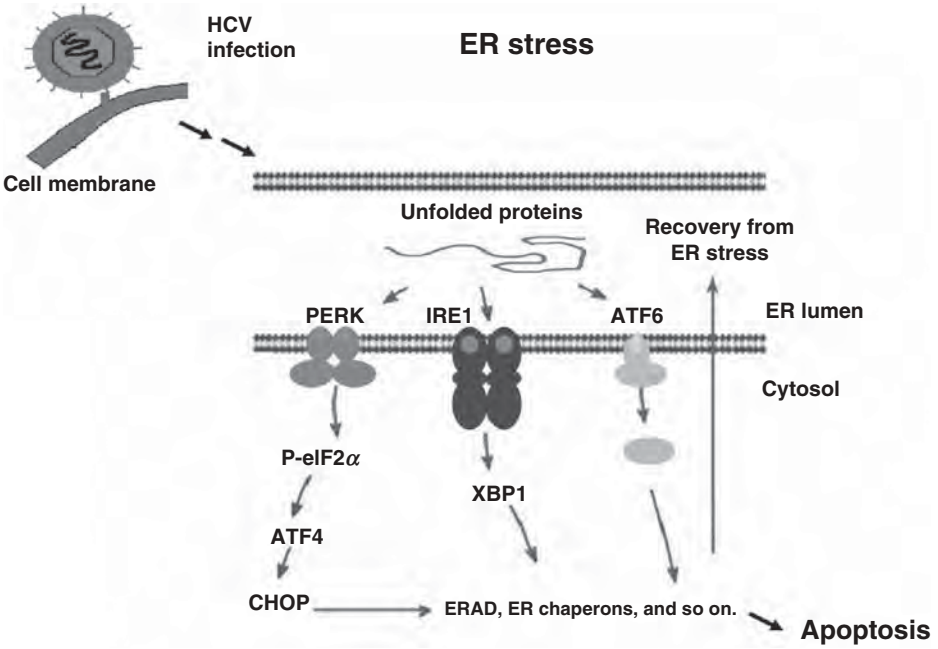

Figure 13.1. ER stress and the unfolded protein response signaling pathways.

AUTOPHAGY

Autophagy is a process by which cells remove long-lived proteins and damaged organelles for degradation and recycling. During autophagy, double- or multiple membrane vesicles, also known as autophagosomes, will form. These membrane vesicles will then fuse with lysosomes to form autolysosomes for the degradation of their contents (Levine and Kroemer, 2008). In addition to maintaining cellular homeostasis, autophagy can also serve as an innate immunity to remove intracellular pathogens (Jackson et al., 2005; Talloczy et al., 2006). Interestingly, several viruses also evolved mechanisms to subvert this innate immune response and use autophagic vacuoles to enhance their replication.

Autophagy may favor or suppress cancer development. Nutrient starvation can induce autophagy for the survival of cells. This is beneficial to growing cancer cells as such cells often find themselves in short supply of nutrients due to their rapid growth and insufficient vascularization in tumor nodules. However, there is also evidence that autophagy can have an inhibitory effect on cancer cells. Beclin-1, a protein involved in the formation of the autophagosomes, is a tumor suppressor protein. Studies have shown that beclin-1 is monoallelically deleted in 40%–75% of cases of human breast, ovarian, and prostate cancers. Mouse studies, investigating defects in beclin-1, show that a decrease in autophagy is linked to an increased propensity for tumor developments (Qu et al., 2003; Yue et al., 2003). These studies show that a malfunction in autophagy can lead to cancer. Thus, autophagy may play dual functions in cancer development.

Recently, it has been shown that HCV induces the accumulation of autophagosomes via the induction of ER stress (Ait-Goughoulte et al., 2008; Sir et al., 2008). Interestingly, this induction of the autophagic response is incomplete, as the formation of autolysosomes is suppressed in HCV-infected cells (Sir et al., 2008). This incomplete activation of the autophagic pathway likely plays an important role in HCV pathogenesis, as it enhances HCV RNA replication and perturbs cellular homeostasis. It has been demonstrated that mice with mutations that prevent lysosomal clearance of autophagosomes show organ injury (Levine and Kroemer, 2008). Thus, the suppression of maturation of autophagic vacuoles by HCV may also lead to chronic liver injury and hepatocarcinogenesis.

APOPTOSIS

Apoptosis is programmed cell death. During apoptosis, cells undergo a series of biochemical and morphological changes, including membrane blebbing, cell shrinkage, nuclear fragmentation, and chromosomal DNA fragmentation. Apoptosis is tightly regulated and can be activated by ER stress, viral infection, and DNA damage. It is important for removing virus-infected cells and for suppressing tumor development.

HCV can induce and also suppress apoptosis. By using a recently developed cell culture system for HCV infection and propagation, it has been demonstrated that HCV infection could induce apoptosis of infected cells (Zhu et al., 2007; Sekine-Osajima et al., 2008). However, many HCV-infected cells are also resistant to cell death, suggesting either a selection for cells that are resistant to apoptosis or the adaptation of the virus to the host cell. Most of the HCV proteins have been shown to affect apoptosis, although sometimes with conflicting results. One notable example is the HCV core protein. The core protein has been shown to alter mitochondrial functions, inducing oxidative stress and thus making cells more susceptible to apoptosis (Li et al., 2007). However, other studies have also shown that the induction of ROS by the core protein lowers consecutive caspase-independent cell death (Machida et al., 2006). HCV core protein can also deregulate the control of apoptosis by inducing ER stress and calcium depletion (Benali-Furet et al., 2005). Further conflicting studies involve the binding of the core protein to p53. This binding has been shown to activate and inhibit p53 activities, leading to either anti- or proapoptotic effects (Ray et al., 1997; Otsuka et al., 2000; Kao et al., 2004; Herzer et al., 2005). In some studies, the core protein inhibits apoptosis by inducing the phosphorylation and activation of STAT3, which induces the antiapoptotic bcl-xL (Otsuka et al., 2002; Yoshida et al., 2002). However, some studies show that the core protein can induce apoptosis through mitochondrial cytochrome C release and indirect activation of proapoptotic bax (Chou et al., 2005; Lee et al., 2005). In addition to the core protein, other conflicting results have been found for all other HCV proteins (for a review, see Fischer et al., 2007). Some of the possible explanations for the controversies could be due to the use of different host cells and/or different HCV genotypes for the studies. If this is indeed correct, then HCV proteins of different genotypes may have different effects on the host cells during different states of the disease.

Enhanced apoptosis by HCV can lead to enhanced compensatory proliferation of hepatocytes (Zheng et al., 2007), which may facilitate the development of HCC. The suppression of apoptosis by HCV may also lead to the growth of damaged cells for tumor formation. In either case, the ability of HCV to affect apoptosis is likely also an important factor in HCV carcinogenesis.

CONCLUSION

Patients with chronic HCV infection have increased risks for HCC and BCL. As reviewed in this chapter, HCV may induce cancer via multiple pathways. A simplified model of these pathways, which are interlinked, is illustrated in Figure 13.2. As shown in this model, HCV infection can induce chronic liver inflammation (step 1). This will lead to the generation of ROS and oxidative stress (step 2), which can also be induced by HCV gene products (step 3). Oxidative stress can lead to DNA damage (step 4), which in turn may lead to apoptosis (step 5). HCV infection can also induce ER stress (step 6), which can in turn lead to oxidative stress (step 7), apoptosis (step 8), and incomplete autophagic response (step 9). All of these downstream effects of ER stress induced by HCV can cause chronic liver injury. In addition to enhancing apoptosis, HCV gene products can also suppress apoptosis (step 10). The ability of HCV to enhance or suppress apoptosis likely depends on the disease states of patients. The suppression of apoptosis by HCV or the selection of apoptosis-resistant cells can lead to abnormal cell growth. In addition to these pathways, HCV can also suppress DNA repair to enhance DNA damage and cause chromosomal instability (step 11). All of these different pathways together can lead to dysregulated cell growth (step 12) and cancer (step 13). Due to these multiple oncogenic pathways, it is difficult to design therapeutic interventions that will effectively prevent the development of HCV-associated cancers in patients. Thus, the best approach to prevent HCV-associated

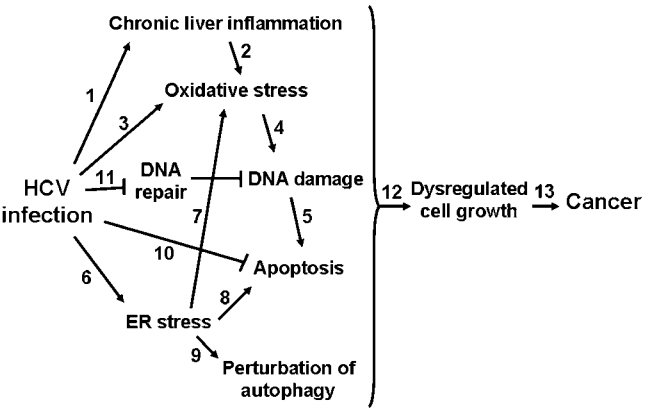

Figure 13.2. Illustration of the HCV oncogenic pathways.

cancers is the eradication of the virus from patients. This will rely on the development of efficacious anti-HCV drugs that have minimal side effects.

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14

CLINICAL ASPECTS OF HTLV-1-ASSOCIATED CANCER

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Since the 1950s, retroviruses have been demonstrated to cause cancers in a variety of animals. However, whether retrovirus induces cancer in humans or not has been a long-lasting enigma. In Japan, Takatsuki's group observed that the frequency of T-cell leukemia was unexpectedly high compared with that in Western countries (Uchiyama et al., 1977; Takatsuki, 2005), where B-cell leukemia is predominant. Such patients with T-cell leukemia in Japan exhibited similar clinical features, for example, frequent skin involvement, presence of characteristic leukemic cells in the peripheral blood (PB), hypercalcemia, and complication of opportunistic infections (Uchiyama et al., 1977). In addition, it was noticed that the birthplace of these patients was clustered in Southwestern Japan (Takatsuki et al., 1977). Therefore, this disease was proposed as a distinct clinical entity, adult T-cell leukemia (ATL); at the time, it was speculated that some pathogens were associated with this disease based on geographic clustering (Uchiyama et al., 1977). Thereafter, human T-cell leukemia virus was found by Gallo's group (Poiesz et al., 1980), and the linkage between ATL and pathogenic virus was reported by Hinuma's group (Hinuma et al., 1981). Thus, ATL has been proved to be the first disease caused by human retroviral infection.

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HUMAN T-CELL LEUKEMIA VIRUS-TYPE 1 (HTLV-1)-INFECTED CELLS IN CARRIER STATE: BEFORE ONSET OF ATL

HTLV-1-infected cells form a virological synapse with uninfected cells, and then the virus transmits to uninfected cells (Igakura et al., 2003). Thus, HTLV-1 transmits mainly cell to cell. Free virions could not be detected in HTLV-1-infected individuals, and infectivity of free virions is pretty poor *in vitro* (Derse et al., 2001). Since infected cells are essential for the transmission of HTLV-1, infection of HTLV-1 occurs by three major routes: (a) mother to infant, (b) sexual, and (c) parenteral transmissions.

After transmission, HTLV-1 increases the number of infected cells by actions of viral genes (Matsuoka and Jeang, 2007). Such an increased number of infected cells facilitates the transmission of this virus to other hosts. HTLV-1 induces clonal proliferation of infected cells after transmission by actions of virus-encoded genes (Etoh et al., 1997; Cavrois et al., 1998). Since HTLV-1 exists as provirus in the host cells and most of the infected cells have one copy of provirus, the quantification of provirus (provirus load) in mononuclear cells is determined by the number of infected cells. Provirus load differs more than 1000 times among infected individuals but is relatively constant in a single infected individual (Etoh et al., 1999). How is this provirus load determined? *De novo* infection seems to have little effect on provirus load since reverse transcriptase inhibitors have no influence on provirus load in both HTLV-1 associated myelopathy (HAM)/tropical spastic paraparesis (TSP) patients (Taylor et al., 2006) and animal models (Miyazato et al., 2006). Therefore, the clonal proliferation of HTLV-1-infected cells is predominant as a determinant of provirus load. Among viral proteins produced by HTLV-1, Tax is considered to play a central role in the proliferation of HTLV-1-infected cells and ATL cells through its pleiotropic actions. Tax can activate nuclear factor κB, activator protein-1, cyclic AMP response element-binding protein pathways along with functional inhibition of p53, and mitotic arrest-defective (MAD) 1. With these actions, Tax likely induces cell proliferation and inhibits apoptosis, resulting in an increase in infected cells. However, since Tax protein is a major target of cytotoxic T lymphocytes (CTLs) in HTLV-1-infected carriers, Tax-expressing cells are rapidly eliminated *in vivo* (Asquith et al., 2007). In addition to Tax, p12, p30, and HTLV-1 bZIP factor (HBZ) are implicated in the proliferation of infected cells (Ding et al., 2003; Silverman et al., 2004; Satou et al., 2006). In particular, the *HBZ* gene is expressed in carriers and ATL patients, indicating that *HBZ* gene functions in infected cells and ATL cells. However, CTLs against HBZ have not been demonstrated yet. It is possible that HBZ is not so immunogenic, which might be a reason that the *HBZ* gene is expressed in carriers and ATL patients.

Persistent HTLV-1 infection in spite of strong immune response to HTLV-1 indicates a mechanism of escape from the host immune surveillance. *In vivo* and *ex vivo* analyses of infected cells show that most of infected cells do not express viral protein, especially Tax, *in vivo*. Analyses of *in vivo* dynamics of HTLV-1-infected cells showed that increased proliferation of CD4+CD45RO+ T cells was correlated with Tax expression, suggesting that Tax expression enhanced both proliferation and elimination by CTLs (Asquith et al., 2007). Administration of valproate, which has inhibitory function to histone deacetylase (HDAC), to HAM/TSP patients decreased provirus load, which suggests that viral gene-expressing cells by the administration of the HDAC inhibitor are eliminated by CTLs *in vivo* (Lezin et al., 2007). Thus, proliferation of infected cells

is determined by the balance between viral gene expression and the host immune system.

Although provirus load remarkably differs among HTLV-1 carriers, it remains relatively constant in individuals, demonstrating that host factors influence provirus load, namely the number of infected cells. Considering that the cellular immune responses are critically implicated in the control of HTLV-1 infection, human leukocyte antigen (HLA) should be a candidate for such a host genetic factor. HLA has been reported to be associated with increased provirus load (Jeffery et al., 1999). Higher provirus load is speculated to be a risk factor for the development of ATL (Hisada et al., 1998). Although provirus load in patients with HAM/TSP is usually high, frequent development of ATL has not been reported. Therefore, high provirus load does not always mean the development of ATL. Since familial clustering has been reported in ATL (Miyamoto et al., 1985), factors other than provirus load should be considered in oncogenesis by HTLV-1. A polymorphism of the *TNF-*α gene was reported to show an association with ATL (Tsukasaki et al., 2001).

CLINICAL CHARACTERISTICS OF ATL

Originally, ATL has been characterized by the following four clinical features: (a) presence of ATL cells with multilobulated nuclei (flower cells: Fig. 14.1a), (b) infiltration of ATL cells into various tissues and organs (skin, lung, liver, gastrointestinal tract, and central nervous system [CNS]) (Fig. 14.2), (c) frequent complication of hypercalcemia (Fig. 14.3), and (d) complicated opportunistic infections. ATL develops predominantly in male (male to female ratio is 1.5:1), whose age at onset ranged from 25 to 94 years, with a median age of 60 years in Japan. On the other hand, the average age of Caribbean and African ATL patients is about 43 years old, suggesting that the onset is influenced by genetic or environmental factors.

Morphological features of ATL cells are characteristic to this disease. Typical ATL cells have lobulated nuclei, which is called a "flower cell" based on its nuclear

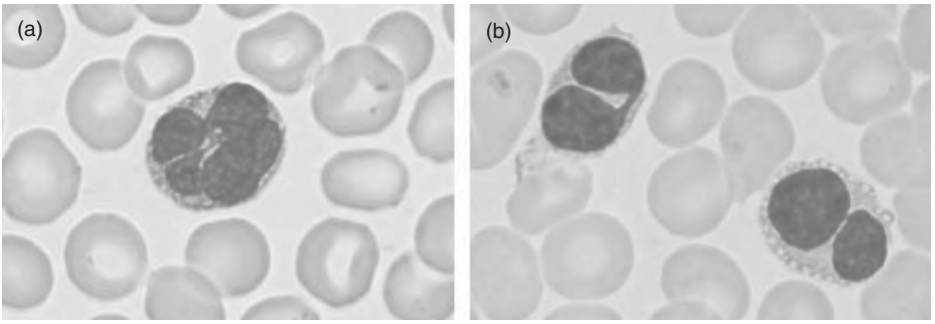

Figure 14.1. Leukemic cells in the peripheral blood of ATL patients. (a) An ATL cell in an acute ATL patient showed lobulated nuclei. (b) ATL cells in a chronic ATL patient. See color insert.

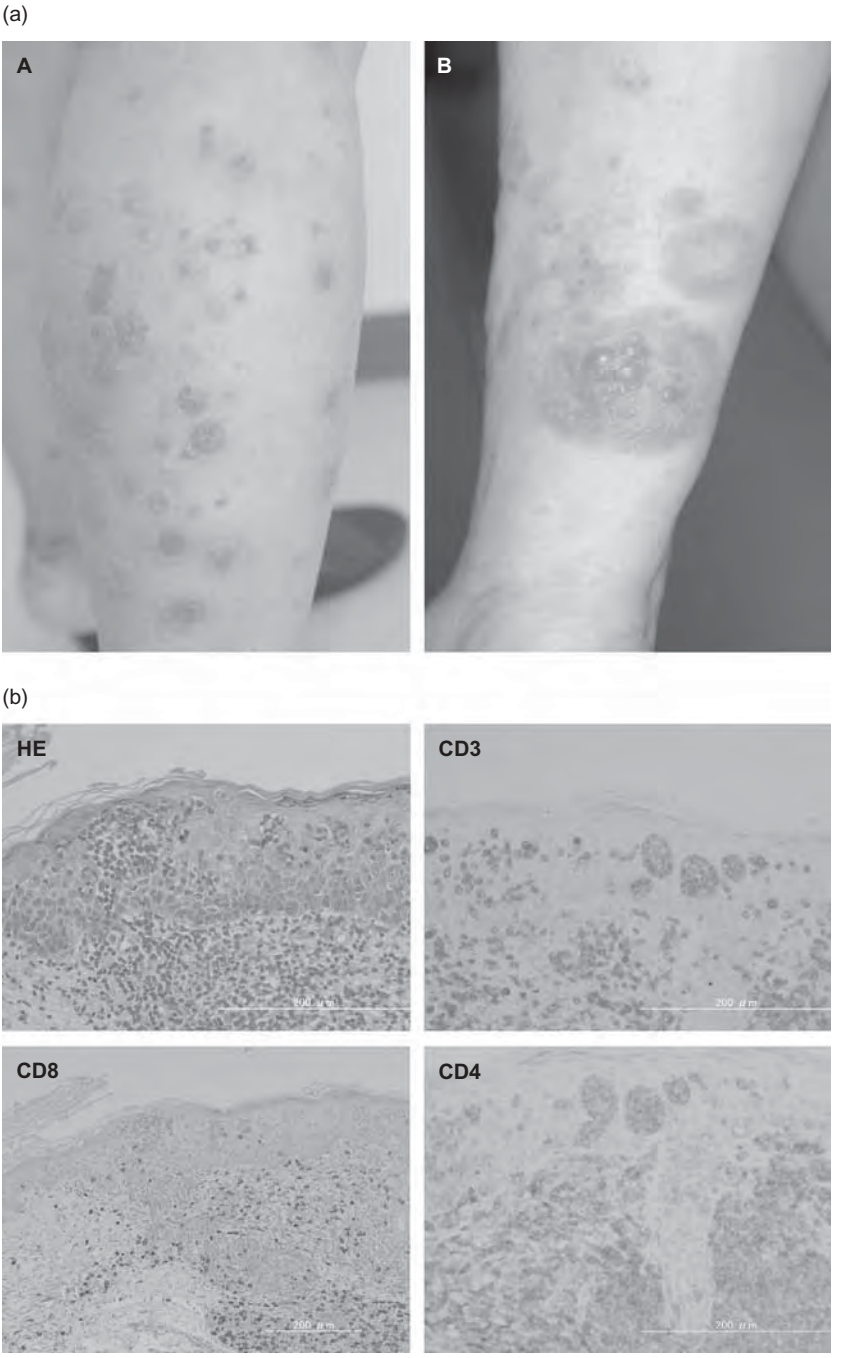

Figure 14.2. Skin involvement in ATL patients. (a) Skin lesions of ATL patients. (b) Immunohistochemical analyses of skin lesions. ATL cells infiltrated into the epidermis and formed Pautorier's microabscess. ATL cells were CD3+, CD4+, and CD8–. (Courtesy of Dr. Wakasugi, Kumamoto University School of Medicine.) See color insert.

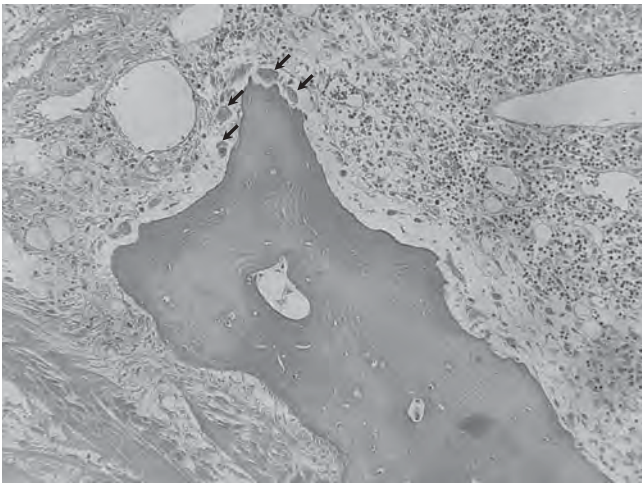

Figure 14.3. Increased osteoclasts in the bone of hypercalcemic ATL patients. Increased osteoclasts are indicated by arrows. See color insert.

morphology (Fig. 14.1a). Deformity of the nuclei is not so remarkable in the leukemic cells in chronic ATL (Fig. 14.1b).

ATL cells are known to have a tendency to infiltrate various organs and tissues. Skin involvement is observed in about 70% of patients with ATL during the entire clinical course. Skin lesions range from tumorous to erythematous (Fig. 14.2a). Histological studies often showed the formation of Pautorier's microabscess in the epidermis (Fig. 14.2b). Such epidermotropic phenotype is attributed to the expression of various molecules on ATL cells. Chemokine (C-C motif) receptor 4 (CCR4) is highly expressed on ATL cells (Yoshie et al., 2002); in addition, its ligands, thymus and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC), are produced from ATL cells (Shimauchi et al., 2005). Since epidermotropic T cells express CCR4, such expression might account for affinity of ATL cells to skin tissue. Increased CCR4 expression is also reported in cutaneous T-cell lymphoma (Ferenczi et al., 2002), which resembles ATL. Infiltration of ATL cells into the lung, liver, and gastrointestinal tract is frequently observed, especially in acute ATL.

Hypercalcemia is a frequent complication in ATL. In hypercalcemic ATL patients, activated osteoclasts increase in the bone, and adsorption of bone is enhanced.

Opportunistic infections, such as fungal, protozoal, and viral infections, are frequently implicated in patients with ATL due to the inevitable impairment of the T-cell function. Pathogens include *Pneumocystis jiroveci*, cytomegalovirus, strongyloidiasis, and a variety of fungi. Prophylactic administration is effective during treatment. Opportunistic malignancies, Kaposi's sarcoma (Greenberg et al., 1990), and Epstein–Barr virus (EBV)-associated lymphoma (Tobinai et al., 1991) have also been reported in patients with ATL, which also indicates a state of immunodeficiency in these patients.

CLINICAL SUBTYPES OF ATL

After HTLV-1 has been shown to be a causative virus of ATL, a variety of clinical features have been clarified. Four clinical subtypes of ATL have been reported: smoldering, chronic, lymphoma, and acute-type ATL. Criteria of diagnosis have been proposed by Shimoyama et al. (Shimoyama, 1991).

1. Smoldering type: 5% or more abnormal lymphocytes of T-cell nature in the PB; normal lymphocyte level ($<4 \times 10^9$ per liter); no hypercalcemia; lactate dehydrogenase (LDH) value of up to 1.5 times the normal upper limit; no lymphadenopathy; no involvement of liver, spleen, CNS, bone, or gastrointestinal tract; and neither ascites nor pleural effusion. Skin and pulmonary lesions may be present. In patients with less than 5% abnormal lymphocytes in PB, at least one histologically proven skin or pulmonary lesion should be present.
2. Chronic type: absolute lymphocytosis of more than 3.5×10^9 per liter; LDH value up to twice the normal upper limit; no hypercalcemia; no involvement of CNS, bone, or gastrointestinal tract; and neither ascites nor pleural effusion. There may be histologically proven lymphadenopathy with or without extranodal lesions, and there may be involvement of the liver, spleen, skin, and lungs, and 5% or more abnormal lymphocytes.
3. Lymphoma type: no lymphocytosis; 1% or less abnormal lymphocytes; histologically proven lymphadenopathy with or without extranodal lesions.
4. Acute type: the most common form of presentation; highly aggressive malignancy that shows lymphadenopathy, hepatosplenomegaly, and skin lesions but does not meet the criteria of the other types.

DIAGNOSIS OF ATL

ATL can be defined as a neoplastic proliferation of HTLV-1-infected cells. Characteristic clinical features (skin involvement, hypercalcemia, presence of typical flower cells in the PB) provide useful clues for the diagnosis of ATL. The presence of antibody to HTLV-1 is a critical factor, although seronegative ATL has been reported. In such case, a generation of antibody might be suppressed. To establish the definite diagnosis of ATL, monoclonal integration of HTLV-1 provirus should be demonstrated in the genome of neoplasms by Southern blot method or inverse polymerase chain reaction (PCR).

PATHOPHYSIOLOGY OF ATL

ATL is a neoplasm of CD4+ T lymphocytes, which play central roles in the control of the immune system. Cytokines produced by ATL cells influence the clinical symptoms and manifestations of the patients (Table 14.1). Interleukin (IL)-5 promotes the

TABLE 14.1. Association of Cytokines and Surface Molecules with ATL

Cytokines or Surface Molecules	Clinical Effects	References
IL-1	Fever?	Wano et al. (1987); Yamamoto et al. (1986)
IL-5	Eosinophilia	Ogata et al. (1998); Yamagata et al. (1997)
IL-6	Inflammation	Mori et al. (1994); Yamamura et al. (1998)
IL-10	Immunosuppression?	Mori et al. (1996)
PTH-rP	Hypercalcemia	Honda et al. (1988); Watanabe et al. (1990)
TGF-β	Immunosuppression	Niitsu et al. (1988)
MIP-1α	Hypercalcemia?	Okada et al. (2004)
GM-CSF	Neutrophilia?	Yamada et al. (1996)
RANK ligand	Hypercalcemia	Nosaka et al. (2002)
CCR4	Skin involvement	Yoshie et al. (2002)

GM-CSF, granulocyte macrophage colony stimulating factor; MIP-1α, macrophage inflammatory protein 1α.

generation of eosinophils in bone marrow, leading to eosinophilia (Ogata et al., 1998). IL-10 is known to be an immunosuppressive factor to cell-mediated immunity. The function of IL-10 involves the inhibition of proinflammatory cytokine secretion and the expression of MHC class II and costimulatory molecules by inactivation of macrophages and dendritic cells (O'Garra and Vieira, 2007). Since IL-10 is produced from ATL cells (Mori et al., 1996), enhanced production of IL-10 might be implicated in mechanisms of immunosuppression observed in ATL patients.

Hypercalcemia is observed in about 70% of ATL patients during their clinical courses (Kiyokawa et al., 1987). In hypercalcemic ATL patients, increased osteoclasts were observed (Fig. 14.3), which resulted in accelerated bone resorption. Bone is constitutively remodeled by osteoblasts (matrix synthesis) and osteoclast (bone resorption). Osteoclasts are derived from hematopoietic precursor cells and belong to the monocyte macrophage lineage. During differentiation of osteoclasts, precursor cells sequentially express c-Fms (receptor of macrophage colony stimulating factor [M-CSF]) followed by receptor activator nuclear factor κB (RANK) (Arai et al., 1999). RANK ligand induces differentiation of osteoclast from hematopoietic precursor cells in cooperating with M-CSF. ATL cells in hypercalcemic patients express RANK ligand on their surfaces and induce differentiation of osteoclasts, indicating that ATL cells expressing RANK ligand directly increase the number of osteoclasts (Nosaka et al., 2002). Parathyroid hormone-related peptides (PTH-rP) cause hypercalcemia in patients with various cancers. ATL cells also produced PTH-rP, which is also implicated in hypercalcemia (Honda et al., 1988; Watanabe et al., 1990).

INTERPLAY BETWEEN HOST IMMUNE SYSTEM AND HTLV-1 INFECTION

Since cell-mediated immunity is severely impaired in ATL patients, opportunistic infections frequently occur, which is a major obstacle in the treatment of ATL patients (Taylor and Matsuoka, 2005). Pathogens of these opportunistic infections include

viruses (cytomegalovirus, varicella-zoster virus, herpes simplex virus, JC virus), fungi (*P. jiroveci*), parasites (*Strongyloides stercoralis*), and bacteria. Humoral immunity remains intact in ATL patients, while cell-mediated immunity is severely impaired. As mechanisms of immunodeficiency, ATL cells produce immunosuppressive cytokines such as IL-10 (Mori et al., 1996) and transforming growth factor (TGF)-β (Niitsu et al., 1988). In addition, ATL cells often express *FOXP3* gene (Karube et al., 2004), which is the master gene of regulatory T cells. Regulatory T cells suppress immune reactions by cell-to-cell contact. One of the molecules that is responsible for immunosuppression in ATL is CTL-associated antigen-4 (CTLA4). ATL cells with FOXP3 expression might suppress the immune system in the same manner of regulatory T cells.

Conversely, immunodeficient states have been reported to promote oncogenesis by HTLV-1. The development of ATL has been reported (Hoshida et al., 2001) following a renal transplantation. Similarly, the development of ATL has been reported in three cases after liver transplantation among eight seropositive recipients (Kawano et al., 2006). All ATL cells were derived from recipient cells, indicating that immunosuppressive agents promote oncogenesis by HTLV-1. On the other hand, the development of donor-derived ATL has been reported shortly after allogeneic bone marrow transplantation from seropositive sibling to ATL patient (Tamaki and Matsuoka, 2006). This clone was identified in the donor, indicating that the proliferation of this clone was controlled in the donor (Tamaki et al., 2007). Although this donor remains an asymptomatic carrier, this clone became ATL in the immunosuppressed recipient. This case also demonstrates the significance of the host's immune system to control the growth of HTLV-1-infected cells and ATL cells. These HTLV-1-induced oncogeneses under immunodeficient conditions are similar to those observed in EBV. These findings show the significance of host immune system and incubation time before the onset of ATL.

In the carrier state, mild immunodeficiency is also reported. As a mechanism of immunosuppression, decreased production of naive T cell has been reported (Yasunaga et al., 2001). In elder HTLV-1 carriers, increased virus load of EBV has been detected, which also reflects immunodeficiency. Onset of ATL in the elder carriers might be due to suppressed immune responses to HTLV-1.

PROVIRUS IN ATL CELLS

The only direct evidence of HTLV-1 infection remaining in ATL cells is the provirus integrated into the genome. Therefore, its analyses are assumed to provide important information on HTLV-1 infection oncogenesis. In most of the ATL cases, one HTLV-1 provirus is integrated in the genome. Since Tax has pleiotropic actions on cell proliferation and oncogenesis, and induces various cancers in the transgenic animals (Grossman et al., 1995; Kikuchi et al., 2002; Hasegawa et al., 2006), Tax is considered to play a critical role in the leukemogenesis. The enigma is that Tax expression is frequently lost in ATL cells. Three mechanisms have been identified: (a) genetic changes of the *tax* gene (nonsense mutation, deletion, and insertion) (Furukawa et al., 2001; Takeda et al., 2004), (b) DNA methylation of 5′ long terminal repeat (LTR) (Koiwa et al., 2002; Taniguchi et al., 2005), and (c) deletion of 5′ LTR (Tamiya et al., 1996; Miyazaki

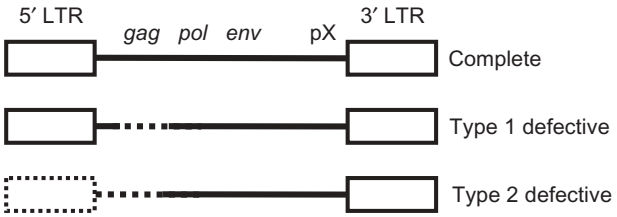

Figure 14.4. Structure of HTLV-1 provirus in ATL cases. There are three types of HTLV-1 provirus in ATL cells: (a) complete type, (b) type 1 defective provirus has 5′ and 3′ LTR lacking internal region, and (c) type 2 defective provirus lacking 5′ LTR and internal region. See color insert.

et al., 2007). Among fresh leukemic cells isolated from ATL patients, about 60% of cases do not express the *tax* gene transcript. Interestingly, ATL cells with genetic changes of the *tax* gene expressed its transcripts, suggesting that ATL cells do not silence the transcription when the *tax* gene is abortive (Takeda et al., 2004). Loss of Tax expression gives ATL cells an advantage for their survival since they can escape from CTLs.

As shown in Figure 14.4, structures of proviruses are categorized into three groups: (a) complete type, (b) defective provirus with 5′ and 3′ LTR (type 1 defective provirus), and (c) defective provirus without 5′ LTR (type 2 defective provirus) (Tamiya et al., 1996). Type 2 defective provirus is frequently observed in aggressive forms of ATL, acute, and lymphoma type, when compared with chronic and smoldering type ATL, indicating that this type of defective provirus is associated with disease progression. Analyses of integration sites revealed that type 2 defective proviruses were generated both before and after integration (Miyazaki et al., 2007). Since the frequency of type 2 defective provirus is higher in ATL cells than in carriers, this defective provirus reflects selection during the leukemogenesis. However, the structure of the *tax* gene is frequently not maintained while the *HBZ* gene is intact in all cases, indicating the significance of *HBZ* gene in the leukemogenesis.

TREATMENT OF ATL

After the clinical entity of ATL has been established, many aspects of HTLV-1 infection and ATL have been clarified. However, the prognosis of ATL patients has not been improved significantly despite intensive chemotherapy. The median survival time of acute or lymphoma-type ATL was reported to be 13 months with the most intensive chemotherapy (Yamada et al., 2001). This poor prognosis is due to resistance of ATL cells to anticancer drugs and frequent opportunistic infections caused by an immunodeficient state (Taylor and Matsuoka, 2005). As described above, cell-mediated immunity is severely impaired in ATL patients. Frequent complication of opportunistic infections is a major obstacle during treatment of ATL patients, which is worsened by chemotherapeutic agents. Regarding the resistance to anticancer drugs, one mechanism is the activated NF-κB pathway in ATL cells (Mori et al., 1999), which increases

the transcription of antiapoptotic genes such as *bcl-xL* and *survivin*. A proteasome inhibitor, bortezomib, is currently used for the treatment of multiple myeloma. One of its mechanisms is suppression of the NF-κB pathway by inhibiting the proteasomal degradation of IκB protein. Several groups have shown that bortezomib is effective against ATL cells both *in vitro* and *in vivo* (Tan and Waldmann, 2002; Mitra-Kaushik et al., 2004; Satou et al., 2004; Nasr et al., 2005). Since the sensitivity to bortezomib is well correlated with the extent of NF-κB activation, the major mechanism of the anti-ATL effect is speculated to be the inhibition of NF-κB. In addition, an NF-κB inhibitor has also been demonstrated to be effective against ATL cells (Mori et al., 2002; Watanabe et al., 2005).

During chemotherapy for ATL, chemotherapeutic agents worsen the immunodeficient state of ATL patients. In this regard, antibody therapy against ATL cells has advantages due to its decreased adverse effects. A humanized monoclonal antibody to CD25 has been clinically administered to patients with ATL (Waldmann et al., 1993, 1995). In addition, a monoclonal antibody to CD2 is at the preclinical stage (Zhang et al., 2003). As described above, most ATL cells express CCR4 antigen on their surfaces, and a humanized antibody against CCR4 is being developed as an anti-ATL agent (Ishida et al., 2004).

Advances in the treatment of ATL were brought by allogeneic bone marrow or stem cell transplantation (Borg et al., 1996; Utsunomiya et al., 2001). Relapse of ATL was linked with the absence of graft-versus-host disease (GVHD), suggesting that GVHD or graft-versus-ATL may be implicated in the clinical effects of allogeneic stem cell transplantation. However, since elder carriers usually develop ATL, the age is a limiting factor in transplantation therapy to ATL patients. Sixteen patients with ATL, who were over 50 years of age, were treated with allogeneic stem cell transplantation with reduced conditioning intensity (RIST) from HLA-matched sibling donors (Okamura et al., 2005). Among nine patients in whom ATL relapsed after transplantation, three achieved a second complete remission after rapid discontinuation of cyclosporine A. This finding strongly suggests the presence of a graft-versus-ATL effect in these patients. In addition, Tax peptide-recognizing cells were detected by a tetramer assay in patients after allogeneic stem cell transplantation (Harashima et al., 2004). In eight patients, the provirus became undetectable by real-time PCR. Among these, two patients who received grafts from HTLV-1-positive donors also became provirus negative by real-time PCR after RIST. Since the provirus load is relatively constant in HTLV-1-infected individuals, this finding indicates that immune response against HTLV-1 is activated after RIST, which suppresses the provirus load. This may account for the effectiveness of allogeneic stem cell transplantation to ATL. However, Tax expression is frequently lost in ATL cells as described above. Many questions arise, such as whether the *tax* gene status is correlated with the effect of allogeneic stem cell transplantation, and whether the effectiveness of the anti-HTLV-1 immune response is against leukemic cells or nonleukemic HTLV-1-infected cells. Nevertheless, these data suggest that potentiation of the immune response against viral proteins such as Tax may be an effective way to treat ATL patients (Kannagi et al., 2004). Such strategies may enable preventive treatment of high-risk HTLV-1 carriers, such as those with familial ATL history, predisposing genetic factors to ATL, or a higher provirus load.

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15

ONCOGENIC POTENTIAL OF THE HTLV-1 TAX PROTEIN

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Although the precise cause of tumorigenesis is unknown for most human cancers, approximately 15% of all cancers have been linked to infection with a specific viral pathogen (Butel, 2000). Typical of most cancers, genomic instability and subsequent multistep tumorigenesis are also hallmarks of viral-associated human cancers. Thus, oncogenic viruses offer an enviable opportunity to investigate molecular and cellular mechanisms that promote the development of cancer. This chapter will discuss the oncogenic potential of the human T-cell leukemia virus-type 1 (HTLV-1) transforming protein, Tax, and the role of Tax in genomic instability and cellular transformation.

HTLV-1

HTLV-1 is the etiologic agent of adult T-cell leukemia (ATL), an aggressive clonal malignancy of mature $CD4^+$ T lymphocytes that develops in 2%–5% of HTLV-1-infected individuals (Gessain et al., 1985; Kondo et al., 1987; Johnson et al., 2001). This virus is endemic in parts of Japan, South America, Africa, and the Caribbean, with estimates of 10–20 million HTLV-1-infected people worldwide (Franchini, 1995). The delayed onset of ATL (two to four decades after infection) is consistent with a multistep

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process of T-lymphocyte immortalization and transformation. Leukemic cells from ATL patients display gross chromosomal abnormalities that are associated with cellular transformation and tumor progression.

THE HTLV-1 ONCOPROTEIN, TAX

The HTLV-1 genome is approximately 9 kb in length and encodes the typical complement of retroviral structural and functional gene products. The 3′ end of the viral genome encodes Tax, a 40 kDa oncoprotein that is essential for HTLV-1 replication and viral transformation (Grassmann et al., 1992). Tax is a potent transactivator of viral gene expression and also regulates the transcription of an ever-growing list of cellular genes. Indeed, microarray analyses have suggested that nearly 300 genes are up-regulated in Tax-expressing cells (Nasr et al., 2003; Pise-Masison and Brady, 2005; De La Fuente et al., 2006).

Early studies demonstrated that the expression of Tax in rodent fibroblasts resulted in morphological transformation, implicating this protein in leukemogenesis (Pozzatti et al., 1990; Tanaka et al., 1990; Smith and Greene, 1991; Feuer and Green, 2005). Subsequently, the delivery of Tax via a transformation-defective *Herpes saimiri* virus vector immortalized primary human T lymphocytes *in vitro*, and these transformed cells resembled the leukemic phenotype of HTLV-1-infected T cells from patients (Grassmann et al., 1989). Tax has also been shown to induce tumors in transgenic animals and to immortalize human T cells (Hinrichs et al., 1987; Nerenberg et al., 1987). Tax-transgenic mice developed neurofibromas and mesenchymal tumors when Tax was ubiquitously expressed in all tissues but developed primary peripheral lymphomas comprised of $CD8^+$ T and natural killer cells when Tax expression was targeted to lymphocytes using the lymphocyte-specific human granzyme B promoter (Hinrichs et al., 1987; Nerenberg et al., 1987; Grossman et al., 1995). Thus, Tax is necessary and sufficient to induce cellular transformation and, consequently, has been implicated as the major transforming protein of HTLV-1.

Despite strong evidence implicating Tax in the process of tumorigenesis, the exact mechanisms of Tax function have yet to be fully elucidated. While all leukemic cells isolated from ATL patients contain integrated copies of the viral genome, only a small percentage of these cells express Tax, and this expression is low and typically only detected by polymerase chain reaction (PCR). Despite low-level expression in transformed cells, Tax is believed to play a central role in driving the initiating events of transformation. There is conflicting data on whether Tax expression is required at some minimum level to maintain transformation. In one study, antisense oligonucleotides that reduced Tax expression by 90% in tumor cell lines from Tax transgenic mice did not affect the rate of cell growth or tumor formation *in vivo* (Kitajima et al., 1992). In contrast, another study showed that HTLV-1-transformed Rat1 cells, which no longer express Tax, lost the ability to form tumors in nude mice, and reintroduction of the *tax* gene restored the ability of these cells to form tumors (Yamaoka et al., 1992), implicating Tax in both initiation and maintenance of cellular transformation. Thus, previous studies have consistently indicated a critical role for Tax

in the initiation of transformation, but determining the potential role of Tax in the maintenance of transformation will require additional studies.

MODULATION OF TRANSCRIPTION BY TAX

Tax was originally described as a potent transactivator of the HTLV-1 long terminal repeat (Sodroski et al., 1984), mediating this effect through cyclic AMP response element-binding protein (CREB) (Franklin et al., 1993; Adya et al., 1994; Low et al., 1994; Adya and Giam, 1995). Subsequently, Tax has been shown to affect at least three other transcription factor pathways: nuclear factor-kappa B (NF-κB) (Ballard et al., 1988; Leung and Nabel, 1988; Ruben et al., 1988; Kanno et al., 1994; Sun et al., 1994), serum response factor (SRF) (Fujii et al., 1991, 1992, 1995), and activator protein-1 (AP-1) (Armstrong et al., 1993; Fujii et al., 2000; Mori et al., 2000). Using these pathways, Tax modulates the expression of many cellular genes including growth factors and their receptors, cytokines, transcription factors, cell cycle regulators, DNA repair proteins, and cell adhesion molecules (Lemoine and Marriott, 2001). Altered transcription factor function features prominently in Tax-mediated cellular transformation.

SPECIFIC CELLULAR GENES REGULATED BY TAX

The transcriptional effects of Tax cause profound perturbations in cell cycle progression and apoptosis. Through the CREB/ATF pathway, Tax up-regulates the expression of Bcl-xl, an antiapoptotic factor, as well as of cyclin D2, whose role in cell cycle progression will be discussed later (Santiago et al., 1999; Tsukahara et al., 1999). In contrast, Tax represses the cyclin A and cyclin D3 genes through a CREB response element (CRE) site via a mechanism that is thought to involve the formation of different complexes on the promoters (Kibler and Jeang, 2001). This repression may affect cell cycle progression through S phase as well as mitotic exit. Tax up-regulates other genes, including ICAM-1, fucosyltransferase, and galectin-3, through CRE elements, and these genes have been shown to play key roles in leukemic cell infiltration and metastasis (Hsu et al., 1996; Tanaka et al., 1996; Hiraiwa et al., 2003).

In addition to activating gene expression, Tax can inhibit the transcription of certain cellular promoters by interacting with CREB binding protein (CBP)/p300. Indeed, Tax represses the expression of the DNA polymerase β gene via an E-box DNA element (Jeang et al., 1990). In addition, Tax has been shown to repress p53, $p18^{INK4C}$, and more recently, the human telomerase (hTERT) promoters through E-box recognition sequences (Akagi et al., 1995; Suzuki et al., 1999a; Gabet et al., 2003). This repression is thought to result from the direct competition between Tax and E-box-binding transcription factors of the basic helix-loop-helix family for the recruitment of CBP/p300 (Suzuki et al., 1999b). Similarly, Tax expression interferes with the binding of p53 to CBP/p300, an interaction required to enhance p53-mediated transcription, resulting in inhibition of the transactivating function of p53 (Ariumi et al., 2000). The ability of Tax to repress p53-mediated transcription is not merely due to sequestration of CBP/p300.

Tax also represses p53-mediated transcription in an NF-κB-dependent manner by inducing p53 phosphorylation, which alters the ability of p53 to interact with a variety of transcription factors (Jeong et al., 2004). Inhibition of p53 function by Tax obstructs the G1/S checkpoint, p53-mediated apoptosis, and DNA repair (discussed in detail below).

NF-κB signaling pathways are common targets of viral infection. In resting cells, NF-κB proteins are sequestered in the cytoplasm by inhibitory partners, including IkBα, IkBβ, p100, and p105 (Chen and Greene, 2004). Cellular stimulation and activation of cellular signaling pathways induce phosphorylation of the inhibitory proteins, targeting them for ubiquitination and subsequent degradation. These modifications unmask the nuclear localization sequence of NF-κB and permit the active form to translocate to the nucleus to activate target genes. HTLV-1-infected cells, as well as cells expressing Tax alone, induce and maintain NF-κB activation as a result of Tax binding to the inhibitory proteins IkBα and IkBβ as well as to the IKKγ subunit of the kinase regulatory complex. This binding disrupts inactive, cytoplasmic NF-κB complexes, allowing nuclear translocation of active NF-κB (Hirai et al., 1992; Beraud et al., 1994; Kanno et al., 1994; Suzuki et al., 1995). In this way, Tax activates a large number of NF-κB-dependent cellular genes that promote lymphocyte growth, activation, and survival. These genes include cytokines and growth factors such as IL-1, IL-2, IL-6, IL-8, IL-15, and transforming growth factor (TGF)-β, growth factor receptors such as IL-2Rα and OX40, and antiapoptotic factors such as Bcl-xl (reviewed in Lemoine and Marriott [2001]). Tax also activates the cellular proto-oncogene, c-*myc*, which can independently mediate cellular transformation via NF-κB regulation (Keath et al., 1984; Adams et al., 1985). Furthermore, NF-κB-dependent stimulation of IL-2 and IL-2 receptor expression creates an autocrine feedback loop that can stimulate the proliferation of infected cells in the absence of growth factors (Ballard et al., 1988; Wano et al., 1988). Overall, NF-κB activation is an essential component of Tax-mediated transformation (Robek et al., 1998; Ross et al., 2000; Sun and Yamaoka, 2005).

An additional mechanism used by Tax to induce cell proliferation involves binding to the cellular transcription factor, SRF, and induction of aberrant expression of several immediate-early nuclear oncogenes including c-*fos*, Fos-related antigen-1 (*fra*-1), early growth response gene-1 (*egr*-1), and *egr*-2 (Alexandre and Verrier, 1991; Fujii et al., 1991, 1992; Suzuki et al., 1993). The interaction of Tax with SRF has been shown to enhance its binding to serum response elements (SREs) located in c-fos, Nur77, and viral promoters, resulting in transcriptional activation of these genes (Winter and Marriott, 2007). Tax also facilitates the association of CBP/p300 with SRF at these cellular promoters as part of the activation process (Shuh and Derse, 2000).

TAX INTERACTS WITH PSD-95/SAP90, DISKS LARGE, AND ZONA OCCLUDENS-1 (PDZ) DOMAIN-CONTAINING PROTEINS

Several viral oncoproteins interact with cellular proteins that contain PDZ domains and facilitate their tumorigenic potential. Cellular PDZ domain-containing proteins such as the human homologue of *Drosophila* disks large tumor suppressor (hDlg) typically lack

catalytic activity and function as scaffolding for large signaling complexes at specialized membrane sites of cell–cell contact (Craven and Bredt, 1998; Fanning and Anderson, 1999a,b; Krummel and Macara, 2006). In fact, PDZ domains act as protein–protein interaction modules that bind specific sequence motifs usually at the carboxyl-terminus of the target proteins (Fanning and Anderson, 1996; Songyang et al., 1997). Tax possesses such a motif, a type 1 carboxyl-terminal PDZ domain-binding motif (PBM) that mediates interactions with cellular PDZ domain-containing proteins including hDlg1 (Lee et al., 1997) and others (Rousset et al., 1998). Disruption of the Tax PBM substantially decreases Tax-mediated cellular transformation and micronuclei (MN) formation as well as HTLV-1-mediated T-cell proliferation *in vitro* and persistence *in vivo* (Endo et al., 2002; Hirata et al., 2004; Xie et al., 2006; Higuchi et al., 2007). In fact, the related Tax protein from HTLV-2 is nontumorigenic due, in part, to the absence of the carboxyl-terminal PBM (Rousset et al., 1998; Suzuki et al., 1999a; Hirata et al., 2004). Interestingly, the activation of the NF-κB and CREB pathways by Tax does not require the PBM (Yamaoka et al., 1996, 1998; Matsumoto et al., 1997; Xie et al., 2006); yet, the activation of the noncanonical NF-κB pathway functions together with the PBM to augment the transforming ability of Tax (Higuchi et al., 2007). HTLV-1 mutants lacking the Tax PBM can transform human T cells, albeit less efficiently than virus with wild-type Tax, but cannot establish persistent infection in rabbits (Xie et al., 2006). These results suggest that the Tax PBM is required for infection *in vivo* but may be dispensable for cellular transformation *in vitro*.

The oncogenic potential of Tax relies on its interaction with the PDZ domain of hDlg1 (Hirata et al., 2004; Tsubata et al., 2005). Tax is thought to promote cellular proliferation by interfering with the binding of the Dlg1 PDZ domain to the adenomatous polyposis coli tumor suppressor protein (Suzuki et al., 1999a). Interestingly, knockdown of hDlg1 expression enhanced the transforming potential of Tax, and less hDlg1 was expressed in human T-cell lines immortalized by HTLV-1 than in control cells, suggesting that the inactivation of hDlg function is crucial for cellular transformation by Tax (Ishioka et al., 2006). In addition, Tax binds to the human homologue of the *Drosophila* tumor suppressor Scribble (hScrib), a protein that interacts with hDlg1 to form complexes involved in T-cell signaling. Tax inhibits the ability of hScrib to attenuate T-cell receptor-induced signaling (Arpin-Andre and Mesnard, 2007). The effects of this dysregulation of T-cell signaling and subsequent proliferation signals by Tax, however, are not yet clear. Overall, the PBM of Tax interacts directly with the PDZ domain of multiple proteins to effectively promote viral survival, abnormal cell proliferation, genetic instability, and cellular transformation.

EFFECTS OF TAX ON CELL CYCLE CHECKPOINTS

In addition to effects on transcription regulation, Tax also interferes with the normal regulation of cell cycle checkpoints via protein–protein interactions. In general, Tax stimulates cellular G1/S entry and blocks the initiation of mitosis (Liang et al., 2002). Cyclin D forms an active complex with Cdk4 or Cdk6 during G1 phase of the cell cycle, and this complex phosphorylates retinoblastoma (Rb), which, in its

hypophosphorylated state, sequesters the transcription factor E2F (Weinberg, 1995; Sherr, 2004). Hyperphosphorylation of Rb releases E2F and, in turn, enables the transcriptional activation of E2F-responsive genes that promote cell cycle progression into S phase (Matsushime et al., 1994; Adams, 2001). Tax stabilizes the cyclin/Cdk complexes via direct interactions with cyclin D3, cyclin D2, Cdk4, and Cdk6 (Neuveut et al., 1998). Tax-expressing cells, therefore, exhibit earlier onset of cyclin D/Cdk4/6 kinase activity and subsequent accumulation of hyperphosphorylated Rb (Neuveut et al., 1998; Haller et al., 2002). Tax also binds directly to hypophosphorylated Rb, and this interaction results in the proteasomal degradation of Rb and release of E2F (Kehn et al., 2005). The early release of E2F in Tax-expressing cells truncates G1 phase, interferes with checkpoint control, and accelerates cell cycle entry into S phase (Lemoine and Marriott, 2001).

In mammalian cells, p53 controls the cell's response to stress such as DNA damage and oncogene activation, making it a crucial player in the maintenance of genome integrity. Due to its pivotal role in guarding against cellular transformation, p53 is a major target for inactivation by viral oncoproteins. While HTLV-1 Tax does not bind p53 (Yamato et al., 1993; Pise-Masison et al., 1998a), it does abrogate the transactivating function of p53 and override p53-mediated cell cycle arrest or apoptosis (Uittenbogaard et al., 1995; Pise-Masison et al., 1998b; Ariumi et al., 2000). One mechanism by which Tax inhibits p53 function involves NF-κB-mediated phosphorylation of p53, which likely alters the interactions of p53 with a variety of transcription factors (Jeong et al., 2004). Tax, as discussed above, also suppresses p53-mediated transactivation by competing with p53 for binding to the transcriptional coactivator CBP/p300, thereby disrupting p53-dependent G1 arrest or apoptosis (Grossman, 2001). Furthermore, the inhibition of p53 target genes blocks the induction of the G1/S checkpoint (Van Orden et al., 1999; Ariumi et al., 2000). As a result, DNA replication proceeds despite the presence of growth-suppressive signals. This unchecked cell cycle progression is likely to contribute to immortalization and cellular transformation.

The repair of DNA damage prior to DNA synthesis in S phase is critical for accurate genome replication. In response to DNA damage, p53 is activated to regulate cell cycle progression by delaying S-phase entry and allowing DNA repair to proceed prior to DNA replication. The p53-dependent transcriptional activation of Cdk inhibitors (CKIs), such as $p21^{WAF1/CIP1}$, $p27^{KIP1}$, $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, and $p19^{INK4d}$, inhibits cyclin–Cdk kinase activities, resulting in cell cycle arrest at the G1/S transition (Peter and Herskowitz, 1994; Morgan, 1995; Sherr and Roberts, 1995). However, the inactivation of p53 function by Tax allows cells to bypass the G1/S checkpoint and enter S phase where damaged DNA can be replicated.

Tax directly binds to and inhibits many CKIs. For example, the binding of Tax to the Cdk4/6 inhibitors, $p15^{INK4b}$ and $p16^{INK4a}$, obstructs their ability to block kinase activity, thereby interfering with G1 growth arrest and disabling the restriction of damaged DNA replication (Low et al., 1997; Hatta and Koeffler, 2002). Tax also represses the transcription of $p18^{INK4c}$ and $p19^{INK4d}$ through E-box elements, resulting in decreased expression and activity of these cell cycle inhibitors (Suzuki et al., 1999a). Together, these effects of Tax stimulate cell cycle progression. CKIs induce cell cycle arrest by regulating cyclin–Cdk kinase activities, consistent with their roles as tumor suppressor

proteins that are frequently deleted in human cancer cells (Sherr and Roberts, 1995; Sherr, 2004). Functional inhibition of CKIs by Tax may mimic the deletion of these genes in cancer cells, thereby promoting constitutive activation of cyclin–Cdk complexes and cell cycle progression.

In contrast to the ability of Tax to inhibit p53-dependent $p21^{WAF1/CIP1}$ expression, Tax actually activates the expression of the CKI $p21^{WAF1/CIP1}$ in a p53-independent manner. These seemingly contradictory roles of Tax are interesting due to the unique features of $p21^{WAF1/CIP1}$ in which increased $p21^{WAF1/CIP1}$ expression is not always inhibitory to the cell cycle. In fact, $p21^{WAF1/CIP1}$ can bind and stabilize Cdk complexes such as cyclin D/Cdk4/6/Cdk2, thereby increasing kinase activity and promoting cell cycle progression from G1 to S (Akagi et al., 1996; Cereseto et al., 1996; De La Fuente et al., 2000; Haller et al., 2002; Hatta and Koeffler, 2002).

DNA damage-induced cell cycle arrest also occurs at the G_2/M checkpoint. Following DNA damage, Chk1 and Chk2 signaling pathways activate several downstream targets including p53 (G1 cell cycle arrest), Cdc25 (S-phase delay), and Cdc25A/C (G_2 arrest). Chk1 phosphorylation targets Cdc25A for proteasomal degradation and inhibits the activation of the Cdk1/2 complex, which is required for the progression through the S-phase and G_2/M checkpoints. Tax directly interacts with Chk1 and inhibits its ability to phosphorylate p53 (Park et al., 2004). As a consequence, the inhibition of Cdc25A degradation in Tax-expressing cells attenuates cell cycle arrest in G_2. In addition, Tax binds to Chk2, and this complex colocalizes at sites of DNA damage (Haoudi and Semmes, 2003), preventing its release from chromatin and attenuating gamma irradiation-induced apoptosis (Park et al., 2005; Gupta et al., 2007). Thus, Tax affects multiple pathways to ultimately promote cell cycle progression despite accumulation of DNA damage.

INHIBITION OF DNA REPAIR AND PROMOTION OF GENOME INSTABILITY BY TAX

Cellular transformation is frequently associated with genome instability. Although no specific type of chromosomal damage has been associated with the development of ATL, HTLV-1-transformed lymphocytes isolated from ATL patients as well as Tax-transformed cells in culture exhibit a wide range of chromosome abnormalities, including duplications, deletions, translocations, rearrangements, and aneuploidy (Miyamoto et al., 1983; Rowley et al., 1984; Whang-Peng et al., 1985; Itoyama et al., 1990).

Altered chromosome numbers, which are a hallmark of all types of tumors, are classified as either aneuploidy, the gain or loss of whole chromosomes, or loss of heterozygosity (LOH), the gain or loss of chromosome sections or single genes (Lengauer et al., 1998). Both of these types of genetic alterations affect the expression and function of oncogenes, tumor suppressors, and/or other essential proteins. Cytogenetic and microsatellite analyses have detected both aneuploidy and LOH in ATL tumors. Although no uniform karyotypic abnormalities have been consistently identified in all ATL tumors (Kao et al., 2000), specific abnormalities including trisomy 3 and 7, amplification of 14q, deletions of 1q and 6q, monosomy of the X chromosome, and loss of

the Y chromosome appear more frequently than others in ATL cells (Zech et al., 1976; McCaw et al., 1977; Fukuhara and Rowley, 1978; Ueshima et al., 1981; Miyamoto et al., 1983; Rowley et al., 1984; Whang-Peng et al., 1985; Sanada et al., 1987; Fujita et al., 1989; Kamada et al., 1992). These chromosomal rearrangements are likely to cause or contribute to cellular transformation and tumor progression.

Tax dysregulates cellular functions but does not directly induce DNA lesions (Kao and Marriott, 1999; Majone and Jeang, 2000). Rather, Tax induces genome instability by interfering with DNA metabolism, cell cycle progression, and apoptosis of cells containing dangerous amounts of DNA damage. Several repair pathways that are used by cells to restore DNA lesions resulting from exogenous genotoxic factors or normal replication processes include nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), homologous recombination (HR), and nonhomologous end joining (NHEJ) (Sancar et al., 2004). Tax appears to promote aneuploidy, genomic instability, and subsequent tumorigenesis by directly inhibiting these DNA repair pathways.

Initial studies on the effects of Tax expression on DNA repair found that Tax represses the transcription of DNA polymerase β, an essential enzyme involved in BER (Jeang et al., 1990). Subsequently, HTLV-1-transformed cells and cells transiently expressing Tax were shown to have defects in BER (Philpott and Buehring, 1999). Tax expression has now been associated with increased mutation frequencies within the cellular genome (Miyake et al., 1999), which likely contributes to cellular transformation.

Tax also targets the most versatile DNA repair pathway, NER, which requires DNA polymerases delta and epsilon and uses proliferating cell nuclear antigen (PCNA) as a cofactor (Riedl et al., 2003). In the presence of DNA lesions, levels of the cyclin-dependent kinase $p21^{WAF1/CIP1}$ increase to interact with PCNA. In this way, DNA replication is blocked without affecting PCNA-dependent repair (Fotedar et al., 2004). Excessive PCNA, however, can overcome the $p21^{WAF1/CIP1}$-induced replication block, and as a result, the polymerase continues to synthesize DNA through template lesions, resulting in nucleotide misincorporation (Li et al., 1994; Mozzherin et al., 1996, 1997). Interestingly, the suppression of NER by Tax correlates with its ability to activate PCNA transcription and increase the levels of PCNA protein (Lemoine et al., 2000). Thus, Tax encourages error-prone DNA repair by altering the BER polymerase and the NER cofactor, PCNA.

Presently, there is no direct evidence that Tax affects recombinational repair; however, several reports suggest that Tax may interfere with the repair of DNA double-strand breaks. Tax-expressing cells form MN that contain whole chromosomes and centric and acentric fragments (Majone et al., 1993). MN are produced as by-products of DNA damage and indicate the presence of unresolved chromosomal breaks. Thus, MN formation reflects an inability to properly repair DNA damage and segregate chromosomes following exposure to specific mutagens. Consistent with observations of chromosomal abnormalities in ATL tumors, the presence of MN in Tax-immortalized or Tax-transfected cells is greater than in cells lacking Tax expression. In fact, cells that express wild-type Tax displayed MN frequencies of 1.5%–1.7%, while cells that express transcriptionally inactive Tax mutants had MN frequencies of only 0.4%–0.7% (Majone et al., 1993). The increased presence of both kinetochore-positive and

-negative MN in these cells demonstrates the combined clastogenic and aneuploidogenic effects of Tax (Majone et al., 1993; Majone and Jeang, 2000). Taken together, these results indicate that Tax regulates cellular processes that repress DNA repair while promoting DNA replication and cell cycle progression in the presence of DNA lesions. Replication of unrepaired lesions could lead to the accumulation of mutations and ultimately contribute to Tax-mediated genomic instability and transformation.

Tax-mediated dysregulation of telomerase (hTERT) provides yet another opportunity to generate chromosomal abnormalities. Specific nucleotide repeat sequences, known as telomeres, are added to the ends of chromosomal DNA by hTERT. Telomeres prevent the fusion of chromosomal ends as well as their degradation by exonucleases. In the absence of hTERT, telomere shortening occurs, which allows end-to-end chromosomal fusions and formation of dicentric chromosomes that are vulnerable to breakage during mitosis. Aneuploidy, chromosomal rearrangements, and translocations can result. Indeed, *in situ* labeling of DNA ends revealed more unprotected DNA ends in Tax-expressing cells than in control cells (Majone and Jeang, 2000). Tax represses hTERT expression during mitogenic stimulation and activates hTERT expression in the absence of mitogenic stimulation (Gabet et al., 2003; Sinha-Datta et al., 2004). Following T-cell stimulation, decreased hTERT expression may cause transient genomic instability that is relieved upon removal of the mitogenic stimulus. Inhibition of telomerase activity may also promote persistent telomere dysfunction, resulting in the accumulation of unbalanced chromosomal rearrangements (Gabet et al., 2003). The biologic advantage of hTERT repression for HTLV-1 remains to be determined; however, Tax-mediated activation of hTERT in the absence of the mitogenic signal may offer a long-term proliferative advantage to cells that have acquired chromosomal abnormalities. Recently, Tax was shown to prematurely attenuate ataxia telangiectasia mutated (ATM) kinase phosphorylation, thereby blunting the cellular response to double-strand DNA breaks (Chandhasin et al., 2008). This effect contributes to premature DNA replication in the presence of genomic lesions, leading to genomic instability and cellular transformation.

Gene amplification occurs at a greater rate in cancer cells than in normal cells (Lengauer et al., 1998) presumably since amplifications occur in response to chromosomal breaks that result from incorrectly resolved DNA recombination (Kuo et al., 1994; Wettergren et al., 1994; Coquelle et al., 1997). These abnormalities are most prevalent when p53 is inactivated in mammalian cells (Yin et al., 1992). In the presence of Tax, gene amplification increased four- to fivefold, and cells failed to undergo a typical, p53-dependent G1 arrest, suggesting that the ability of Tax to alter cell cycle arrest depends on its ability to repress p53 activity (Lemoine and Marriott, 2002). Therefore, Tax-mediated repression or inactivation of p53 and DNA repair mechanisms discussed above may contribute to the induction of gene amplification.

Aneuploidy is a general characteristic of cancer cells and is commonly detected in ATL cells. Alterations in chromosome number arise as a result of mitotic perturbations and are generally considered to be a cause, rather than a consequence, of transformation (Rasnick, 2002). Cells use the mitotic spindle assembly checkpoint (MSC) to ensure accurate partitioning of replicated chromosomes during mitosis and to avoid aneuploidy. This checkpoint halts the progression from metaphase to anaphase in the presence of spindle damage or segregation errors. Several protein groups are required for

the proper functioning of the MSC complex: monopolar spindle 1 (Mps1), budding uninhibited by benzimidazole (BUB-1, -2, and -3), mitotic arrest defective (MAD-1, -2, and -3), and the anaphase-promoting complex (APC) (Hoyt et al., 1991; Li and Murray, 1991; Cohen-Fix et al., 1996; Abrieu et al., 2001; Jallepalli and Lengauer, 2001). Malfunction of the MSC can result in defective sister chromatid segregation and lead to loss or gain of genetic material. In fact, the disruption of the MSC is associated with altered chromosome structures and numbers (Lavia et al., 2003). Several HTLV-1-transformed cell lines have been shown to lack MSC integrity following exposure to nocodazole, a microtubule depolymerizing agent (Kasai et al., 2002). In transfected mouse embryo fibroblasts, Tax localizes at the centrosomes during M phase (Peloponese et al., 2005). Indeed, Tax-expressing cells accumulate an abnormal number of centrosomes, which are associated with MN formation and aneuploidy (Nitta et al., 2006). Consistent with these observations, Tax binds and inactivates the centrosomal hsMAD1 protein and sequesters both MAD-1 and -2 in the cytoplasm, preventing the latter from proper kinetocore localization (Jin et al., 1998). In addition, Tax stimulates cellular G1/S entry and blocks mitosis (Liang et al., 2002). The ability of Tax to disable these critical cellular functions results in chromosome missegregation during mitosis.

Recently, Tax was shown to interact directly with the APC, a multiprotein E3 ubiquitin ligase complex that controls the transition from metaphase to anaphase (Liu et al., 2005). This interaction induces premature activation of the APC resulting in delayed mitotic transition and multinucleation. Early APC activation also causes premature degradation of cyclin A, cyclin B1, securin, and Skp2; mitotic aberrations; rapid senescence; and cell cycle arrest in HeLa and yeast cells (Liu et al., 2005; Kuo and Giam, 2006). Tax-induced rapid senescence (*tax*-IRS) in HeLa cells is primarily mediated by stabilization of $p27^{KIP1}$ and is accompanied by a substantial surge in $p21^{WAF1/CIP1}$ mRNA and protein levels. This senescence is causally associated with APC activation (Kuo and Giam, 2006) as Tax mutants that fail to activate the APC do not induce G1 arrest (Merling et al., 2007). Interestingly, HTLV-1-transformed T cells express low levels of $p27^{KIP1}$, which allows them to evade permanent arrest (Kuo and Giam, 2006). Taken together, the functions of Tax provide an environment favorable for the development of aneuploidy in ATL cells.

SUMMARY

For several decades, RNA tumor viruses, including HTLV-1, have been intensely studied because of their association with human cancers and their usefulness as model systems for the study of cellular transformation. The HTLV-1 Tax oncoprotein contributes to tumorigenesis by interfering with cellular transcription regulation, tumor suppressor function, cell cycle regulation, and DNA repair. This review has discussed mechanisms that lead to increased genome instability and cellular transformation mediated by the HTLV-1 Tax oncoprotein. Tax expression is associated with the formation of specific types of genomic alterations that are frequently associated with ATL, including increased point mutations, altered chromosome number, chromosomal translocations, and gene amplification. Although the mechanisms used by Tax to

transform cells and promote tumor progression are not fully understood, the effects of Tax on pathways that regulate the development of genomic alterations have been discussed here.

While changes in transcription do not directly lead to cellular transformation, they can induce a highly proliferative state, which is generally thought to be a *sine qua non* condition for oncogenesis. These transcriptional changes affect the ability of the host cell to properly respond to genotoxic agents by altering the concentration and, possibly, localization of proteins involved in the repair of DNA damage, cell cycle control, and apoptosis, ultimately inducing genomic instability and promoting oncogenic transformation. Mammalian cells have evolved mechanisms to maintain genomic integrity by inducing cell cycle arrest in response to DNA damage. Such checkpoint mechanisms allow time to repair DNA damage before the cell commits to DNA replication and cell division. It appears that the functions of Tax have evolved to promote DNA replication and cellular proliferation in order to ensure efficient replication of the proviral genome in the host cell. As an apparently unintended consequence, Tax promotes the proliferation and accumulation of cells that are genetically unstable, some of which may contain transforming mutations. It is almost certain that continued study of mechanisms of Tax-mediated transformation will provide new insight into viral and cellular processes that regulate transformation, and may reveal new therapeutic interventions for the treatment and/or prevention of ATL.

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16

HIV-1-ASSOCIATED MALIGNANCY: BASIC AND CLINICAL ASPECTS

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BACKGROUND

The widespread use of highly active retroviral therapy (HAART) in 1996 for treating human immunodeficiency virus, type 1 (HIV-1), has led to a dramatic decrease in opportunistic infections and in morbidity and mortality rates. Its impact on HIV-associated non-Hodgkin's lymphoma (H-NHL), however, has been variable. There have been some studies that have reported an increase (Jacobson et al., 1999; Mocroft et al., 2000; Bonnet et al., 2006), and some that have reported a decrease (Ledergerber et al., 1999a,b; Grulich et al., 2001; Franceschi et al., 2003) in H-NHL incidence (Fig. 16.1). Others have also claimed that the incidence has not changed but contributes to a greater percentage of first acquired immunodeficiency syndrome (AIDS)-defining illnesses. Additionally, they claim that nadir $CD4^+$ T-cell count, high HIV viral load, increased age, no prior HAART use, and male gender are predictors in the development of H-NHL (Matthews et al., 2000; Cheung et al., 2005). The discrepancies among reports could be a reflection of differences in population groups and the underestimation of incidence rates by the Center for Disease Control (CDC). The CDC claims that in the United States, individuals afflicted with HIV are 60 times at greater risk of contracting NHL than the general population, with an incidence rate of H-NHL being 2.9% (Beral et al., 1991; Knowles, 2003). However, the

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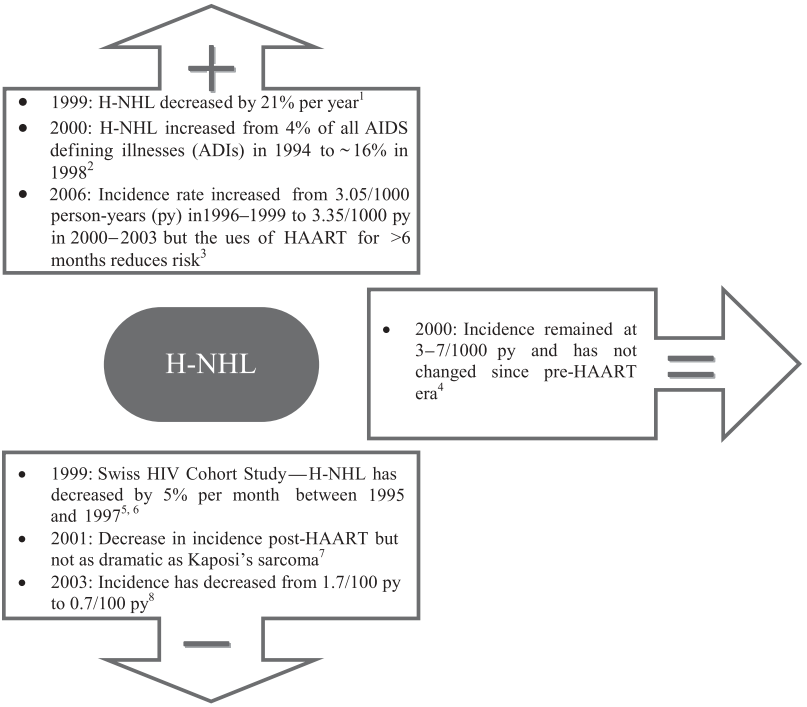

Figure 16.1. Discrepancies in HIV-associated non-Hodgkin's lymphoma (H-NHL) incidence rates. The pattern of increases (+), decreases (–), or unchanged (=) number of cases documented in various parts of the world as the use of HAART increased. [1]Jacobson et al., 1999; [2]Mocroft et al., 2000; [3]Bonnet et al., 2006; [4]Franceschi et al., 2003; [5]Ledergerber et al., 1999a; [6]Ledergerber et al., 1999b; [7]Grulich et al., 2001; [8]Matthews et al., 2000.

wide spectrum of HIV-related illnesses results in illnesses other than H-NHL being diagnosed; thus, realistic incidence rates lay between 4% and 10% (Biggar and Rabkin, 1992; Levine, 2000; Knowles, 2003). Inconsistencies in reporting, diagnosing, access and adherence to therapy, drug resistance, and improvements in HIV therapy have made reporting the true incidence extremely challenging (Lim and Levine, 2005).

Behind Kaposi's sarcoma (KS), H-NHL is the second most common neoplasm occurring in the adult HIV-infected population. It is particularly more prevalent in intravenous drug users (IVDU) and individuals with hemophilia (Beral et al., 1991; Levine, 1992; Knowles and Pirog, 2001). Approximately 10% of all NHL occurring in the United States is attributed to H-NHL (Beral et al., 1991; Biggar and Rabkin, 1992; Rabkin, 1994). In contrast to adults infected with HIV, H-NHL is the most common neoplasm occurring in children (0–12 years) and adolescents (13–19 years) in the United States and Europe. Similar to H-NHL estimates in adults, incidence rates in children and adolescents are possibly underreported. Biggar et al. found that among the 100 childhood H-NHL cases in their study, only 35% were recorded in both AIDS and cancer registries, while 37% were only found in the AIDS registry and 28% were only found in the cancer registry (Biggar et al., 2000).

To obtain a better grasp on the situation, numerous linkage studies that link AIDS and cancer registries have been attempted. If these registries can reliably identify the serostatus of patients, they can provide a significant contribution to H-NHL research. Since the California cancer registry requires the HIV serostatus to be included in NHL incidence reports, Diamond et al. compared the registry status against medical records and found 8% false positives, 3% false negatives, a positive predictor value of 93%, and a negative predictor value of 97%. The results from their evaluation concluded that the use of cancer registries is a valid research practice (Diamond et al., 2007).

In 1982, the CDC defined KS and primary central nervous system lymphoma (PCNSL) as AIDS-defining malignancies, which was subsequently modified to include NHL and invasive cervical cancer (Centers for Disease Control and Prevention, 1992, 1994; Cheung et al., 2005). In addition to the aforementioned AIDS-defining malignancies such as KS and H-NHL, other cancers including Hodgkin's lymphoma (HL), invasive anal carcinoma, multiple myeloma, leukemia, lung cancer, and cancers involving the oral cavity, lip, esophagus, stomach, liver, pancreas, larynx, heart, vulva, vagina, kidney, and soft tissues were also found to occur in HIV-infected individuals (Goedert et al., 1998; Frisch et al., 2001; Gallagher et al., 2001; Grulich et al., 2002; Cheung et al., 2005). Of adults infected with HIV, 4%–5% identified H-NHL as their AIDS-defining illness, whereas only 2.5% of HIV-infected children below 13 years of age attributed their AIDS-defining illness to malignancy, with the majority being H-NHL (Burkitt's lymphoma [BL], diffuse large B-cell immunoblastic [IBL] lymphoma, PCNSL).

It has been speculated that the risk for developing H-NHL is 100-fold after being infected with the virus for 7–8 years and 165-fold within 3 years of an AIDS diagnosis when compared with the general population, with the relative risk highest in high-grade lymphomas (652-fold) and in intermediate lymphomas (261-fold) (Biggar and Rabkin, 1992; Lim and Levine, 2005). The typical clinical spectrum of H-NHL includes systemic NHL, PCNSL, and primary effusion lymphoma (PEL). The general trends of H-NHL coinciding with the HAART era that have been published in literature are displayed in Figure 16.1. Due to the differences in resources and in reporting criteria throughout the world, a contrasting picture has emerged that has made the understanding of the pathogenesis and prognosis of H-NHL difficult and challenging. There have been several studies that reported a significant decrease in H-NHL in the HAART era (International Collaboration on HIV and Cancer, 2000; Kirk et al., 2001a,b; Franceschi et al., 2003; Bonnet and Morlat, 2006; Bonnet et al., 2006; Busnach et al., 2006; Crum et al., 2006; Diamond et al., 2006; Engels et al., 2006; Biggar et al., 2007) with the exception of a slight increase in Burkitt H-NHL (International Collaboration on HIV and Cancer, 2000; Diamond et al., 2006). This decrease was found to be extremely dependent on prolonged exposure (6 months or greater) to HAART, whereas uncontrolled HIV RNA viral load could produce a much greater risk (Besson et al., 2001; Kirk et al., 2001b; Bonnet et al., 2006). In contrast, others have reported no change in H-NHL and that HAART has not yet been proven to have a profound effect on H-NHL (Buchbinder et al., 1999; Jacobson et al., 1999; Ledergerber et al., 1999a,b; Levine et al., 2000; Matthews et al., 2000; Serraino et al., 2000), which could be the reflection of inadequate follow-up time following widespread use of the therapy (Buchbinder et al., 1999). Of particular interest is the epidemiology of H-NHL in HIV-infected children, in

which the prevalence of HIV infection and cancer incidence rates are disproportionately lower (Biggar et al., 2000; Dal Maso et al., 2001). In comparison to adults, with three to eight cases of H-NHL per 1000 person-years, the incidence in children ranges from 0.66 cases per 1000 children to less than 4 per 1000 children in the United States and Italy, respectively. The pathology observed in children is similar to that experienced in adults, with the exception of more leiomyosarcoma reported in children (Biggar et al., 2000; Serraino et al., 2000; Besson et al., 2001).

Currently, it is unknown why epidemiology reports of H-NHL have been inconsistent; however, it has been attributed to factors such as HAART and its effect on the immune system, the direct and indirect role of HIV, study design limitations, population differences, and access and adherence to treatment (Caselli et al., 2000; Levine et al., 2000; Gates and Kaplan, 2002; Lim and Levine, 2005). Analogous to the inconsistent reports in epidemiology, variable successes in treatment responses to chemotherapy and immunomodulators have also been documented (Sparano, 1995; Sandler and Kaplan, 1996; Levine, 2000; Kersten and Van Oers, 2001; Tirelli et al., 2002; Spina et al., 2003). These observed differences might reflect the etiologies of the subtypes of H-NHL as well as the advances in supportive protocols and constant improvements that occur with chemotherapy and antiretroviral therapy (Cheung et al., 2005).

Despite many of the potential advancements in the understanding of H-NHL, many important gaps remain, which may be attributed to limited detailed data on long-term cancer trends, the fact that elevated risks that occur in other non-AIDS-defining cancers are not thoroughly evaluated, possible differential involvement of the immune function in H-NHL development, and the inexistence of data concerning the overall cancer risk that will have a simultaneous increase with the survival rates of HIV-infected individuals (Scadden, 1999; Engels et al., 2006). Better understanding of the pathogenic mechanisms that lead to H-NHL will help to address these concerns, as well as facilitate in providing enhanced prevention and improve the targeted treatment strategies. Recent progress in pathogenesis, epidemiology, and treatment of H-NHL has shed more optimism for individuals diagnosed with H-NHL. The impact and role of HIV and influence of HAART have confounded the advances made in this area. The interplay between immunodeficiency caused by HIV and immune restoration resulting from HAART creates a challenging environment in which H-NHL develops. While much progress is required in further elucidating the pathogenesis and improving prognosis, the evolving picture of H-NHL over time, since the use of HAART, illustrates the complex nature of this disease, which is influenced by HIV, the immune system, and various therapeutic interventions. This chapter summarizes recent advances made in our understanding of H-NHL.

CLASSIFICATION AND PATHOLOGY

H-NHL consist of a heterogeneous group of malignant disorders that ranges from aggressive high, to intermediate, to indeterminate-phenotype lymphomas. It includes small, noncleaved cell lymphoma (Burkitt or Burkitt like) and diffuse large cell lymphoma, along with some lesser common subtypes such as T-cell NHL, PCNSL, mucosa-associated lymphoid tissue (MALT) neoplasm, and PEL (Gaidano et al., 1994; Mueller and Pizzo, 1996; Levine, 2000; McClain et al., 2000; Bower and Fife, 2001; Knowles and Pirog, 2001; Sparano, 2001).

TABLE 16.1. HIV-Associated Non-Hodgkin's Lymphoma Classification

Type	% of H-NHL
Systemic lymphoma	50–80
Aggressive/high grade	45–70
Burkitt's lymphoma	10–50
Classic	5–30
Plasmacytoid differentiation	5–20
Burkitt like	<2
Diffuse large B-cell lymphoma (DLBCL)	35–50
Immunoblastic (IBL)	10–20
Centroblastic (CB)	25–30
Intermediate grade	10–33
Low grade	<1
Primary CNS lymphoma (PCNSL)	20–25
Primary effusion lymphoma (PEL)	2–4
Classic: body cavity based	<4
Solid: extracavitary	<4
T-cell lymphoma	<2

All percentages are approximations that are based on published data; variations may reflect differences in population groups.

Data from Batchelor et al., 2003; Carbone et al., 2003; Mbulaiteye et al., 2003; Engels et al., 2006; Biggar et al., 2007.

Classification of H-NHL is divided among three broad categories and is based on its perceived anatomical origin: systemic lymphoma (nodal and extranodal), PCNSL, and PEL, also referred to as body cavity-based lymphoma (Table 16.1).

Systemic NHL accounts for the largest percentage of H-NHL (50%–80%) and can be further classified into aggressive, intermediate, and low-grade lymphomas. The two most documented systemic lymphomas are BL and diffuse large B-cell lymphoma (DLBCL), both of which are aggressive. BL is noted for being infected with Epstein–Barr virus (EBV) in 30%–50% of cases, having *c-myc* gene alterations in 100% of cases and p53 gene alterations in 50%–60% of cases. There are three different types of BL: classic, plasmacytoid differentiated, and atypical, also known as Burkitt like. On the other hand, DLBCL is divided into DLBCL with centroblastic (CB) features and DLBCL with IBL features. CB DLBCL is infected with EBV in 30% of cases and has *bcl-6* gene alterations in 20% of cases. IBL DLBCL is infected with EBV in 90% of cases and express latent membrane protein-1 (LMP1) in 90% of cases. Furthermore, 85% of systemic lymphomas have extranodal involvement, with the central nervous system (CNS) being the most common site followed by the gastrointestinal tract, bone marrow, and liver. These lymphomas can originate or present virtually any site, such as the orbit, mandible, skin, salivary glands, oropharynx, heart, lungs, liver, rectum, muscles, bones, kidneys, gonads, and adrenal glands, many of which are unconventional NHL sites in non-HIV-infected individuals (Knowles, 2003).

PCNSL comprises 20%–25% of H-NHL, and it is estimated that the HIV-infected population is 1000 times more susceptible to acquiring PCNSL (Beral et al., 1991). EBV infection occurs in 100% of PCNSL cases, with LMP1 expressed in 90% of cases. PCNSL typically contains IBL features.

A rare but fascinating subset of H-NHL is PEL, accounting for 2%–4% of all H-NHL. PEL is unique, in that it is typically diagnosed in individuals infected with HIV and proliferates independently to tumor mass primarily in the pleural, pericardial, or abdominal cavities as lymphomatous effusions (Knowles et al., 1989). These cells often contain Kaposi's sarcoma herpesvirus (KSHV)/human herpesvirus-8 (HHV8) and EBV in 100% and 70% of cases, respectively, and they typically do not express CD20.

A minimal number (less than 2%) of H-NHL consist of T-cell lymphomas, although it is rather uncommon as the majority of tumors are derived from B cells (Knowles, 1996; Levine et al., 2000).

PATHOGENIC MECHANISMS

Although the exact etiology of H-NHL is currently undetermined, several preexisting factors are thought to play a role in lymphoma development. These factors include the disruption of immune status with compromised immune surveillance, virus-induced immunosuppression, chronic antigen stimulation, cytokine release and dysregulation, genetic abnormalities, and coinfection with EBV or other pathogenic viruses (Gates and Kaplan, 2003). The existence of infectious agents distinct from HIV is also plausible. EBV and KSHV/HHV8 are the primarily studied pathogenic agents as they have been shown to be associated with H-NHL (Table 16.2). They most likely act in concert during the transformation and differentiation process producing the various histological H-NHL subtypes. Insight into the pathways of H-NHL development can be gained through histological H-NHL characterization (Boshoff and Weiss, 2002; Sparano, 2003).

During B-cell development, naive B cells enter the germinal center (GC) and emerge as memory B cells or plasma cells that secrete immunoglobulin. GCs are specialized compartments in peripheral lymphoid tissues, where high affinity antibody-producing cells are generated (Ranuncolo et al., 2007). Upon T-cell activation, naive B cells enter GCs and become centroblasts and mature into centrocytes after several rounds of proliferation. During this process, the centroblasts simultaneously undergo rapid cell proliferation, class switch recombination, somatic hypermutation of the immunoglobulin variable genes (IgV), and *bcl-6* mutation. The resultant centrocytes then migrate into a heterogeneous zone composed of T cells, macrophages, and follicular dendritic cells (FDCs). Prior to exiting the GC as memory B cells or plasma cells, centrocytes must first undergo a successful selection process by FDCs (Ranuncolo et al., 2007). Furthermore, the heavy and light immunoglobulin genes accumulate point mutations as rearranged genes. B-cell transformation can take place during any B-cell differentiation stage, producing the various subtypes of H-NHL. Based on this phenomenon of B-cell maturation, genotypic and phenotypic markers can be used to classify lymphomas into GC-derived or post-GC-derived B-cell lymphomas. Genotypic markers

TABLE 16.2. Genetic Mutations and Viral Infections Associated with H-NHL

Type	EBV Infection (%)	*c-myc* Mutation (%)	*p-53* Mutation (%)	*bcl-6* Mutation	LMP1 Expression (%)	KSHV/ HHV8 Infection (%)
Systemic lymphoma	40–50	NA	NA	NA	NA	NA
Aggressive/high grade						
Burkitt's lymphoma	30–50	100	50–60	NA	NA	NA
Diffuse large B-cell lymphoma (DLBCL)						
Immunoblastic (IBL)	90	NA	NA	NA	90	NA
Centroblastic (CB)	30	NA	NA	NA	NA	NA
Primary central nervous system lymphoma (PCNSL)	100	NA	NA	NA	90	NA
Primary effusion lymphoma (PEL)	70	NA	NA	NA	NA	100

EBV, Epstein–Barr virus; LMP1, latent membrane protein-1; KSHV/HHV8, Kaposi's sarcoma herpesvirus/ human herpesvirus-8; NA, current data shows no association.

Data from Subar et al., 1988; Carbone et al., 2003; Knowles et al., 2003.

include IgV and *bcl-6* mutations that are indicative of a GC- or post-GC-derived B cell (Carbone, 2003). Phenotypic markers include the expression of *bcl-6*, MUM1, and CD138/syndecan-1. As noted above, *bcl-6* expression is indicative of a GC B cell and MUM1, and CD138/syndecan-1 expression is indicative of a post-GC B cell. BL and CB DLBCL contain the *bcl-6*-positive/MUM1-negative/syndecan-1-negative profile as expressed in GC B cells (Carbone et al., 2001). In contrast, IBL DLBCL, PCNSL, PEL, plasmablastic lymphoma, and HIV-HL contain the *bcl-6*-negative/MUM1-positive/ syndecan-1-positive profile as expressed in late centrocytes and post-GC B cells (Carbone et al., 2001) (Fig. 16.2).

Since coinfection with other pathogenic agents such as EBV and KSHV/HHV8 often occur, the presence of one or both of these viruses also contribute to the development of a characteristic profile for the different H-NHL subtypes. EBV is the most common virus as it is associated with 40%–50% of systemic H-NHL and 50%–70% of DLBCL (Subar, 1988; Carbone, 2003; Knowles, 2003). Conversely, PEL often contains both viruses with KSHV/HHV8 in 100% of its cases and EBV in 70% of its cases.

Although there are similarities between the EBV and KSHV/HHV8, significant differences between the two exist and may account for the different disease patterns associated with each. KSHV/HHV8 is a 140 kb gammaherpesvirus with sequence

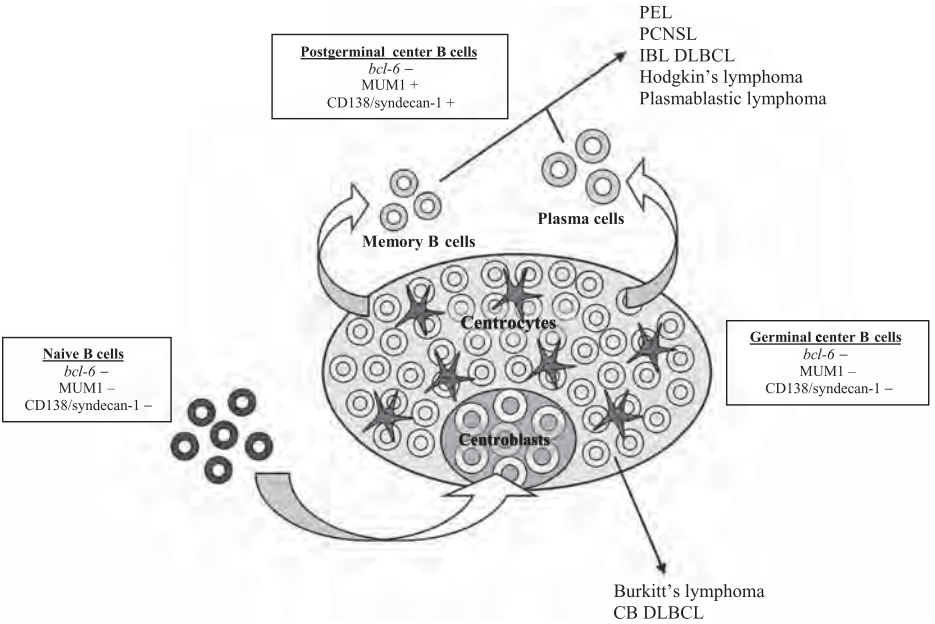

Figure 16.2. Representative pathways leading to H-NHL. Upon T-cell activation, naive B cells enter the germinal center (GC), become proliferating centroblasts, and mature into nonproliferating centrocytes, which is associated with somatic hypermutation of the immunoglobulin variable genes, class switch recombination, and *bcl-6* mutations. Centrocytes undergo selection process by follicular dendritic cells (FDCs) before emerging as memory B cells or plasma cells. Markers such as *bcl-6*, multiple myeloma oncogene (MUM1), or CD138/syndecan-1 are used to identify histological subtypes. H-NHL Burkitt's lymphoma (BL) and centroblastic diffuse large B-cell lymphoma (CB DLBCL) express the *bcl-6*+/MUM1–/syndecan– profiles as shown in GC B cells. Primary effusion lymphoma (PEL), primary central nervous system lymphoma (PCNSL), immunoblastic diffuse large B-cell lymphoma (IBL DLBCL), Hodgkin's lymphoma, and plasmablastic lymphoma contain the *bcl-6*–/MUM1+/syndecan+ profile as shown in post-GC B cells.

homology to EBV and *Herpes saimiri* virus. Due to the fact that KSHV/HHV8 is consistently present in PELs, it is possible that this virus is critical in PEL pathogenesis and development, but the exact etiology remains unknown. In contrast, EBV infection is associated with the majority of H-NHLs but is more common in H-NHL subtypes containing post-GC B-cell characteristics than in H-NHL subtypes containing GC B-cell characteristics (Ambinder, 2001). Thus, EBV is detected in virtually all PCNSL but is detected in only 50%–70% of all DLBCL. The expression of the EBV-encoded oncoprotein, LMP1, is primarily found in post-GC H-NHL variants and is unusual in GC variants (Hamilton-Dutoit et al., 1993; Ambinder, 2001; Carbone, 2002). These defining characteristics play a crucial role in the diagnosis of H-NHL subtypes. It has been speculated that resting B cells serve as EBV reservoirs and that patients with compromised immune systems are at risk for infection leading to polyclonal B-cell

activation and aberrant B-cell regulation. A second hit produces *c-myc* gene rearrangements, inactivating the p53 tumor suppressor gene and ultimately results in lymphoma development through monoclonal emergence (Klein and Klein, 1985; Knowles, 2003). Because the EBV genome is only detectable in 50%–70% of all DLBCL, other factors have been implicated in the etiology, including impairment of T-cell surveillance and polyclonal B-cell expansion, both of which may also produce gene rearrangements.

Gene alterations involving oncogenes and immunoglobulin genes have been extensively studied in H-NHL. Although the exact impact of these alterations on H-NHL is still unclear, analyses of immunoglobulin genes, *c-myc*, *bcl-6*, and *ras* regions strengthen our understanding of the molecular events that lead to H-NHL. As previously described, marked B-cell proliferation in the setting of HIV-associated immunosuppression may be an important factor in the pathogenesis of H-NHL. These genetically unstable hyperstimulated B cells are likely to undergo translocation during Ig rearrangement. Translocation of a part of the *c-myc* gene on chromosome 8 and the immunoglobulin gene on chromosome 14, or the kappa or lambda light chain genes on chromosomes 2 and 22, occurs with defective recombination during isotype switching of the constant regions with preferential V_H Ig usage. In some cases, translocation can cause the *bcl*-6 gene to become juxtaposed onto non-Ig genes (Ohno, 2004). As a result, *c-myc* deregulation ensues, and differentiation of GC B cells into plasma cells occurs; the plasma cells are then predisposed to neoplastic transformation (Ohno, 2004). Despite the majority of studies that have repeatedly demonstrated that H-NHL cases exhibit clonal immunoglobulin gene rearrangements, there have been reports of "polyclonal lymphomas" that are characterized by mixed immunophenotypes, undetectable EBV, and no evidential *c-myc* rearrangement (McGrath et al., 1991; Herndier et al., 1992; Shiramizu et al., 1992; Ng et al., 1997; Rodriguez-Alfageme et al., 1998; McClain et al., 2000; Knowles and Pirog, 2001; Pinkerton et al., 2002). Furthermore, with the absence of the EBV receptor, CD21 could be a factor in the absence of EBV (Shiramizu et al., 1992). Based on these observations, it is speculated that these unique polyclonal lymphomas could be a unique entity to HIV-infected individuals; however, more studies are needed to establish its significance in H-NHL (Shiramizu et al., 1992).

Mutations and deletions in the p53 tumor suppressor gene on chromosome 17 have been found in one-third of H-NHL, particularly in Burkitt and Burkitt-like NHL (Ballerini et al., 1993; Nakamura et al., 1993; De Re et al., 1994). These genetic alterations in p53 affect its role in cell cycle regulation and may be of importance to the lymphomagnetic process in H-NHL. In general, p53 mutations are frequently found in human tumors and are not specific for H-NHL (Nakamura et al., 1993). In addition, *bcl-6* rearrangements have been found in 20% of systemic H-NHL, primarily CB DLBCL. *bcl-6* is required for the initiation of naive B cells into centroblasts through its entrance into the GC (Ranuncolo et al., 2007). Studies have shown that mutations to *bcl-6* at its promoter sites cause it to be constitutively active, a phenomenon that is the most common genetic lesion in B-cell lymphomas and found to induce DLBCL in mice (Pasqualucci et al., 2003; Ranuncolo et al., 2007). Furthermore, it has recently been shown that *bcl-6* serves as a transcriptional repressor that represses the ATR gene, which encodes a critical DNA-damage sensor protein, allowing for the evasion of ATR-mediated checkpoints in the cell cycle (Ranuncolo et al., 2007). Bypassing cell

cycle checkpoints allows the GC B cells to maintain DNA damage that arises with somatic hypermutation (Jardin et al., 2007). Additionally, it has been demonstrated that *bcl-6* suppresses p53, protecting the cells from DNA-damage-induced apoptosis and contributing to its survival (Phan and Dalla-Favera, 2004). Hence, the dysregulation of GC formation through *bcl-6* rearrangements may be of great significance to the pathological mechanisms of the H-NHL subtypes that contain these mutations. In a similar fashion to *bcl-6*, single nucleotide substitutions in the *ras* gene cause it to be constitutively active—an occurrence that is unusual to conventional NHL but is seen in 15% of systemic H-NHL. The majority of these point mutations occur in *n-ras*. Therefore, *ras* mutations, an event that is unique to H-NHL, may facilitate in producing a possible scenario of a tumorigenic pathway. Additional genetic characteristics have also been described including PAX5, TP53, RHO/TTF, and PIM1 (Carbone, 2003), but more studies need to be completed to determine if a true H-NHL association exists.

Since genetic abnormalities are not present in all H-NHL types, other factors must be considered for the development of a schematic pathogenesis model. A multifactorial model involving immune immunosuppression, impaired immune surveillance, and cytokine release has been speculated to provide an explanation for the heterogeneous molecular characteristics of H-NHL. This model theorizes that HIV-induced immunodeficiency causes inadequate B-cell control by T cells, thus causing a polyclonal proliferation of B cells through B-cell stimulation by HIV or coinfecting virus (EBV or KSHV/HHV8). This produces an environment whereby populations of B cells are at risk for accumulating a second hit that would ultimately lead to the malignant transformation and outgrowth of a B-cell clone. The multifactorial model is strongly supported by earlier molecular analyses of the immunoglobulins produced by systemic H-NHLs, showing polyclonal B-cell proliferations that progresses toward an outgrowth of a transformed B-cell clone (Ng et al., 1994; Bessudo et al., 1996; Przybylski et al., 1996). Consequently, the earlier described polyclonal lymphomas could be representative of the early and middle stages of this model, and monoclonal lymphomas of the final stages.

HIV promotes B-cell proliferation through its induction of cytokine production and release. Common cytokines include interleukins (IL) 1, 2, 4, 6, 7 and 10, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α). It has been shown that IL-6 and IL-10 are directly linked to HIV lymphomagenesis of EBV-infected B cells (Knowles, 2003).

In addition, it is speculated that HIV could directly influence the transformation and proliferation of a cancer cell through a mechanism called insertional mutagenesis. Recent studies have shown that the once perceived notion of random HIV integration may in fact occur less randomly and integrate in close proximity to a gene such as a transcription factor, causing for its activation and resulting in transformation (Killebrew and Shiramizu, 2004).

HIGHLY ACTIVE ANTIRETROVIRAL THERAPY AND H-NHL

As previously described, since the introduction of HAART in the early 1990s, there has been a marked decline in opportunistic infections, mortality rates, and AIDS progression. A decline in KS incidence has also been observed; however, reports for

H-NHL have been variable. This inconsistency could be attributed to the effect of HAART on the different H-NHL subtypes. For example, the survival of patients with DLBCL has improved during the post-HAART era, whereas the survival of patients with BL or PCNSL remains poor and unchanged (Robotin et al., 2004; Lim et al., 2005; Behler and Kaplan, 2006; Mounier et al., 2006). In fact, since the introduction of HAART, patients have been found to have higher CD4 cell counts at the time of H-NHL diagnosis, and some have also documented an increase in H-NHL as the initial AIDS-defining illness (Matthews et al., 2000; Besson et al., 2001; Robotin et al., 2004). The former suggests that H-NHL presents early during HIV disease and at a less advanced stage (Robotin et al., 2004). Thus, it is not surprising that a continuous rise in H-NHL is seen in countries with limited resources (Chokunonga et al., 1999; Gatphoh et al., 2001; Newton et al., 2001; Sanpakit et al., 2002).

In 1993, the International Prognostic Index (IPI) was created to predict the prognosis of NHL patients based on age, serum lactate dehydrogenase (LDH) levels, performance status, tumor stage, and extranodal site involvement. Using the Chelsea and Westminister HIV cohort, Bower et al. found IPI risk groups and CD4 cell counts to be independent predictors of death in the post-HAART era, but the study was limited by a small sample size and different HAART regimens (Bower et al., 2005). However, there are generally no differences in initial presentations at the time of H-NHL diagnosis during the pre- or HAART era with respect to age, gender, histology, and stage (Besson et al., 2001).

To determine the importance of HAART in the prevention of cancer, the rates of AIDS-defined malignancies were compared between continuous and interrupted antiretroviral therapy in the Strategies for Management of Antiretroviral Therapy (SMART) trial. Through this study, it was shown that higher rates of lymphoma occurred in the arm where antiretroviral therapy was interrupted (Silverberg et al., 2007). It has also been implicated that survival rates increase when HAART is initiated at CD4 cell counts greater than 350 cells/mm^3, but these results are limited to injection drug users only (Wang et al., 2004). However, zidovudine (AZT) is not recommended as part of the HAART regimen due to its myelosuppressive effects (Cheung et al., 2005; Behler and Kaplan, 2006).

The impact of HAART on the incidence of H-NHL remains unclear, and its effect on the prognosis for treatment is unknown. Many challenges remain in deciphering how combating of HIV will improve H-NHL prognosis.

THERAPEUTIC STRATEGIES FOR H-NHL

Treatment for patients diagnosed with H-NHL in the HAART era depends on the general type of H-NHL as noted in the Classification and Pathology section of this chapter: systemic lymphoma, PCNSL, or PEL.

The clinical characteristics observed in those diagnosed with PCNSL typically have low CD4 cell counts (<50 cells/mm^3) and poor prognosis with a median survival of 3 months (Levine et al., 1991; Gates and Kaplan, 2002; Tiwari et al., 2002; Scadden, 2003; Robotin et al., 2004). In contrast, systemic lymphomas and PEL are associated with higher mean CD4 cell counts (208 cells/mm^3) and better prognosis (Sparano

et al., 1996; Kaplan et al., 1997; Levine, 2000; Sparano, 2001; Spina et al., 2003; Robotin et al., 2004). Overall, treatment strategies for non-HIV-infected NHL cases are often used as guidelines in developing treatment strategies for H-NHL individuals. Many of the reported studies on systemic lymphoma use standard chemotherapy modalities such as cyclophosphamide, doxorubicin, vincristine, prednisone (CHOP); etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin (EPOCH); cyclophosphamide, doxorubicin, etoposide (CDE); doxorubicin, cyclophosphamide, vindesine, bleomycin, prednisone (ACVBP); cyclophosphamide, etoposide, methotrexate (CVM); methotrexate, bleomycin, cyclophosphamide, doxorubicin, vincristine (m-BACOD); or combinations of any of the above drugs (Sparano et al., 1996; Kaplan et al., 1997; Levine, 2000; Sparano, 2001; Spina et al., 2003; Cheung et al., 2005; Lim et al., 2005; Mounier et al., 2006). Radiotherapy administered independently or in conjunction with other therapy is often used for local control and PCNSL, respectively.

Recent successes in the use of the anti-CD20 monoclonal antibody, rituximab, in treatment protocols of HIV-negative individuals with NHL have led to its consideration in H-NHL treatment strategies, and recent attempts for the inclusion of rituximab in H-NHL therapy have been somewhat successful. It has been demonstrated that the use of rituximab in combination multichemotherapy regimens is safe and effective (Tirelli et al., 2002; Spina et al., 2005; Boue et al., 2006; Intragumtornchai et al., 2006; Weiss et al., 2006). However, an AIDS Malignancies Consortium (AMC) study completed by Kaplan et al., claimed that there was no significant improvement in H-NHL patients treated with CHOP in combination with rituximab (R-CHOP) in comparison to CHOP alone, and that an observable increase in treatment-related infectious deaths occurred in the R-CHOP group (Kaplan et al., 2005). Due to the above circumstances, the addition of rituximab to H-NHL treatment protocols remains controversial, and further investigations are warranted to verify its safety and efficacy.

Another controversial issue is concomitant HAART and chemotherapy. Several studies have shown that concomitant HAART and chemotherapy are associated with increased response rates and survival in comparison to chemotherapy alone and that the toxic effects are tolerable (Vaccher et al., 2001; Weiss et al., 2006). Despite these encouraging results, the potential drug interactions and overlapping toxicities of antiretroviral therapy and chemotherapeutic agents have caused many uncertainties. To combat these issues, many have considered other treatment approaches, such as HAART suspension during chemotherapy and risk-adapted intensive chemotherapy. However, the continuation of HAART for viral control is feasible as it decreases viral replication and maintains high CD4 concentration. In addition, the low tolerability to chemotherapy has led to the development of reduced-dose chemotherapy approach, or risk-adapted intensive chemotherapy. Mounier et al. analyzed the effects of traditional versus low-dose chemotherapy on survival and found no difference (Mounier et al., 2006). This is supported by other studies that demonstrate similar results; however, more studies are needed to determine combined toxicities and drug interactions (Gisselbrecht et al., 1993; Vaccher et al., 2001; Sparano et al., 2004; Sparano, 2007).

A decrease in treatment-related complications can be achieved through the use of various supportive care measures, including hematopoietic stimulators such as

granulocyte colony-stimulating factor (G-CSF), which stimulates the production of white blood cells in the bone marrow (Mounier et al., 2006; Weiss et al., 2006). Prophylactic intrathecal chemotherapy is also commonly used for patients with EBV-positive NHLs to prevent leptomeningeal spread (Cheung et al., 2005).

Of all systemic H-NHL, BL is the most difficult to treat and is often associated with poor outcome in comparison to DLBCL when treated with standard chemotherapy methods (Lim et al., 2005). The complications with BL treatment most likely stem from its aggressive nature and rapid progression. However, close monitoring and a multidisciplinary approach with intensive combination chemotherapy has greatly improved the prognosis for these patients (Astrow et al., 2003; Wang et al., 2003; Lim et al., 2005).

In comparison, the experience with PCNSL and PEL has not been as encouraging as in systemic B-NHL. PEL usually presents in advanced HIV disease and is not succumbed by the effects of chemotherapy (Behler and Kaplan, 2006), but it is moderately affected by high-dose methotrexate-based methods (Boulanger et al., 2003). Although the survival outcomes and prognosis for PCNSL patients have been suboptimal, high-dose methotrexate and radiation have provided some hope for patient survival. Still, much progress is needed to reach the level of success that is seen in other H-NHL.

Very young children with PCNSL are usually only treated with chemotherapy as radiation may have detrimental effects on brain development. In older children (>3 years of age), multimodality approaches are used in attempts to improve prognosis (Formenti et al., 1989). The treatment protocols used for adult systemic H-NHL have been tested in children but have been slightly altered to become short, but dose-intensive, regimens that are more tolerable in children (Mueller and Pizzo, 1996); Caselli et al., 2000). Overall, children respond fairly well to treatment (Mueller and Pizzo, 1996).

In some instances, there are patients who initially respond well to treatment but later experience a recurrence and are in need of a more aggressive approach. A possible option is stem cell transplantation, which has had variable success in relapsed patients (Molina et al., 2000; Krishnan et al., 2001; Re et al., 2003; Gabarre et al., 2004; Serrano et al., 2005).

Further investigations need to be done on strategies that include antiviral interventions. Therapeutic targets against HIV, EBV, and HHV8/KSHV may be beneficial in the inhibition of pathogenic mechanisms that are associated with these viruses.

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17

HIV-RELATED LYMPHOMA

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ETIOLOGY AND GENETICS

Several mechanisms of pathogenesis have been reported, yet the reason why non-Hodgkin's lymphomas (NHLs) are more common in human immunodeficiency virus (HIV) than in other forms of immunodepression is not completely understood (Gaidano et al., 1998; Knowles, 1999). There are several schools of thought to account for this difference in both clinical and molecular behavior, each implicating different biologic aspects. Some favor HIV targeting of specific genes. Others have suggested local immune deregulation involving both alteration in cell profile and alteration of cytokine profiles, whereas others again favor direct, viral–viral interactions. HIV infection is associated with a reduction of cell-mediated immunity, and the failure of CD4-helper T cells to recognize clones of abnormal proliferating cells may be one of the reasons behind the development of acquired immunodeficiency syndrome (AIDS)-associated malignancies (Dalgleish and O'Byrne, 2002).

The humoral predominant chronic immune activation seen in AIDS may also be important in providing the necessary environment for oncogenic viruses to induce

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lymphoma. The possible mechanisms and pathways responsible for the development of this heterogeneous group of lymphomas include chronic B-cell stimulation induced by HIV itself, as well as other coinfecting viruses, such as Epstein–Barr virus (EBV) and human herpesvirus-8 (HHV8), genetic aberrations, and cytokine deregulation. Disruption of the cytokine network leading to high serum levels of interleukin 6 (IL-6) and interleukin 10 (IL-10) is a feature of HIV-related lymphomas associated with EBV or HHV8. EBV is identified in the neoplastic cells of approximately 60% of AIDS-related lymphoma (ARL), but detection of EBV varies considerably with the site of presentation and histological subtype. EBV infection occurs in almost all of the cases of primary central nervous system lymphoma (PCNSL) and primary effusion lymphoma (PEL), 80% of diffuse large B-cell lymphoma (DLBCL) with immunoblastic features, and 30%–50% of Burkitt's lymphoma (BL). Nearly all cases of Hodgkin's lymphoma (HL) in the setting of HIV infection are associated with EBV. HHV8 is specifically associated with PEL, which often occurs in late stages of the disease in the setting of profound immunosuppression.

Polyclonal B-cell expansion from chronic antigenic stimulation, driven by HIV, coinfecting viral agents, and deregulated cytokine production, results in populations of proliferating cells at risk for acquiring genetic mutations that may lead to malignant transformation (Fig. 17.1). In particular, recent work has shown that infection of B cells, with several oncogenic viruses, results in the expression of activation-induced cytidine (AID), a DNA-mutating enzyme that plays a central role in DNA-modifying

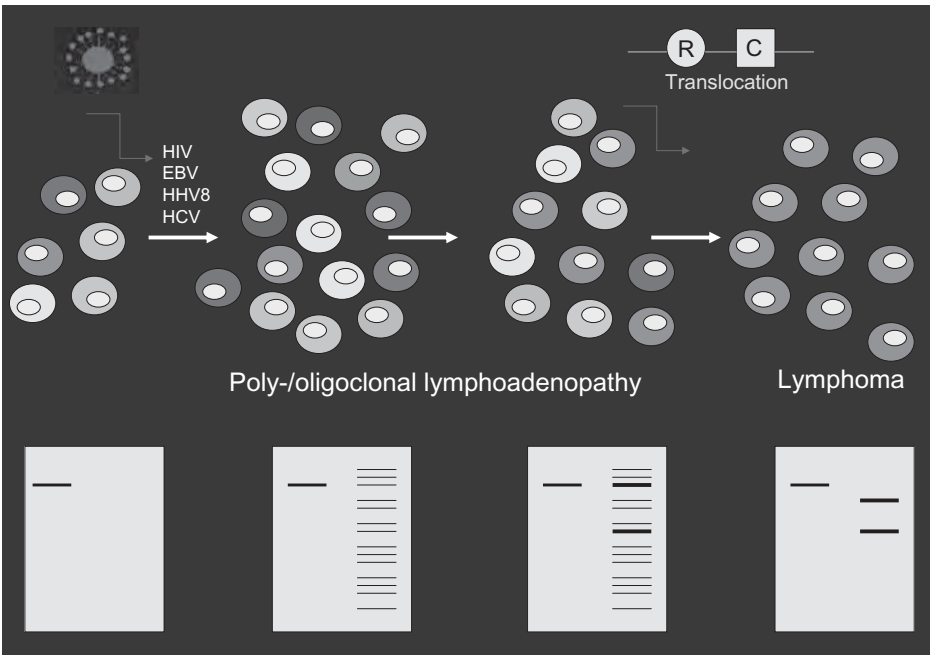

Figure 17.1. A model for virus-linked lymphomagenesis (see text). See color insert.

events. The virus-driven AID expression was associated with a progressive increase in the mutation rate of oncogenes that are associated with ARL, providing a novel mean by which EBV or other oncogenic virus infection of B cells could contribute to the pathogenesis of ARL. Common oncogenes involved in DNA-modifying events include the classic *c-MYC* oncogene in BL and BCL6 mutations in DLBCL, as well as abnormalities of tumor suppressor genes.

The pathway of tumor progression is determined by specific cell cycle checkpoint aberrations, and, as previously reported, one mechanism by which cell cycle control may be altered is by interaction with viral oncoproteins. DNA viruses may stimulate G1 progression and S-phase entry for successful replication of their genomes, thus resulting in disruption of the normal cell cycle control regulatory mechanisms. In addition, loss of control at the G1/S checkpoint allows the accumulation of numerous small genetic changes, leading to unchecked progression through G2/M. Loss of tumor suppressor genes, referred to as the loss of the heterozygosity (LOH) pathway, and genetic instability at the microsatellite (MSI) loci are among these genetic changes, both are involved in tumor progression in AIDS-associated malignancies. Defects in the mismatch repair (MMR) genes, which prevent them from repairing slippage errors that occur during replications, are believed to cause MSI. An increased rate of MSI has been described in several HIV-related malignancies including Kaposi's sarcoma (KS), NHLs, anal intraepithelial neoplasia (AIN), and HIV-associated lung cancers (Bedi et al., 1995). It has been suggested that HIV may target the MMR genes or other repair pathways.

However, the HIV genome has not been detected in tumor samples, and, in those studies where it has been detected in low copy numbers, it has been attributed to HIV in background inflammatory cells. The hypothesis that HIV might use a "hit and run" mechanism, modifying the cell without detection of the viral genome, should also be considered (Clarke and Chetty, 2002). Alternatively, a viral protein may specifically bind to the DNA or cellular proteins, corrupting the replication process.

Tat protein of HIV is also a likely candidate to contribute to tumor pathogenesis in HIV-infected patients (Ensoli et al., 1994; Fiorelli et al., 1998; Altavilla et al., 1999; Kundu et al., 1999). Tat protein is an early nonstructural protein necessary for virus replication, which is secreted by infected cells and taken up by uninfected cells (Rubartelli et al., 1998). Extensive evidence indicates that Tat is a cofactor in the development of AIDS-related neoplasms (Noonan and Albini, 2000), and the protein has also been found to have an oncogenic role *in vitro* and *in vivo* (Kim et al., 1992; Corallini et al., 1993, 1996; Kundu et al., 1999). However, the molecular mechanism of Tat-mediated tumorigenesis is not clear at present. There is experimental evidence to suggest a potential role of Tat-mediated chemotaxis and invasion in the pathogenesis of AIDS-related malignancies (Barillari and Ensoli, 2002). Deregulation of cellular genes and functions by Tat can also cause abnormalities that may contribute to AIDS pathogenesis and to the development of AIDS-associated disorders (Kundu et al., 1997, 1998; Kashanchi et al., 2000). Extracellular Tat is able to regulate many cellular genes, which are involved in cell signaling and translation and ultimately controls the host proliferation and differentiation signals (Li et al., 1995; Zauli et al., 1995; de la Fuente et al., 2002). The molecular mechanism underlying Tat's pleotropic activity may include the generation of functional

heterodimers of Tat with cell cycle proteins (Kashanchi et al., 2000). In particular, Tat protein of HIV has also recently been shown to physically interact with the RB2/p130 tumor suppressor gene product (Lazzi et al., 2002) and E2F4 (Ambrosino et al., 2002), resulting in uncontrolled cell proliferation (De Falco et al., 2003). The interaction of Tat with cell cycle regulatory proteins alone may not be sufficient for neoplastic transformation *in vivo* and other cofactors may be required. This is also consistent with the finding that Tat cannot induce cell growth unless cells are previously activated with inflammatory cytokines (Ensoli et al., 1990; Barillari et al., 1992; Albini et al., 1995; Fiorelli et al., 1998). Nevertheless, these results open a window on AIDS-related oncogenesis and suggest a reevaluation of HIV itself as an oncogenic virus.

Another mechanism, through which Tat may influence HIV-mediated transformation, is by hyperactivation of transcription by interacting with chromatin remodeling complexes. Though viral transcription is fully dependent upon host cellular factors and the state of host activation, recent findings indicate a complex interplay between viral proteins and host transcription regulatory machineries, including histone deacetylases (HLACs), histone acetyltransferases (HATs), cyclin-dependent kinases (CDKs), and histone methyltransferases (HMTs). The chromatin structure presents a significant barrier to transcription. Various complexes, including HATs, covalently modify nucleosomal histone proteins through acetylation, while ATP-dependent chromatin remodelers alter the chromatin structure via ATP hydrolysis. These modifications and alterations of chromatin structure increase DNA accessibility to transcription factors and activators, thus promoting transcription initiation and efficient elongation. A more current view is that activators must first recruit chromatin remodelers, in order to create a chromatin environment permissive for preinitiation complex (PIC) assembly. Many reports in the last several years have linked Tat transactivation to chromatin remodeling *in vitro* and *in vivo*. Recently, it has been demonstrated that Tat is able to target HATs to the promoters, is able to form a ternary complex with several HAT complexes (Kamine et al., 1996; Hottiger and Nabel, 1998; Weissman et al., 1998; Kiernan et al., 1999; Col et al., 2001; Vendel and Lumb, 2003), and is acetylated by these complexes (Kiernan et al., 1999; Deng et al., 2001; Bres et al., 2002; Dorr et al., 2002), thus increasing the acetylation of nucleosomal histones. Such enhancement of histone acetylation may be due to the fact that acetylated Tat binds with higher efficiency to nucleosomal DNA and changes the conformation, resulting in the accessibility of histone tails to HATs (Deng et al., 2001). The acetylated form of Tat is released from transactivating responsive sequence (TAR) RNA, which can now bind to the SWI/SNF chromatin remodeling complex (Agbottah et al., 2006), facilitating the remodeling of downstream nucleosomes allowing for further transcription of DNA (Fig. 17.2).

CLINICAL FEATURES

Both NHL and Hodgkin's disease in AIDS are characterized clinically by the presence of B symptoms at diagnosis in ~90%, advanced clinical stage with extranodal involvement in >60% at presentation, and clinical aggressiveness with poor therapeutic outcomes (Knowles et al., 1988; Matthews et al., 2000; Levine et al., 2000; Sparano, 2001; Aboulafia et al., 2004).

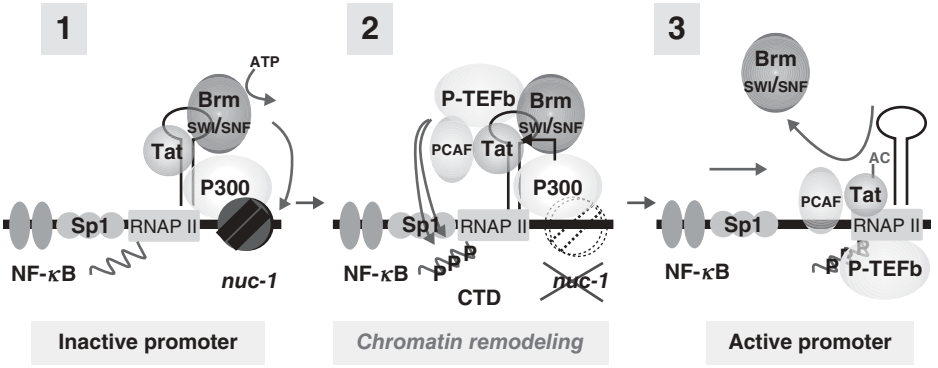

Figure 17.2. Role of Tat in chromatin remodeling (see text). Courtesy of Dr. Stephane Emiliani, Institut Cochin Department of Infectious diseases, Paris, France. See color insert.

The systemic B symptoms include unexplained fever, drenching night sweats, and/or weight loss in excess of 10% of normal body weight. In the setting of HIV infection, lymphoma tends to present in multiple, extranodal sites. Bone marrow involvement is diagnosed in approximately 20% of patients at initial presentation (Seneviratne et al., 2001). Median hemoglobin in patients with marrow involvement is 10.6 mg/dl, which does not differ from the mean hemoglobin level in patients without bone marrow involvement. In the setting of lymphomatous involvement of the bone marrow, approximately one-third of patients present with platelet counts <100.000/mm^3. Greater than 50% bone marrow involvement is associated with decreased survival in these patients. The gastrointestinal tract is involved in lymphoma in approximately one quarter of reported patients, who may present with abdominal pain, anorexia, nausea, and vomiting, or a change in bowel habits. Abdominal distension or an abdominal mass may be noted. Involvement of the rectum is not unusual, presenting as a rectal mass or pain on defecation. Lymphoma in the liver is also relatively common; these patients may present with jaundice, systemic B symptoms, abdominal pain, or anorexia. Hepatitis C infection is not associated with an increased likelihood of ARL, and patients who are infected with hepatitis C appear to tolerate chemotherapy similarly to those who are hepatitis C virus negative (Levine et al., 1999). Leptomeningeal involvement by lymphoma occurs approximately in 20% of patients with newly diagnosed systemic HIV-related lymphoma (Levine, 1992). These patients may be entirely asymptomatic in terms of the central nervous system (CNS). When symptoms do occur, the most common include altered mental status, cranial nerve palsies, or headache.

PCNSL represents a distinct extranodal presentation of DLBCL in HIV infection, usually of the immunoblastic type, that is associated with severe immunosuppression (CD4 <50/mm3), EBV positivity, and a poor prognosis. Lymphomatous involvement in HIV-infected persons with PCNSL is typically confined to the craniospinal axis without systemic involvement (Stebbing et al., 2004). Imaging alone cannot distinguish between PCNSL and cerebral toxoplasmosis, the latter being the most common cause

of focal cerebral lesions. Presence of EBV within the cerebrospinal fluid (CSF), as detected by Epstein-Barr virus nuclear antigen 1 (EBNA-1) by polymerase chain reaction (PCR), may also be used to diagnose HIV-related PCNSL as well as in the case of lymphomatous meningitis in patients with systemic HIV-related lymphoma. Hodgkin's disease (HL) may be seen with increased frequency in persons infected with HIV. HL tends to have a more aggressive course and more likely to be stage III or IV at presentation (82%), to have bone marrow involvement, to have noncontiguous spread of tumor, to have numerous Reed–Sternberg cells, and to show an association with EBV infection, in patients infected with HIV (Spina et al., 2003; Thompson et al., 2004).

Intravenous drug users constitute the risk group most frequently affected. Patients with HIV-associated HL are more likely (70%–96%) to have "B" symptoms including fever, night sweats, and/or weight loss of >10% of normal body weight. HL in HIV-infected persons is more likely to be accompanied by anemia, leukopenia, or thrombocytopenia (Spina et al., 2003; Thompson et al., 2004).

Generalized lymphadenopathy is likely to be present, and the clinical picture may resemble persistent generalized lymphadenopathy (PGL). However, mediastinal lymphadenopathy is less frequent in HIV-infected patients with HL (Spina et al., 2003; Thompson et al., 2004).

LYMPHOMAS ALSO OCCURRING IN IMMUNOCOMPETENT PATIENTS

BL (Fig. 17.3)

Classical variant shows morphological features of classical BL with a monomorphous, medium-sized cell proliferation; sometimes the cells exhibit greater polymorphism in the size and shape. It represents 30% of all ARLs. EBV is positive in about 30%–50% of cases (Raphael et al., 2008).

BL with plasmacytoid differentiation is a feature relatively unique to AIDS patients and represents about 20% of NHL cases. It is characterized by medium-sized cells with abundant basophilic cytoplasm and an eccentric nucleus, often with one centrally located prominent nucleolus. EBV is positive in about 50%–70% of cases (Raphael et al., 2008).

HIV-associated BL, like other cases of BL, express B-cell-associated antigens (e.g., CD19, CD20, CD79a), CD10, and BCL6. The cells are negative for CD23, CD44, CD138, and TdT. For instance, aberrant phenotypes have been described, and CD5 and BCL2 may be expressed in rare cases (Raphael et al., 2008).

DLBCL (Fig. 17.4)

The majority of these lymphomas contain numerous centroblasts admixed with a variable component of immunoblasts. EBV is present in 30% of cases. Cases containing more than 90% immunoblasts and usually exhibiting plasmocytoid features are classified as the immunoblastic variant. They represent about 10% of ARL and are associated

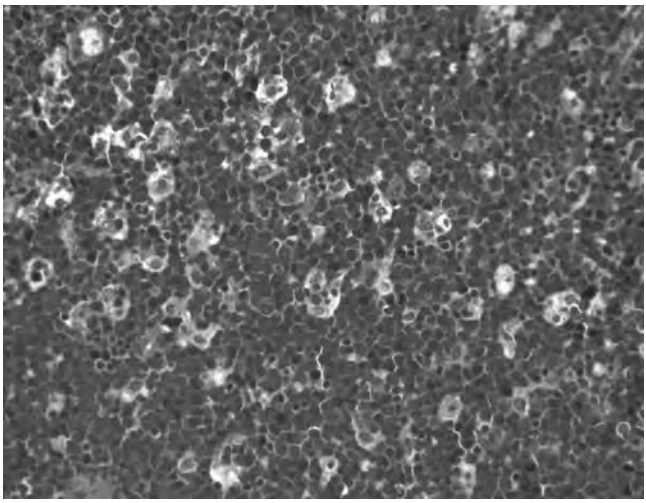

Figure 17.3. Burkitt's lymphoma. The cells are uniform in size and shape with multiple small nucleoli. The nuclei of the tumor cells approximate in size those of the admixed starry-sky histiocytes (20×). See color insert.

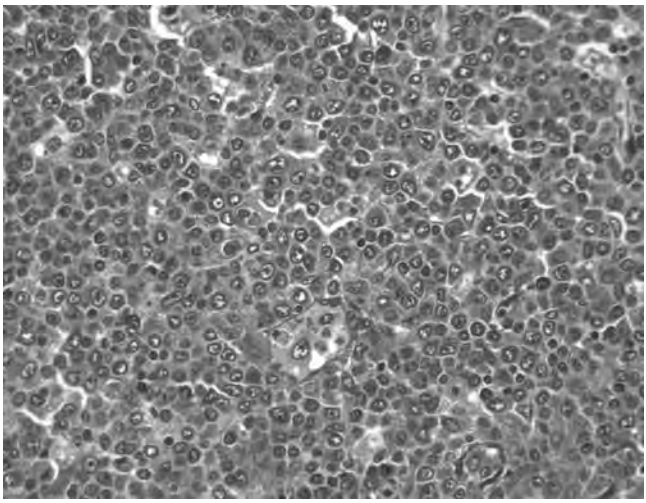

Figure 17.4. Diffuse large B-cell lymphoma. The medium- to large-sized tumor cells have a polymorphic and polylobulated appearance with vesicular nuclei, fine chromatin, and two to four membrane-bound nucleoli (20×). See color insert.

with EBV in 90% and often occur late in the course of HIV disease. PCNSLs are usually of the immunoblastic type (Raphael et al., 2001).

AIDS-related DLBCL has a more variable immunophenotype compared with DLBCL in immunocompetent patients. In addition, AIDS-associated DLBCL tend to have an intermediate germinal center-activated B-cell phenotype, suggesting a unique pathophysiology, when compared with non-AIDS DLBCL, which expressed a distinct immunophenotype germinal center or activated B cell (Madan et al., 2006).

HL

Most cases correspond to either the mixed cellularity or lymphocyte-depleted forms of classical HL. Some cases of nodular sclerosis HL are also seen. HIV-related HL is associated with EBV in nearly all cases; the cells express latent membrane protein-1 (LMP1) and are EBV-coded RNA (EBER) positive (Raphael et al., 2001). The immunophenotype of HL in HIV-positive patients is similar to that of the HL in the general population. In contrast to that observed in HL in the general population, where Reed–Sternberg cells are derived from germinal center cells, Reed–Sternberg cells of HL in the HIV setting consistently display BCL6/CD138-positive phenotype and therefore reflect postgerminal center B cell (Carbone et al., 1999).

Other Lymphomas (Rare)

Rare cases of mucosa-associated lymphoid tissue neoplasm (MALT) lymphoma have been described in both pediatric and adult patients with HIV infection. Rare cases of peripheral T-cell and natural killer lymphoma can also occur (Raphael et al., 2001).

LYMPHOMAS OCCURRING MORE SPECIFICALLY IN HIV-POSITIVE PATIENTS

PEL

Two variants of HIV-associated PEL have been described (Komanduri et al., 1996; Raphael et al., 2001; Chadburn et al., 2004). These include classic PEL or "body cavity-based lymphoma," which has a unique tropism for serous body cavities, and extracavitary or solid PEL, which is an extraserous lymphoma reported in HIV-positive patients with or without associated effusions (Raphael et al., 2001).

In classic PEL, lymphomatous effusions may involve the pleural, pericardial, and/or peritoneal body cavity, whereas solid PEL primarily involves extraserous sites such as the large bowel, skin, lung, and lymph nodes (Chadburn et al., 2004). With Wright or May–Grünwald–Giemsa staining performed on cytocentrifuge preparations, the cells exhibit a range of appearances, from large immunoblastic or plasmablastic cells to cells with more anaplastic morphology. Nuclei are large, round to more irregular in shape, with prominent nucleoli. The cytoplasm can be very abundant and is deeply basophilic with the presence of vacuoles in occasional cells. A perinuclear hof consistent with plasmacytoid differentiation may be seen. Some cells can resemble

Reed–Sternberg cells. The cells often appear more uniform in histological sections, than in cytospin preparations.

The neoplastic cells are positive for Kaposi's Sarcoma-associated Herpesvirus (KSHV)/HHV8 in all cases. Most cases are coinfected with EBV (Raphael et al., 2001).

Lymphoma cells usually express leukocyte common antigen (CD45) but are usually negative for pan B-cell markers such as CD19, CD20, and CD79a. Surface and cytoplasmic expression of immunoglobulin is, likewise, often absent. Activation and plasma cell-related markers such as CD30, CD38, and CD138 are usually demonstrable. Aberrant cytoplasmic CD3 expression has been reported. Because of the markedly aberrant phenotype, it is often difficult to assign a lineage with immunophenotyping (Raphael et al., 2001).

Plasmablastic Lymphoma of the Oral Cavity (Fig. 17.5)

Plasmablastic lymphoma is another unique lymphoma subtype that typically involves the jaw and oral cavity of HIV-infected patients (Raphael et al., 2001; Colomo et al., 2004). More recently, HIV-related plasmablastic lymphoma has been documented in other sites such as the anorectum, nasal and paranasal regions, skin, testes, bones, and lymph nodes (Chetty et al., 2003; Hichman et al., 2004). The neoplasm is highly associated with EBV infection (50%), but no association with KSHV/HHV8 has been detected. The tumors display a diffuse pattern of growth interspersed by macrophages. The tumor cells are large blasts with eccentrically located nuclei and usually single, centrally located, prominent nucleoli. The cytoplasm is deeply basophilic with para nuclear hof.

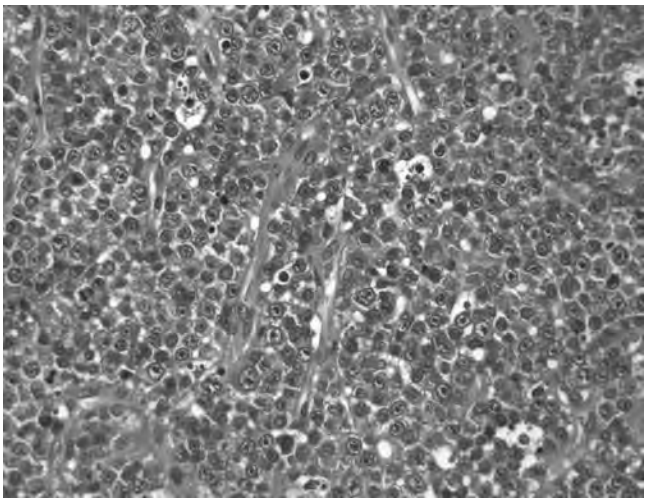

Figure 17.5. Plasmablastic lymphoma of the oral cavity. The tumor cells are large blasts with eccentrically located nuclei and usually single, centrally located, prominent nucleoli. The cytoplasm is deeply basophilic with para nuclear hof (20×). See color insert.

Tumor cells express the B-cell markers such as CD19 and CD79a, but characteristically, CD20 is negative. Cytoplasmic immunoglobulins and other markers of plasmacytic differentiation, such as CD138 and CD38, are also detected in plasmablastic lymphomas of the oral cavity (Raphael et al., 2001).

LYMPHOMAS OCCURRING IN OTHER IMMUNODEFICIENT STATES

Polymorphic Lymphoid Proliferations Resembling Posttransplant-Associated Lymphoproliferative Disease (PTLD)

PTLD may be seen in adults and also in children but is much less common than in the posttransplant setting, comprising less than 5% of HIV-associated lymphomas. These conform to the criteria of polymorphic B-cell PTLD. The infiltrates contain a range of lymphoid cells from small cells, often with plasmacytoid features, to large cells having the features of immunoblasts, with scattered large bizarre cells expressing CD30. EBV is often present, but some cases may be EBV negative (Raphael et al., 2001).

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18

BIOLOGY AND EPIDEMIOLOGY OF HHV-8

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Human herpesviruses are large, double-stranded DNA viruses that are ubiquitous in nature. There are currently eight known human herpesviruses belonging to three subfamilies: Alpha-, Beta-, and Gammaherpesvirinae. They are subdivided based on morphology, biologic properties, genome structure, and sequence homology (Roizman and Pellet, 2001). This diversity of hosts indicates that these viruses have developed successful and efficient ways to infect different kinds of cells. Human herpesvirus-8 (HHV-8), also known as Kaposi's sarcoma-associated herpesvirus (KSHV), is the most recently described human herpesvirus. HHV-8 was codiscovered by Chang and Moore in 1994, from lesions of a Kaposi's sarcoma (KS) patient by representational differential analysis (Chang et al., 1994). HHV-8 DNA sequences in the KS tissue, which were not found in normal skin tissue, could then be amplified. This review discusses the biology, disease associations, detection, and transmission of HHV-8.

HHV-8 BIOLOGY

Although it is difficult to obtain infectious virus from culture explants of KS tissue, cell lines derived from primary effusion lymphomas (PELs), such as BC3, KS-1,

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BCBL-1, and BC1, proved to be very valuable for studies on HHV-8 (Ablashi et al., 2002). HHV-8 particles are about 100–150 nm in size with a lipid envelope and an electron dense core (Renne et al., 1996). The genome of HHV-8 contains approximately 140 kb of long unique DNA flanked by two terminal repeat regions, 25–35 kb each (Russo et al., 1996). It encodes over 80 open reading frames (ORF) and has significant homology to the *Rhadinovirus* genus of the gamma herpesvirus subfamily, all of which are known to infect lymphocytes (Moore et al., 1996). HHV-8 is the only member of the genus *Rhadinovirus* known to infect humans but is closely related to another human gamma herpesvirus, Epstein–Barr virus (EBV), which belongs to the genus *Lymphocryptovirus*. EBV is well known to transform human B cells in culture and is also etiologically linked to human cancers, such as Burkitt's lymphoma, central nervous system (CNS) lymphoma, and nasopharyngeal carcinoma.

A distinct feature of herpesviruses is their ability to establish a latent, lifelong infection, which is interrupted by frequent reactivation leading to lytic replication. The latent phase provides a relatively stable and immunologically silent mode of persistence, whereas the lytic phase allows virions to be shed and transmitted to new hosts. In latency, HHV-8 persists predominantly in B cells and is characterized by a restricted pattern of viral gene expression that facilitates the avoidance of immune surveillance (Zhong et al., 1996; Staskus et al., 1999). While the host immune response generally prevents disease, it does not prevent viral persistence. During this phase, several viral-encoded cellular homologues of known cytokines or cytokine receptors involved in signal transduction are also up-regulated. These include interleukin (IL)-6, IL-8 receptor, G-protein coupled receptor (GPCR), and interferon regulatory factors that are important for HHV-8 to evade innate and adaptive immune responses (Ensoli and Sturzl, 1998). The latent virus can be induced to undergo lytic reactivation, possibly due to loss of immunogenic control or upon stimulation by cellular factors, leading to the spread of the virus from the lymphoid reservoir to other cell types contributing to pathogenesis (West and Wood, 2003). This highly successful strategy has ensured human herpesvirus infection in a majority of the adult human population globally. A number of proteins expressed in the lytic phase have been identified to elicit antibody responses in infected individuals and will be discussed in the section regarding the diagnosis of HHV-8 infection. Preliminary reports have indicated that specific neutralizing antibodies against HHV-8 virion proteins may be able to limit the spread of HHV-8 or be involved in the control of viral load in infected individuals (Dialyna et al., 2004). The virion proteins that elicit a neutralizing antibody response have not yet been identified. The natural history of HHV-8 infection is still not very clear. There have been very few reports that document primary HHV-8 infection and its associated symptoms (Oksenhendler et al., 1998; Luppi et al., 2000; Andreoni et al., 2002). In a study from Egypt, primary HHV-8 infection was associated with febrile illness and maculopapular rash (Andreoni et al., 2002). It is still not known whether these symptoms are similar in all age groups and if the severity of disease is dependent on the immune status.

CLINICAL MANIFESTATIONS ASSOCIATED WITH HHV-8

There is a strong evidence linking HHV-8 to at least three malignancies namely, KS, multicentric Castleman's disease (MCD), and PEL (Chang et al., 1994; Cesarman et al., 1995; Soulier et al., 1995). During the early stages of the AIDS epidemic, KS was the most common AIDS-defining illness. In fact, it was the sudden appearance of KS and, shortly thereafter, the appearance of high-grade non-Hodgkin's lymphoma (NHL) in a handful of young homosexual men who otherwise were in good health that signaled the start of the AIDS epidemic (Ziegler et al., 1984). For most immunocompetent individuals, infection by HHV-8 remains subclinical as shown by the low incidence of HHV-8-associated malignancies.

KS

The most important clinical manifestation of HHV-8 infection is KS. KS was originally described by Moritz Kaposi in 1872 as an idiopathic, multipigmented sarcoma of the skin (Kaposi, 1872). Four epidemiological forms of KS have been defined (classic, endemic, iatrogenic, and AIDS associated), and the histological features of KS are indistinguishable. KS is a proliferative condition with lesions comprised of spindle cells surrounding vascular slits (Ablashi et al., 2002). There are three distinct histological stages of KS, which include the patch, plaque, and nodular forms. Though KS is most commonly localized to the skin, organ involvement can occur and is not uncommon in AIDS-associated KS (Wong and Damania, 2005).

Classic KS is a rare disease, and geographic location, ethnicity, age, and gender heavily influence the incidence rate (Iscovich et al., 2000). This is the least aggressive form of KS and is a slow or nonprogressing condition that generally presents as a disease of the limbs, and occurs primarily among older men of Mediterranean and Jewish descent (Reynolds et al., 1965; Iscovich et al., 2000). The male to female ratio has been reported to be 15:1 with age of onset usually greater than 50 years of age and rarely (4%–8%) prior to 30 years of age (Biggar et al., 1984; Dictor and Attewell, 1988; Geddes et al., 1994). The incidence of classic KS in North America, Northern Europe, and Asia is low (DiGiovanna and Safai, 1981; Iscovich et al., 1998a,b, 2000; Guttman-Yassky et al., 2006).

Recognition of KS as a common cancer in parts of Africa became apparent from a report in the 1950s showing high prevalence of KS among Bantu tribesmen of South Africa (Oettle, 1962). The highest incidence of KS was observed in a belt-like path stretching from Cameroon, the former Zaire, to Uganda and downward through Tanzania, north and eastern Zambia, Zimbabwe, and northern South Africa (Cook-Mozaffari et al., 1998). KS was soon recognized to be one of the most common neoplastic diseases in equatorial Africa (Lothe and Murray, 1962; Oettle, 1962; Olweny, 1984). Although the other African subvariants of KS have a male to female ratio similar to that of classic KS and a mean age of onset of 48 years, the lymphadenopathic form is seen mostly in prepubertal children with no sex predilection. Rapid visceral involvement can cause early death, and skin lesions are sparse (Friedman-Kien and Saltzman, 1990). Studies of endemic KS are now difficult to perform in the setting of the African AIDS epidemic.

The iatrogenic form of KS appeared in 1960s and 1970s, following advances in transplantation medicine. With the introduction of immunosuppressive regimens used to prevent graft rejection, posttransplant patients developed disseminated KS as early as 2 months and up to 8 years after initiation of therapy (Friedman-Kien and Saltzman, 1990). This form of KS often resolves when immunosuppressive therapy is stopped, calling attention to immune deficiency as an etiologic cofactor. Among transplant recipients, KS develops mostly in those with preexisting HHV-8 infection, and seropositive recipients have a 28- to 75-fold higher KS risk than seronegative recipients (Parravicini et al., 1997; Mbulaiteye and Engels, 2006).

KS and primary central nervous system lymphoma (PCNSL) were recognized as AIDS-defining conditions when they were first reported in the 1980s (Friedman-Kien et al., 1982). Among homosexual men with human immunodeficiency virus (HIV) infection at the beginning of the AIDS epidemic, the risk of developing KS approached 50%, and approximately 40% of patients who received a diagnosis of AIDS presented with KS (Biggar and Rabkin, 1996). AIDS-associated KS is a very aggressive form that often disseminates to visceral organs (Beral et al., 1990). It has been noted that among HHV-8/HIV coinfected males, the 10-year risk of developing KS is 39%–50% (Martin et al., 1998; O'Brien et al., 1999). AIDS-KS incidence decreased after the highly active antiretroviral therapy (HAART) introduction in 1996, probably due to enhanced immune reconstitution and perhaps a direct effect of protease inhibitors as antiangiogenic factors.

PEL

HHV-8 DNA sequences have also been identified within a distinct subgroup of AIDS-related NHL localized in body cavities and presenting as pleural, peritoneal, and pericardial lymphomatous effusions (Cesarman et al., 1995). PEL remains a rare lymphoma, comprising approximately 3% of AIDS-related lymphomas (Gaidano and Carbone, 2001). The prognosis of PEL is poor with a median survival of less than 6 months (Carbone et al., 1996). These lymphomas usually contain large amounts of viral DNA, ranging from 40 to 80 copies per cell. Most PELs are thought to originate from post-germinal center B cells, since they have hypermutation of the immunoglobulin genes. The cytological morphology bridges large-cell, immunoblastic lymphoma and anaplastic large-cell lymphoma (Ablashi et al., 2002). Since PELs are uncommon, even in populations in which the seroprevalence of HHV-8 is relatively high, it is evident that infection by this virus represents only one of several genetic events involved in PEL development.

MCD

MCD is a poorly understood atypical lymphoproliferative disorder thought to be related to immune dysregulation. The population prevalence rate of MCD is unknown. A large retrospective study from a cancer center in 1996 estimated that the total number of cases in the United States ranges from 30,000 to 100,000 (Casper, 2005). MCD presents in two distinct histopathologic subtypes with different clinical characteristics. The first and more common is the hyaline vascular type, which presents as a solitary mass that

is usually curable surgically. The second is plasma cell type, which is associated with more generalized lymphadenopathy and immunologic abnormalities (Ablashi et al., 2002). Patients with MCD frequently develop malignancies, most commonly KS and NHL (Soulier et al., 1995).

HHV-8 EPIDEMIOLOGY

Most studies on HHV-8 epidemiology are cross-sectional in design and report prevalence data in a population. There have been very few longitudinal studies conducted that report incidence in the general population. Table 18.1 summarizes some of the prominent studies in different regions of the world that have been conducted to gather information about HHV-8 prevalence in the general population. They are all cross-sectional in design and cannot be directly compared due to differences in the population that was sampled, assays used, and other factors such as prevalence of HIV-1 infection present in the population. In general, most of these studies showed that geographic differences in HHV-8 seroprevalence exist in different regions of the world and that HHV-8 seroprevalence is high in geographic areas where classic or endemic KS is more common. This includes the KS belt in Africa and the region around the Mediterranean Sea. In sub-Saharan Africa, seroprevalence is generally high. Our studies in Zambia have demonstrated that HHV-8 seroprevalence among adolescent and childbearing women in Zambia is more than 48% (He et al., 1998). Our laboratory was the first to follow a cohort of Zambian women and children to study HHV-8 incidence in this population. In healthy HIV-uninfected children, we observed an incidence of 12 infections/100 child-years within 4 years after birth (Minhas et al., 2008a). This incidence was five times higher in HIV-infected children even though there is no evidence that the prevalence of HHV-8 has changed in Africa since the spread of HIV (Dedicoat and Newton, 2003). In countries surrounding the Mediterranean Sea, the seroprevalence averages 10%, although there are regions where higher prevalence can be found such as Sardinia and Sicily (Dukers and Rezza, 2003), where classic KS is diagnosed more often (Angeloni et al., 1998; Chatlynne et al., 1998; Almuneef et al., 2001). HHV-8 infection is uncommon in the general population in North America, United Kingdom, and Western Europe. Besides the studies summarized in Table 18.1, several other studies have been conducted in countries with low prevalence of HHV-8 that have HHV-8 infection confined to high-risk groups. It is observed that higher seroprevalence is found in groups such as homosexual men, HIV-1-infected immunocompromised patients, and immunosuppressed transplant patients. In the United States, prevalence was found to be between 5% and 20%, and in HIV-1-infected patients, prevalence can be between 20% and 50% (Gao et al., 1996; Lennette et al., 1996; Simpson et al., 1996). Engels et al. reported that HHV-8 seroprevalence in homosexual men in the United States was six times higher than heterosexual men (Engels et al., 2007). Prevalence in Asia has not been studied extensively but appears to be low. In South America, studies have demonstrated low HHV-8 seroprevalence in the general population (Zago et al., 2000; Caterino-De-Araujo et al., 2003; Perez et al., 2004). The exceptions are the Amerindian tribes in Brazil, Ecuador, Peru, and French Guiana, which have demonstrated high

TABLE 18.1. Representative Epidemiological Studies Conducted in Different Parts of the World Depicting the Seroprevalence of HHV-8 in the General Population

Study	Region	Population Screened	Seroprevalence	Notes	Reference
	North America				
1	United States	Children (6–17 years)	0.4%, 1.1%	Prevalence based on latent and lytic antibodies, respectively	Anderson et al. (2008)
2	United States	Children (6 months to 17 years)	4%		Martro et al. (2004)
3	United States	Adults (>18 years)	1.6%, 1.5%	More specific cutoff used. Prevalence varies with assay used	Engels et al. (2007)
			7.1%, 7.4%	Less specific cutoff used. Prevalence varies with assay used	
4	United States	Children (<15 years)	<4%		Lennette et al. (1996)
		Adults	18%–28%	Prevalence varied in different age groups	
	South America				
5	Brazil	Children (0–12 years)	7.6%		Avelleira et al. (2006)
6	Brazil Amerindians	All ages	53%	Amerindians residing in seven different villages were sampled	Biggar et al. (2000)
7	French Guiana Amerindians	All ages	23%	Samples collected from all six tribes	Kazanji et al. (2005)
8	Ecuador Amerindians	Two different tribes sampled	63%, 100%	Prevalence based on lytic and latent antibodies, respectively	Whitby et al. (2004)
9	Peru	Adult blood bank donors	56.25%		Mohanna et al. (2007)
10	Argentina	Adult blood bank donors	4.0%		Perez et al. (2004)
	Brazil		2.8%		
	Chile		3.7%		
	Asia				
11	South Korea	Children (1–15 years)	3.5%		Han et al. (2005)
12	China	Age not specified	13%	Prevalence based on screening for ORF65 antibodies only	Zhu et al. (2008)

	Europe				
13	Italy	Adult blood bank donors	13.8%	Reported prevalence was higher in Southern Italy especially in Sardinia (35%)	Whitby et al. (1998)
14	Israel	Adults	6.1%		Iscovich et al. (2000)
15	Greece	Adults (16–69 years)	7.6%	Population screened for lytic K8.1 antibodies only	Zavitsanou et al. (2006)
16	Spain	Adult blood bank donors	6.5%		Gambus et al. (2001)
17	Sweden	Adults (>30 years)	16.2%		Tedeschi et al. (2006)
2	Germany	Children (6 months-17 years)	3%		Martro et al. (2004)
18	Balkan region—Albania	All ages	28.8%	Rural and urban population sampled	Chironna et al. (2006)
	Balkan region—Kosovo	All ages	18.0%		
	Africa				
19	Egypt	Children (1–12 years)	44.7%, 8.5%	Prevalence based on lytic and latent antibodies, respectively	Andreoni et al. (1999)
20	Egypt	Children	14.3%	Rural Egyptian population screened for K8.1 lytic antibodies only	Mbulaiteye et al. (2008)
		Adults	24.2%		
21	South Africa	Children (0–16 years)	15.9%	Large cohort of women and children including HIV-positive patients	Malope et al. (2007)
		Adults	29.7%		
22	South Africa	Children	12%, 7%	Large cohort of women and children including HIV-positive patients	Dedicoat et al. (2004)
		Adults	40%, 14%	Prevalence based on lytic and latent antibodies, respectively	

TABLE 18.1. *Continued*

Study	Region	Population Screened	Seroprevalence	Notes	Reference
2	Nigeria	Children (6–38 months)	14%		Martro et al. (2004)
23	French Guiana	All ages (>2 years)	13.2%	Noir-Marron is an ethnic group of African origin	Plancoulaine et al. (2000)
24	Uganda	Children (1–16 years)	21%		Mbulaiteye et al. (2005)
25	Uganda	All ages (0–24 years)	52%	Prevalence based on presence of either latent or lytic antibodies	Mayama et al. (1998)
26	Tanzania	Children (0–17 years)	56.5%		Mbulaiteye et al. (2003)
27	Cameroon	Children (<20 years)	27.5%		Gessain et al. (1999)
28	Cameroon	Children (5–10 years)	39.8%, 25.5%	Prevalence based on latent and lytic antibodies, respectively	Rezza et al. (2000)
		Adults (30–40 years)	61.8%, 21.8%	Prevalence based on latent and lytic antibodies, respectively	
29	Zambia	Adults (>16 years)	39.8%	Large cohort of women including HIV-positive patients	Klaskala et al. (2005)
30	Zambia	Adult	48.4%	Large cohort of antenatal women	He et al. (1998)
31	Ghana	Children (<20 years)	45%	HIV seronegative healthy individuals	Nuvor et al. (2001)
		Adults (>20 years)	31.4%		
32	Central African Republic	Adult blood bank donors	22.5%		Belec et al. (1998)

HHV-8 seroprevalence and have identified a new subtype of HHV-8 in this population (subtype E) (Biggar et al., 2000; Whitby et al., 2004; Kazanji et al., 2005).

HHV-8 genomes are now classified into five major subtypes (A–E) that reflect genetic sequence heterogeneity in the highly variable ORF K1 (Hayward and Zong, 2007). K1 gene encodes for a 46 kDa type 1 glycoprotein, which has been used to study the evolution and worldwide distribution of HHV-8. Subtype A HHV-8 is found in Northern Europe and America; subtype B (thought to be the most ancient) is from Africa; and subtype C, often associated with classical KS, is found in Mediterranean countries (Stebbing et al., 2006; Hayward and Zong, 2007). HHV-8 subtype D (mainly from South America and the Pacific Islands) and, more recently, subtype E (South America) have also been described (Kazanji et al., 2005). Based on ORF75 gene, subtype N has been identified in South Africa (Alagiozoglou et al., 2000).

TRANSMISSION AND ASSOCIATED RISK FACTORS FOR HHV-8 INFECTION

The modes of transmission of HHV-8 may be different in different parts of the world, depending on the endemicity of that region, and are still being investigated, but both horizontal and vertical transmissions have been reported (Kedes et al., 1996; Mantina et al., 2001; Mbulaiteye et al., 2003). HHV-8 can be found in the peripheral blood mononuclear cells (PBMCs), saliva, oropharyngeal mucosa, semen, and prostate glands, which may represent the source of both vertical and horiozontal transmissions (Chen and Hudnall, 2006).

Vertical Transmission

Vertical transmission of HHV-8 has been documented but is believed to occur infrequently. Reactivation of herpesvirus infections occurs during pregnancy, but very little is known about HHV-8 infection and pregnancy. Rare cases of KS in newborns have been described (Gutierrez-Ortega et al., 1989; McCarty and Bungu, 1995). Our laboratory has also reported that HHV-8 DNA can be detected in a small percentage of infants born to HHV-8 seropositive mothers in Zambia (Mantina et al., 2001). HHV-8 is often found in cervicovaginal secretions of HIV-1 and HHV-8 coinfected women, suggesting that HHV-8 viral load in the female genital tract might influence vertical transmission (Lisco et al., 2006). Reported data also showed that the rates of detection are higher among African women from areas of nonendemicity or subendemicity (Viviano et al., 1997; Calabro et al., 1999; Whitby et al., 1999; Boivin et al., 2000; Lampinen et al., 2000). Together, these findings lead credence to the theory that *in utero* or intrapartum HHV-8 infection might, albeit rarely, occur in countries where HHV-8 is endemic.

Horizontal Transmission

Horizontal transmission is likely to occur during childhood in endemic countries and during adulthood in nonendemic countries. In Africa, primary infection seems to occur during early childhood and seroprevalence increases with age (DeSantis et al., 2002;

Martro et al., 2004; Plancoulaine et al., 2004). Our cohort studies in Zambia show that more than 10% of children can be infected by 12 months of age (Minhas et al., 2008a). Recent reports indicate that in Africa, transmission from mother to child and between siblings accounts for a substantial proportion of infections in childhood (Mbulaiteye et al., 2003; Plancoulaine et al., 2004). Saliva is now emerging as the likely candidate that mediates childhood transmission. A study conducted in South Africa has reported the presence of HHV-8 DNA in breast milk of seropositive mothers with high-titer antibodies (Dedicoat et al., 2004). Breast milk of mothers has been reported to contain herpesviruses such as cytomegalovirus (CMV), EBV, and HHV-7 (Dworsky et al., 1983; Ahlfors and Ivarsson, 1985; Junker et al., 1991; Fujisaki et al., 1998; Hamprecht et al., 1998). However, our studies in Zambia have failed to detect any viral DNA in breast milk (Brayfield et al., 2004). Therefore, evidence of breast milk transmission remains controversial, but it does not seem to be an important route of transmission to young infants. Very little is known about the transmission of HHV-8 in children in developed countries including the United States, and the results of these reports are conflicting (Martro et al., 2004). Vieira et al. reported that HHV-8 DNA recovered from saliva was similar to HHV-8 DNA recovered from the cultured 293 cells that had been infected with saliva fluid (Vieira et al., 1997). The transmission of HHV-8 from mother to child and between siblings most likely occurs via saliva, given that HHV-8 DNA has been detected in saliva in high copy number (Plancoulaine et al., 2000; Brayfield et al., 2004; Dedicoat et al., 2004; Mbulaiteye et al., 2006). It has been reported that there is an increased prevalence of HHV-8 antibodies in children of mothers shedding high number of viral DNA copies per milliliter of saliva, suggesting that large amounts of HHV-8 in maternal saliva may be associated with the transmission to the child (Alkharsah et al., 2007). All these studies suggest that HHV-8 can be transmitted via the saliva like other human herpesviruses (EBV, CMV, HHV-6, HHV-7, and HSV-1) (Lucht et al., 1998). The specific practices that expose an individual, especially in the case of young children, to salivary transmission still remain to be explored.

In addition, the transmission from contaminated blood products has also been suggested because viral DNA can be detected in 10%–15% of PBMCs of healthy HHV-8 seropositive individuals (Whitby et al., 1995; Dollard et al., 2005; Zavitsanou et al., 2006). Though the risk for such transmission events appears to be very low, a recent study has shown conclusively that the transmission of HHV-8 is possible by blood transfusion, but this risk diminishes as the period of blood storage increases (Hladik et al., 2006). Transmission of HHV-8 via this route is not efficient because of the cell-associated nature of the virus. Also, low and/or intermittent viremia among seropositive donors and low level of circulating virus in asymptomatic donors may contribute to the low rate of transmission.

Epidemiological studies have revealed that the development of KS is more frequent in HIV-1 seropositive homosexual men, suggesting that HHV-8 might be a sexually transmissible agent. HHV-8 can be detected in semen samples in HHV-8 endemic areas (Blackbourn and Levy, 1997). Specific intimate behaviors that increase the risk of transmission are still not clear. Therefore, sexual transmission was believed to be the most common route of HHV-8 transmission in adult immunocompetent populations in countries of low seroprevalence, even though viral loads found in

vaginal, seminal, and prostatic secretions are much lower than in saliva (Bagasra et al., 2005; Edelman, 2005). Varying detection rates have been reported in cervical or vaginal samples (Viviano et al., 1997; Calabro et al., 1999; Whitby et al., 1999; Boivin et al., 2000; Lampinen et al., 2000). Sexual risk factors associated with HHV-8 seropositivity include HIV-1 infection, high number of homosexual partners, history of sexually transmitted infections (STIs), orogenital insertive and receptive sexual practices, use of amyl nitrates, and unprotected sex with commercial sex workers (Melbye et al., 1998; Cannon et al., 2001; Malope et al., 2008). In endemic areas, HHV-8 seropositivity was also associated with increasing age, history of STIs, not using condoms, and having a seropositive spouse (Baeten et al., 2002; Mbulaiteye et al., 2003; Klaskala et al., 2005). Despite these associations shown by previous studies, several recent studies are now questioning the importance of sexual transmission. A recent study from South Africa and our studies in Zambia show that there is no association of HHV-8 infection with any of the sexual risk factors (Malope et al., 2008) (manuscript in preparation). A large cohort-based study from the United States also did not observe any heterosexual transmission to women (Engels et al., 2007). While examining the role of sexual transmission, it is difficult to distinguish whether the transmission event occurred via kissing or via cervicovaginal or seminal secretions. Simple associations without controlling for confounding variables may result in spurious associations. Therefore, sexual transmission needs to be further examined in order to draw any meaningful conclusions.

An uneven geographic distribution of HHV-8 and its familial clustering raises the question as to the existence of environmental or host genetic factors that predispose to HHV-8 infection, increased viral replication, and/or development of KS (Hudnall, 2004; Simonart, 2006). A study performed in the United States indicated that the incidence of classic KS in Jews born in Eastern European and Mediterranean countries was higher than second generation American Jews, suggesting that environmental factors are important (Ross et al., 1985). Recently, an "oncoweed hypothesis" was proposed to implicate environmental cofactors present in KS endemic regions, which cause frequent reactivation of HHV-8, leading to increased viral shedding and transmission. This leads to increased prevalence of HHV-8 in a region as well as increased viral load levels and higher antibody titers (Whitby et al., 2007). This study has identified 184 plant extracts from 38 countries that can cause HHV-8 lytic replication *in vitro*. This is a new hypothesis, and further studies are needed to fully delineate the role of environmental cofactors.

In summary, the exact risk factors that predispose an individual to HHV-8 infection are still not known. These routes may vary in different populations and risk groups, and more extensive studies need to be conducted.

SEROREVERSION OF HHV-8 ANTIBODIES

Upon following a cohort of Zambian mothers and infants for several years, we have frequently observed fluctuations in titers or complete loss of antibody titers against HHV-8 (Minhas et al., 2008a). Other reports have documented similar observations

where even clinically diagnosed KS patients undergo seroreversion after remission (Albrecht et al., 2004; Zavitsanou et al., 2006).

Seroreversion may be either partial or complete. Partial seroreversion results from a loss of detectable titer to one or more antigens, while complete seroreversion results from a total loss of all detectable specific antibodies. We believe that the observed seroreversions in our cohort are both partial and complete seroreversions. Prolonged virus suppression could hypothetically lead to an incomplete antibody response or antibody seroreversion. Seroreversions have been reported in patients after undergoing HCV treatment, and it has been postulated that this is due to the lack of antigenic stimulation after viral eradication (Lefrere et al., 2004). Rare reports of seroreversion in HIV-1 patients with acute infection in whom antiretroviral threrapy was initiated early have also raised the question whether this could be due to a loss of HIV antigenic stimulation, though complete viral eradication is not possible in HIV-1 infection (Amor et al., 2006).

The observed phenomenom of seroreversion of HHV-8 antibodies could be due to several reasons. An absence of antigenic stimulation after the establishment of latency or viral clearance by immunocompetent individuals, who are able to efficiently control further reactivation after primary infection, may cause the antibody titers to drop below detection levels. It has been proposed that HHV-8-specific antibodies might not be readily detectable due to a slow broadening of epitope recognition after primary HHV-8 infection (Biggar et al., 2003). Therefore, the duration of infection may be an important factor that determines the level of antibody titers in infected individuals. HIV-1-related immunosuppression has also been proposed to be a factor responsible for HHV-8 seroreversion but requires further studies before this association can be established. It is also possible that seroreversions could be introduced due to imperfect assays. Different assays and their sensitivity will be discussed further in the next section.

Therefore, there is a need to conduct more extensive longitudinal studies in which infected individuals are followed over a period of time, to get an accurate picture of the persistence of antibodies against HHV-8. Single-point observations as noted in cross-sectional epidemiological studies may be underestimating the seroprevalence of HHV-8 in a population as seroreversion cases will be missed, and reactivation of latent infection to restimulate the antibody response may be misinterpreted as primary infection.

DIAGNOSIS OF HHV-8 INFECTION

Although HHV-8 has now been identified for more than a decade, accurate screening for infection in asymptomatic individuals is still a challenge. A number of techniques have been employed for detecting HHV-8 infection in individuals and include both molecular and serological methods. HHV-8 has now been identified in cells, tissues, and body fluids using different approaches individually and in combined formats.

Polymerase chain reaction (PCR) amplification has been used for the detection of HHV-8 DNA, and initial studies have demonstrated that at least 95% of KS lesions harbor HHV-8 DNA that can be amplified (Chang et al., 1994; Cesarman et al., 1995;

Huang et al., 1995; Sarid et al., 1999; Iscovich et al., 2000). However, the detection rate of HHV-8 DNA in peripheral blood samples from patients without KS is substantially lower and is often not detected even by PCR (Spira et al., 2000; Cannon et al., 2003). Therefore, the utility of PCR as a tool for definitive diagnosis in the general population is limited and has not proven to be a reliable method for large-scale population surveillance of HHV-8 infection.

Different assays or algorithms reported for HHV-8 screening have been summarized in Table 18.2. For the serological detection of HHV-8, immunofluorescence assay (IFA) was one of the first assays to be reported (Lennette et al., 1996). Cell lines derived from PEL and chronically infected with HHV-8, expressing mainly latent and a low level of lytic antigens have been used for latent or lytic IFAs. The level of lytic antigens can be increased by induction with tetradecanoyl phorbol acetate (TPA) or sodium butyrate, and virus can be harvested from the supernatant. Using sera of KS patients,

TABLE 18.2. Assays or Algorithms Developed for Determining HHV-8 Serostatus Summarizing the Type of Assay and Antigen Used and the Reported Sensitivity and Specificity of the Assay

Assay (Cell Line)	Antigen	Reported Sensitivity	Reported Specificity	Reference
IFA (BCBL-1)	Latent and lytic	NR	NR	Lennette et al. (1996)
ELISA	Whole virus	NR	NR	Chatlynne et al. (1998)
EIA	Whole virus	94%	95%	Martin et al. (2000)
ELISA	ORF65, LANA	81.7%	90.8%	Goudsmit et al. (2000)
EIA	ORF65, K8.1	Variable	Variable	Engels et al. (2000)[a]
EIA	Whole virus	88%	97%	Casper et al. (2002)
IFA (BHK21 cells)	ORF73, K8.1	94%–100%	93%–100%	Inoue et al. (2000)
EIA	ORF65, K8.1 peptides	NR	NR	Sergerie et al. (2004)
ELISA	LANA1 (baculoviral), ORF65, and K8.1 peptides	95.6%	94%	Laney et al. (2006)[b]
ELISA	Latent and lytic	81.8%	96%	He et al. (2007)
IFA (BC3 and insect cells)	ORF65, LANA, K8.1	94%	96%	Minhas et al. (2008b)

[a]The sensitivity and specificity reported varied across different populations that were tested.
[b]For a high specificity algorithm, the reported sensitivity and specificity were 93.3% and 98.0%, respectively.
NR, not reported.

several latent and lytic proteins were then identified as highly reactive antigens (Chandran et al., 1998). Of these proteins, ORF59, K8.1A, ORF65, and ORF73 have been used in the development of enzyme immunoassays (EIAs) and reported to be good candidate antigens (Katano et al., 1999, 2000; Enbom et al., 2000; Goudsmit et al., 2000; Casper et al., 2002; Sergerie et al., 2004; De Baets et al., 2007). Also, two commercial EIAs using whole virus lysate and synthetic peptides are now available (Advanced Biotechnologies Inc, Columbia, MD, and Biotrin, Dublin, Ireland). IFA utilizing HHV-8-infected cells is still a commonly used method and is reported as one of the most sensitive assays to screen for HHV-8 antibodies in patients (Schatz et al., 2001). One drawback of the HHV-8-positive cell lines is that they lack matched HHV-8-negative controls.

In spite of developing IFA, enzyme linked immunosorbent assay (ELISA), and Western blotting for detecting HHV-8 antibodies, none of these tests have optimal sensitivity, specificity, and ease of use. Most serological assays employed to detect HHV-8 antibodies do not have high concordance among themselves, which makes it a challenge to identify truly infected and uninfected individuals, especially when the antibody titers against HHV-8 in asymptomatic individuals are considerably lower as compared with KS or PEL patients (Rabkin et al., 1998; Pellett et al., 2003). The use of nonstandardized, "in-house" assays also makes it difficult to directly compare and interpret the results from seroepidemiological studies. Therefore, conclusive diagnosis of HHV-8 has remained a challenge because of the lack of detectable virus in the peripheral blood, discordance between the currently available serological assays, lack of a gold-standard assay, seroreversions, and lack of true seronegative and seropositive group of patients (Ablashi et al., 2002). Nevertheless, for large-scale epidemiological studies, serology still remains a reliable method to identify HHV-8 infection in individuals, to understand the route of transmission and for routine monitoring of "at-risk" individuals (Albrecht et al., 2004). To address these drawbacks, our laboratory has recently developed a screening algorithm using two different IFAs (Minhas et al., 2008b). Samples are screened by IFA utilizing BC3 cells and confirmed on IFA utilizing Sf9 insect cells that express ORF73, ORF65, or K8.1A antigens. Cells expressing glutathione S-transferase (GST)-tag serve as matched negative controls. Whether this screening algorithm can be easily adapted for large-scale seroprevalence studies needs to be determined.

CONCLUSIONS

Our knowledge of HHV-8 epidemiology and transmission has increased significantly in the past 15 years, but several important questions still remain to be answered. Strong epidemiological studies are required to validate previous reports of the association of HHV-8 with other disease conditions. HHV-8 epidemiology clearly shows that immunosuppressed individuals are at a higher risk for developing HHV-8-associated diseases. The ongoing HIV epidemic makes it essential to expand our knowledge about the complex interplay between the two viruses because a significant number of HIV-infected patients are coinfected with HHV-8. Further studies are also required to better understand the route of transmission and the risk factors associated with HHV-8 transmission that is specific to age groups and to different geographic areas.

But to achieve this, there is an urgent need for reliable diagnosis by using standardized assays. Only then can one attempt to introduce this assay for routine screening of transplant patients. The detection of primary HHV-8 infection by detecting IgM antibodies for primary infection is another area where very little research has been conducted. Longitudinal epidemiological studies that incorporate follow-up of patients will be helpful in determining when a primary infection occurs and in determining the incidence of infection, seroreversions, and risk factors associated with transmission.

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THE ROLE OF KSHV IN PATHOGENESIS OF KAPOSI'S SARCOMA

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INTRODUCTION

Our goal here is to focus on what we judge to be the most important information known about the biology, cell biology, and pathogenesis of Kaposi's sarcoma (KS) and the novel herpesvirus Kaposi's sarcoma herpesvirus (KSHV) or human herpesvirus-8 (HHV8) that was first discovered to be associated with it by Chang and Moore in 1994 (Chang et al., 1994). Some perspectives derived from contrasts between KSHV in KS and in lymphomas or with other human tumor viruses are included. Of necessity, the quotations of work from colleagues in this field are highly selective and incomplete (especially older studies), and it is not our intent to catalog every known interaction of the numerous viral-encoded proteins that have been implicated as key players in these processes. Instead, the interpretations and judgments presented are based primarily on the most recent critical published findings of others, although they are inevitably influenced also by our own published and unpublished observations.

Even just over the past 5 years up to March of 2008, there have been numerous reviews on all aspects of research on KSHV, and there are also many excellent older reviews that are not listed here. These include two comprehensive compilations (Dourmishev et al., 2003; Greene et al., 2007) from the already extensive literature

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about KS (over 10,800 citations in Pubmed, with 3500 on KSHV virology). There have also been many additional shorter evaluative reviews focused on either KSHV-associated lymphomas (Du et al., 2007; Carbone and Gloghini, 2008), multicentric Castleman's disease (MCD) (Stebbing et al., 2008), connecting KSHV molecular genetics with pathogenesis (Schulz et al., 2002; Viejo-Borbolla et al., 2003; Henke-Gendo and Schulz, 2004; Ganem, 2006; Schulz, 2006; Dittmer and Damania, 2007; Nicholas 2007), on endothelial- and lymphocyte-based *in vitro* systems (McAllister and Moses, 2007), KSHV signaling (Brinkmann and Schulz, 2006; Montaner et al., 2006; Sullivan, 2007), KSHV-related immune evasion (Moore and Chang, 2003; Rezaee et al., 2006; Coscoy, 2007; Douglas et al., 2007), targeted therapy and treatment (Raggo et al., 2005; Dittmer and Krown, 2007; Hansen et al., 2007), risk factors for classic KS (Brown et al., 2006), or KS disease in Africa (Akanmu, 2006; Mbulaiteye and Goedert, 2008).

COMPARISON OF VIRAL ONCOGENIC MECHANISMS

Among the six viruses unambiguously implicated in human cancers, two are herpesviruses. The Epstein–Barr virus (EBV) is primarily known for its causal involvement in both Burkitt's lymphoma (BL) in B cells and also in nasopharyngeal carcinoma (NPC) in epithelial cells. KSHV causes KS in endothelial cells (EC) as well as primary effusion lymphoma (PEL) plus some forms of MCD, which both involve B cells. These are both gamma subfamily herpesviruses, but the other six human alpha and beta herpesviruses are not believed to be oncogenic in humans. Although the role of hepatitis C in hepatocellular carcinoma is primarily inflammatory, the other three small human tumor viruses all encode one or more specific viral "oncogene" proteins that contribute necessary functional effects to transformation or tumor development at a variety of levels and by multiple mechanisms. These necessary dysregulatory functions are associated with the pluripotent pX protein of hepatitis B virus in hepatocellular carcinoma, with TAX for human T-cell leukemia virus-type 1 (HTLV-1) in adult T-cell leukemia (ATL) and with E6 and E7 for papillomaviruses in cervical carcinoma. The much larger genomes of EBV and KSHV each encode several unrelated potential oncogenes, but there are many more viral genes involved and they participate in even more complex virus–host interaction pathways. In EBV, the implicated culprits are primarily limited to just three latent state proteins: the nuclear antigen called EBNA2 and the two latent membrane proteins known as LMP1 and LMP2, whereas for KSHV, at least six genes have been strongly implicated, including those encoding the latent state proteins latency-associated nuclear antigen (LANA), viral cyclin (vCYC), and viral Flice inhibitory protein (vFLIP), and the lytic cycle proteins viral interleukin six (vIL6), variable ITAM protein (VIP or K1) and viral G-protein coupled receptor (vGPCR).

Two common features about HTLV-1 and the four human oncogenic DNA viruses are first, that the genes most directly involved in the oncogenic processes all encode early lytic or latent state regulatory proteins. Second, it is generally interpreted that in none of these viral infections is progression to tumorigenicity considered either ubiquitous or inevitable. In fact, the development of viral-associated cancer usually occurs only rarely and involves additional irreversible genetic insults to the host cell that are not required for the virus life cycle *per se*. Many of the effects of these viruses are

essential components of the process by which the virus takes control of the host cell. These may include promoting proliferation or S-phase events by interfering with normal cell cycle controls, by inducing signal transduction, blocking apoptosis, regulation of transcription, DNA synthesis, or DNA repair. In addition, all mammalian viruses promote and then evade interferon-mediated innate immunity or antiviral responses, and have mechanisms to avoid cellular T-cell and humoral adaptive immune responses. In some cases, especially with KSHV, reactive paracrine inflammatory and angiogenic responses are also invoked. Furthermore, many of these events are very similar to the dysregulation processes that also occur in nonviral-related cancers, and hence, they have historically provided and are still currently producing many insightful paradigms about oncogenic processes in general.

However, for most viruses, these types of dysregulatory genetic programs alone are evidently not sufficient for tumorigenicity. Consider that most other retroviruses and DNA viruses also produce similar altered cell cycle and inflammatory effects on the host cells, but nevertheless do not produce tumors in humans naturally. These include human immunodeficiency virus (HIV), adenoviruses, papovaviruses, and human alpha and beta herpesviruses, some of which even encode classical DNA virus oncogenes such as T antigen of papovaviruses or E1A/E1B of adenoviruses, which both act primarily to disrupt pRB and p53 cell cycle checkpoints in association with viral DNA replication. Therefore, the particular circumstances in which virus infection leads to cancer are both very subtle and specific and probably, to varying degrees, accidental. Certainly, although there may well be benefits to the virus for the host cells to become long lived and immortalized, there is no selective advantage to the virus for the viral genome-carrying cells to become cancerous and fatal to the host. Indeed, it is arguably true that in every case where this happens, it is an evolutionary dead end for the virus itself. This is also the case even for classical murine and avian retroviruses when they capture and modify cellular proto-oncogenes and become defective helper-dependent viruses by simultaneously losing essential viral genes or functions. Only the notable and rare exception of the *Rous sarcoma* virus retains its infectability after acquiring a cellular proto-oncogene. Of course, this principle is also true even for the cancer cells themselves, except in the curious case of a recently described clonal infectious tumor in dogs. Whether or not the effects of HTLV-1 Tax on preventing the normal G2/M checkpoint induced apoptosis in cells that are undergoing aneuploidy has a survival advantage for the virus is a matter for debate, although it clearly has survival value for the tumor cells and is likely to be a necessary contributing event for the progression of ATL.

An important question in relation to the above is whether the cancer cells themselves might still be able to produce infectious virions. Traditionally, both papillomavirus and hepatitis B have been thought to involve integration events that inactivate the lytic parts of the virus genome, and the lack of this pathway to inactivate progression of the cell killing effects of the lytic cycle may be a major reason that papovaviruses and adenoviruses do not manifest as tumors in humans, whereas they can both do so in nonpermissive animal hosts where viral gene expression is limited just to the early "oncogene" expressing stages. However, although documented for the *E2* gene in bovine papillomaviruses (BPVs), this inactivating integrative process does not seem to occur routinely for human papillomaviruses (HPVs). Curiously, inactivating point mutations in key viral genes that may potentially lead to equivalent effects also do not

seem to occur in any of these systems. Furthermore, although there are exceptions, it is often a characteristic feature of virus-driven cancer cells that the cellular tumor suppressor genes such as p53 or cRAS that are typically mutated in nonviral tumors remain intact, even if their functions are suppressed by the virus. Insertional mutagenesis has been documented in a small number of human cases of retrovirus vector-mediated gene therapy for children with X-linked SCID leading to LTR-activated LMO oncogene triggered lymphomas. But no other natural cases of viral insertional mutagenesis are known in humans, although obviously specific activating chromosome 8/14 translocations of *c-myc* are a consistent defining feature of EBV-associated BL.

In both EBV and KSHV, an alternative latent state viral program effectively serves to shut down the lytic phase and is intimately associated with at least the progenitor cells of the tumor. However, in both cases, the effect is programmed and reversible, and the virus lytic cycle can be reactivated to yield infectious progeny virions. In EBV, the normal reversible latent state occurs in lymphoblastoid cell lines (LCLs) that are immortalized by the virus but are not yet tumorigenic. Whether virus can be properly reactivated after *c-myc* translocations occur in BL or after the unknown additional events that lead to transformation in infected epithelial cells destined to form NPC is not clear. A good recent evaluation of the status and role of EBV in oncogenic disease is provided by Kelly and Rickinson (2007). In contrast, in KSHV, there are established PEL tumor-derived cell lines from which infectious virus can be recovered, even though the PEL cells themselves have numerous genetic abnormalities (Gaidano et al., 1999; Nair et al., 2006). PEL cells are mature plasma cell transformants that express clonal rearranged lambda immunoglobulin light chain proteins, whereas perhaps uniquely so, the KSHV latently infected spindle cells that form the bulk of nodular cutaneous KS lesions are often polyclonal or oligoclonal (Duprez et al., 2007) and might in fact be just a reversible proliferating neoplasia not necessarily involving cellular mutations or oncogenic transformation events. Nevertheless, the more aggressive clonal variants that can become disseminated and invasive at later stages of AIDS-KS probably do suffer additional cellular genetic insults. Herpes simplex virus (HSV) also establishes a long-term latent state, but the differences are presumably that this occurs in postmitotic neurons that do not become immortalized and there are no viral proteins expressed.

DISCOVERY OF THE ETIOLOGIC AGENT CAUSING KS LESIONS

The disease entity given his name was first described by the Hungarian dermatologist Moritz Kaposi in 1872. This classical form of the disease usually manifests as rare slowly progressing angiogenic skin lesions, either in plaque, patch, or nodular form, on the lower extremities in elderly males, especially those of Mediterranean, East European, and Jewish heritage. Before the advent of the AIDS epidemic, another more aggressive form called endemic KS was also found in young African males living in the so-called malaria belt regions throughout the 1920s to 1950s. For still unknown reasons, all forms display high male to female preferences, although this is much reduced in AIDS-KS. The presence of herpesvirus particles thought to be human cytomegalovirus (HCMV) as well

as HCMV DNA and antigens in KS lesions was reported initially by Boldogh et al. (1981), but a role for HCMV in KS was discounted by later more specific molecular hybridization studies (Ambinder et al., 1987).

Beginning in the early 1980s, epidemic KS in young homosexual males (now referred to as men who have sex with men or MSM) was one of the defining features of the early days of the AIDS epidemic, especially in New York and San Francisco. This form was even more aggressive and could become lethally disseminated to internal organs. In 1990, Beral et al. (1990) published a landmark epidemiological study showing that KS rates were increased 20,000-fold in HIV-positive patients, especially those who had acquired HIV sexually, rather than through intravenous (IV) drug usage or in hemophilia patients receiving therapeutic blood products. This began the search for another infectious agent other than HIV as the cause of KS, which was successfully accomplished 4 years later by a representational difference hybridization procedure within AIDS-KS tissue DNA (Chang et al., 1994). The same two small novel herpesvirus DNA fragments found there were also soon identified by polymerase chain reaction (PCR) in greater than 90% of African and classical KS cases and in most cases of body cavity-based lymphoma (later known as PEL), as well as in MCD (Cesarman et al., 1995; Dupin et al., 1995; Soulier et al., 1995). KSHV DNA was also detected in the iatrogenic form of KS that was known to appear at 500-fold increased rates in immunosuppressed renal transplant patients, especially within Saudi Arabia and in some North American hospitals with high Mediterranean or Jewish immigrant patient populations. One interesting characteristic of iatrogenic KS was that it regressed up to 50% of the time if the therapeutic immunosuppressive regimen was stopped. In concert with the obvious role of HIV, this led to the suspicion that the development of KS may have a large reactive inflammatory component to it. This idea was also supported by evidence from the Ensoli group that tissues from KS patients contained high levels of VEGF, bFGF, and inflammatory cytokines such as IL6 (Samaniego et al., 1998).

Obviously, for a virus that causes human disease, the classical Koch's postulates have not and cannot all be fulfilled for KSHV in either KS or PEL. Of course, as discussed by Moore and Chang (1998), the criteria for causality in the modern molecular age have changed greatly. Overall, as described in detail below, the evidence is now overwhelming, and no one seriously doubts causality here. In fact, so much so that we and some other authors have been omitting the word "associated" from the original descriptive name for KSHV. The principal lines of evidence are that (a) in the early studies of MSM AIDS patients without KS, seroconversion to KSHV was highly predictive of subsequent development of KS in up to 50% of cases; (b) the viral genomes and expressed viral proteins such as LANA are nearly universally present within the majority of tumor cells in both KS and PEL *in vivo*; (c) infectious KSHV virions derived from established PEL tumor cell lines can be titered in primary vascular EC cultures, and the infection process converts these cells into proliferating colonies with a spindle phenotype that morphologically closely resembles that of the defining spindle cells of KS lesions; (d) KS-like angioproliferative lesions have been observed after the inoculation of whole virus or KSHV-infected cells into mice (Mutlu et al., 2007) and very recently also in marmosets (J. Jung, pers. comm.); (e) KSHV encodes a large number of viral proteins that are individually and redundantly capable of transforming

fibroblasts in classic focus formation assays, or of inducing growth or inflammatory responses, S-phase progression, cell signaling pathways, and angiogenesis; and finally, (f) a number of inhibitory or therapeutic agents that selectively target either viral genes themselves or the cellular pathways that are known to be altered by viral gene products, lead to apoptotic responses in PEL cells *in vitro* and, in some cases, to KS remission in patients.

DISTRIBUTION, TRANSMISSION, AND EPIDEMIOLOGY OF KSHV

A necessary role for KSHV in KS tumorigenesis became obvious very quickly after its discovery, largely because of the rarity of infection in the general population. KSHV infections were detected in peripheral blood by both PCR and the presence of specific antibodies in up to 50% of MSM AIDS patients even those without obvious KS disease, but seroconversion itself proved to be highly predictive for the development of KS within just a few years (Whitby et al., 1995; Gao et al., 1996a). Furthermore, the overall seropositivity to KSHV was found to correlate well with the rates of classic or endemic KS in most populations (Gao et al., 1996b), being, by far, the highest in many parts of Africa (50%–70% beginning and increasing throughout childhood), then next in Mediterranean countries including localized parts of Italy at 10%–15% (Whitby et al., 1998), and finally lowest in Northern Europe, Asia, and North America, where the penetrance is probably no more than 1%–3% and possibly lower depending on cutoffs used in the assays. This global distribution pattern is very distinctive and different from latent infections with all other human herpesviruses, which are all ubiqitious at a very young age in third world populations, although primary infections by some of them (HSV2, HCMV, and EBV) tend to be delayed in more developed Western countries. Currently, KS is known to be the most frequently diagnosed of all human tumors in Southern Africa (Chokunonga et al., 1999; Dedicoat and Newton, 2003), and it is evident that, although KSHV was already previously endemic in that population, KS rates were relatively low until dual infections caused by the rapid spread of HIV have vastly increased the KS incidence. Conversely, the use of highly active antiretroviral therapy (HAART) to reduce and control the progression of HIV disease in recent years has also greatly reduced the rates of AIDS-associated KS in Western countries, but it does still occur there, and of course KS remains prevalent in AIDS patients in underdeveloped countries.

Although KSHV infections were obviously transmitted by sexual contact within the early AIDS epidemic, most authorities now believe that the primary source of infection is through saliva (Koelle et al., 1997; Vieira et al., 1997), and that the natural mode of transmission in the most heavily penetrated parts of Africa is familial from mother to child and among siblings (Mbulaiteye and Goedert, 2008). KSHV genomes and seropositivity (but without any known KS lesions) have both been found in Brazilian Amerindians (Biggar et al., 2000), and there have been at least two confirmed cases of classical skin KS within elderly Polynesian men (Duprez et al., 2006). The typical titer of KSHV in peripheral blood of KS patients in Uganda has been measured at 4×10^6 genomes (or virus particles) per million PBMCs (Nsubuga et al.,

2008). However, most of the viral load is eliminated, and the KS usually goes into remission after immune reconstitution with HAART therapy, independent of whether the mode of HIV inhibition involves reverse transcriptase (RT) or protease inhibitors (PIs), and also independently of the CD4 levels. Recent studies in renal transplant patients have also shown dramatically reduced rates and increased clinical remissions when therapy with the calcineurin inhibitor cyclosporine-A is replaced by treatment with the proliferation signal inhibitor rapamycin/sirolimus instead (Stallone et al., 2005). In some cases, a rapid reemergence of KS occurs if the latter treatment is stopped. All of these suggest a major role for some form of immunosurveillance in controlling KS in healthy individuals, and conversely of lymphocyte activation or inflammatory stimulation by HIV infection, perhaps via the TAT protein in AIDS patients. The rates of KS development before AIDS was measured per 100,000 persons per year were between 0.5 and 2 in Italy and up to 10 in West Central Africa, but only at 0.014 in one study in the United Kingdom. This closely reflects the relative seropositivity in these three locations, but it may also be true that KS rates are much lower in West Africa than elsewhere in Africa, despite having similar seropositivity rates; some have claimed that this reflects a difference in the ability of HIV-1 versus HIV-2 to act as a cofactor. At its peak in the AIDS epidemic in 1990, the incidence in the United States also reached 10 per 100,000 persons per year, but it has since dropped nearly 10-fold.

VIROLOGY AND GENOME STRUCTURE OF KSHV

The entire 137,000 bp unique double-stranded DNA genome of two strains of the virus found in PEL and KS was completed in 1996 (Russo et al., 1996) and 1997 (Neipel et al., 1997). These and other studies (Nicholas et al., 1997a,b) revealed the presence of nearly a dozen novel and unique captured or "pirated" cellular cDNA genes (such as vIL6, the viral chemokine ligands (vCCL1,2,3), viral modulators of immune responses (vMIR1,2), viral interferon response factors (vIRF1,2,3,4) and vGPCR), but otherwise, KSHV turned out to be a member of the gamma-2 or rhadinovirus genus of herpesviruses (Fig. 19.1). This genus includes several other old world primate gammaherpesviruses such as rhesus rhadinovirus 1 (RRV1) and the prototype rhadinovirus of squirrel monkeys, *Herpesvirus saimiri* (HVS), which causes T-cell lymphoma in marmosets and can immortalize human primary T cells. Nevertheless, like HVS, RRV1 is also biologically quite distinct from KSHV, being highly lytic in many cell types in culture and not causing any KS-like disease. However, there is a second lineage of rhesus and old world primate rhadinoviruses, which are genetically closer to KSHV and do not grow well in cell culture, but are associated with retroperitoneal fibromatosis, an angioproliferative tumor (Rose et al., 2003). Small segments of the POL gene of three viral genomes closely related to KSHV and RRV1 have also been detected in chimpanzees (Lacoste et al., 2001). The linear KSHV genomes packaged into virions are over 165 kb in size and include about 30 tandem repeats of an 800 bp G plus C-rich element encompassing the *cis*-acting latent state Ori-P motifs at the termini (Russo et al., 1996; Hu et al., 2002; Wong and Wilson, 2005). There are also two inverted copies of 1000 bp Ori-Lyt domains

close to or within the domains where the novel cellular genes were inserted as presumably multistep ancient evolutionary cDNA capture events (Nicholas et al., 1998; AuCoin et al., 2002; Lin et al., 2003). In established PEL cell lines, the viral genome persists as a multicopy unintegrated circular plasmid form that, at least in one case examined (BC1/HBL6), contains an extra one-third partial duplication to a total of about 230 bp in size (Russo et al., 1996).

Electron micrographs of KSHV capsids and enveloped particles (although they are very rare) in KS lesions and PEL cells proved that the viral genomes are largely intact and functional (Orenstein, 1992; Orenstein et al., 2000). The 80 genes or open reading frames (ORFs) encoded by KSHV have been numbered in a system matching that of HVS except that the 15 extra genes that are not related to any found in HVS are referred to by K numbers (Fig. 19.1 and Table 19.1). Well-characterized proteins were assigned additional more functional names by consensus agreements at the fourth and fifth International KSHV workshops in 2000 and 2001 (which are used here), although many alternative unapproved names also still persist. The virus is thought to primarily infect ECs and B cells (as exemplified by PELs) and, typically for herpesviruses, exhibits both latent and lytic gene expression patterns, with the latent pathway tending to be the default, although the switch into lytic is capable of being manipulated in culture by the use of inducers such as the phorbol ester TPA/PMA or sodium butyrate or a combination of both (Fig. 19.2).

Unlike HCMV for example, the genomes of KSHV found in KS patients in Africa or in the indigenous native peoples of South Asia and the Pacific Rim differ from each other and from those in Eurasia and North American Caucasians in a distinctive three-branched pattern. This clustering pattern accurately reflects the genetic divergence of modern human hosts into the three major diasporas created by founder effects and isolation associated with the "out of Africa" migrations over the past 100,000 years (Hayward, 1999; McGeoch and Davison, 1999; Zong et al., 2002; Hayward and Zong, 2006). These ethnically restricted strain differences are most strongly exhibited by the VIP or K1 protein that varies by as much as 30% at the amino acid level, so much so that even lower level branching clusters created by the radiation of Eurasian hosts out from a small number of ice age refuges about 10,000 years ago are mirrored in the viral VIP protein clustering patterns (Zong et al., 1999, 2002; Hayward and Zong, 2006). The TMP or K15 protein also comes as two major subtypes or alleles that differ by 70% at the amino acid level, each of which has undergone its own "out of Africa" divergence into three distinct forms. The terminal membrane protein (TMP or K15) also is not "hypervariable" in the same way that VIP is, and the minor or M allele is just an old "exotic" form that diverged from the prototype or P allele at least 20 million years ago (Poole et al., 1999; Hayward and Zong, 2006). The right-hand side M-allelle regions were most likely acquired by multiple step ancient recombination events with other KSHV-like viruses during humanoid evolution, and persists today only as a chimeric P/M genome within about 30% of Eurasian KSHV genomes and in a much smaller fraction of African and Pacific Rim KSHV genomes. Both of these variable genes encode functional membrane-bound tyrosine kinase signaling proteins, and they are probably both under some form of positive biologic selection, with the VIP gene currently evolving at the extraordinarily rapid rate (for a herpesvirus gene) of about one amino acid change every 1000 years.

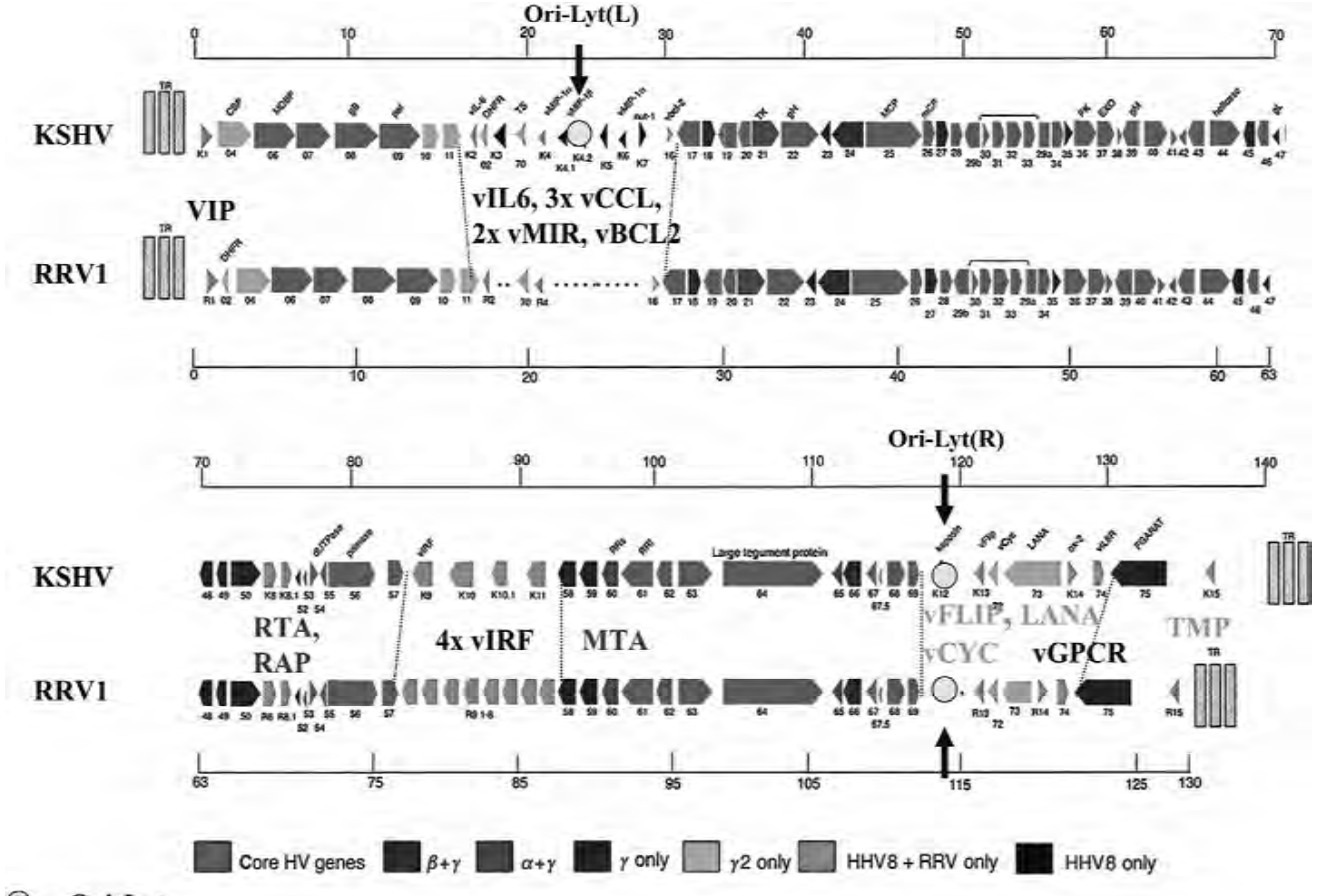

Figure 19.1. Genome map of KSHV and RRV1. The genomic organization of human KSHV compared with that of the related rhesus rhadinovirus RRV1 is depicted. All ORFs are shown as left- or right-oriented arrows of various colors representing gene classes. The duplicated Ori-Lyt domains (yellow circles) are indicated as well as the map locations of the selected critical latent and early lytic genes that are referred to in the text. The *cis*-acting, latent state Ori-P domains lie within the terminal tandem repeats. See color insert.

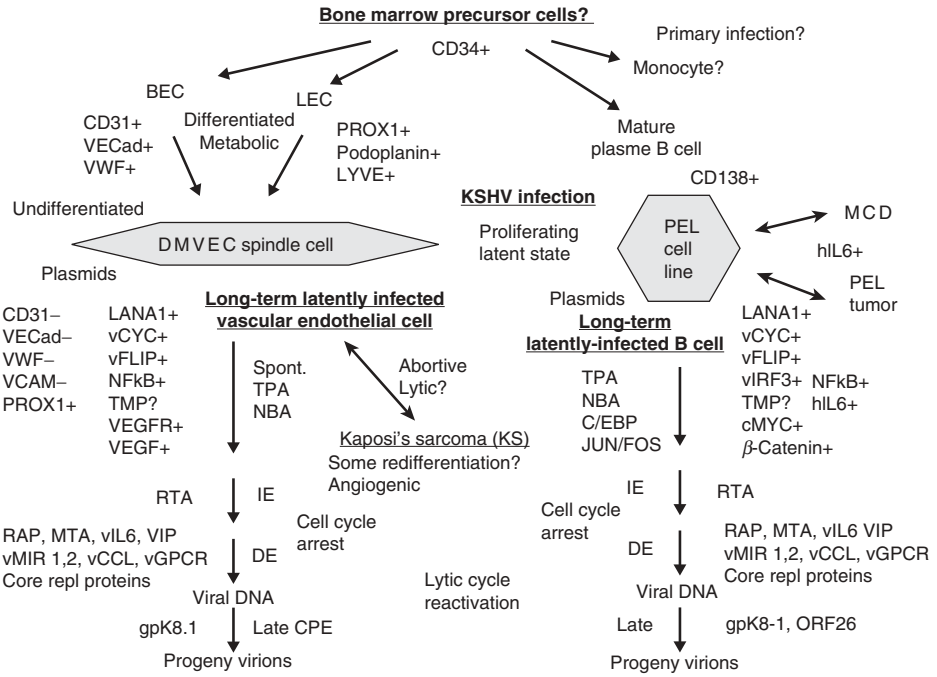

Figure 19.2. Summary of KSHV infection pathways in endothelial cells (ECs) and B cells. The primary target cells for *in vivo* infection by KSHV are unknown, but both ECs and B cells are expected to be involved is sustaining long-term latent infection (upper section of the diagram). The two models available for studying KSHV pathogenesis in cell culture involve KSHV-infected primary or immortalized vascular ECs (lower left-hand side of the diagram) or established B-lymphoblast cell lines derived from PEL tumors that carry multicopy KSHV plasmid genomes (lower right-hand side of the diagram). Major features of the latent and lytic cycle programs in both models are indicated, and manipulations that can induce partial switch into the productive lytic cycle in PELS are depicted. Older KS cell lines derived from KS lesions have lost their KS genomes and are unlikely to be valid models. The essential KSHV-mediated events that lead to KS lesions or PEL are not yet known but are assumed to be complex and multifactorial. Similarly, whether or not additional cellular gene mutational events are required for KS to produce aggressive disseminated visceral disease is not yet resolved. See color insert.

THE SPINDLE CELLS IN KS LESIONS

The most characteristic features of KS lesions are the large bundles of elongated spindle cells that coordinately align into bundled fascicles and predominate within nodular forms of KS. The spindle cells are much less abundant in the earlier flat patch and plaque forms of KS, but all forms also contain other cell types including

especially patches of vascular slits representing immature neovascular areas and often with associated extravasated red blood cells (RBCs), as well as many infiltrating inflammatory myeloid and lymphoid cells. The nature and type of the ECs was debated extensively for many years prior to detection of KSHV, with most authors concluding that they were endothelial in origin, primarily because the expression of several endothelial specific proteins including Factor VIII and VEGF receptors were considered characteristic histological features of the lesions. Some others considered them fibroblasts or derived from smooth muscle cells, and others reported the presence of CD68, indicating myeloid features. A number of "immortalized" cell lines reputed to be of KS origin were also established and studied extensively. These were unusually dependent on soluble factors derived from activated T-cell supernatants for growth, but we now know that none of them retain the virus, and their actual connection to KS spindle cells is unclear.

After the discovery of KSHV, the presence of virus in KS spindle cells was confirmed by immunohistochemistry (IHC) with specific antibodies to detect the punctate major LANA (Dupin et al., 1999). Indeed, the presence of LANA is a marker for spindle cells in all KS lesion samples. The 20–30 punctate LANA nuclear bodies are very characteristic features of KSHV latency, and their number correlates exactly with the number of extrachromosomal plasmid viral genomes present. Early-stage lesions tend to have fewer LANA-positive cells but also have many fewer spindle cells, and it is only in later stage nodular KS where the spindle cells predominate in the characteristic fascicular bundling pattern. These latter lesions also have high levels of KSHV latency gene transcripts and display much lower levels of apoptotic cells than early-stage lesions (Staskus et al., 1997).

Additional characteristic features of nodular KS lesions are high proliferation rates, high levels of VEGF, and expression of the KDR/FLT3 and FLT4 VEGF receptors (Masood et al., 1997), as well as lymphatic EC markers such as PROX1 and podoplanin. Indeed, the expression of nuclear PROX1 occurs in all LANA-positive spindle cells of highly nodular KS lesions at least. PROX1 is a homeobox class protein that initiates the transcriptional program of lymphatic ECs. Several high-quality cellular gene array studies have confirmed higher levels of many but not all lymphatic EC pathway transcripts in KS samples compared with control normal skin-derived samples (Carroll et al., 2004; Hong et al., 2004; Wang et al., 2004a). Despite the fact that typical functional vascular EC proteins such as PECAM1(CD31), VE-cadherin (CD144), and Von Willebrandt factor (VWF) were all reported as characteristic markers for KS lesions in much of the early IHC literature, we have recently reevaluated the situation by double-label IHC in a variety of KS samples and found that none of these three key metabolic EC proteins are expressed in the LANA-positive spindle cells themselves, whereas they are still abundant in the many mature as well as immature ECs forming the microvessel walls in the slit-like neovascular areas that are intermixed throughout the spindle cell bundles (D. Alcendor, Q. Huang, and G.S. Hayward, unpublished data). There are also frequent HLA-, CD34-, and CD68-positive cells scattered about, but again these are not the same as the LANA-positive cells.

EFFECTS OF KSHV INFECTION ON PRIMARY VASCULAR ECS IN CULTURE

Many early attempts to grow either an unknown virus or specifically KSHV from KS tissue samples in a variety of cell culture types were largely unsuccessful (Renne et al., 1998), although it was later shown that some support latent infection only (Bechtel et al., 2003). The major breakthrough came after several KSHV-positive PEL cell lines were established in culture that could be induced to produce the lytic cycle and progeny virus particles to various degrees after treatment with TPA or butyrate (Renne et al., 1996; Cannon et al., 2000), but it has been extraordinarily difficult to infect primary B cells or lymphoblasts with KSHV. However, a biologic colony/plaque assay for enumerating infectious KSHV virions was demonstrated in primary human adult dermal microvascular endothelial cells (DMVEC) by Ciufo et al. (2001), with the measured titers of KSHV from filtered cell culture supernatants from either the TPA-treated PELs or from the DMVECs both reaching 5×10^3 cfu/ml. Most importantly, only those cells in the culture that expressed LANA were converted into spindle cells. Equally significantly, all of the spindle cells in the culture expressed LANA, and there were no paracrine effects on the morphology of adjacent contact-inhibited "cobblestone" monolayers of uninfected DMVECs in the same cultures. The spindle colonies demonstrated high mitotic indexes with the LANA protein decorating the metaphase chromosomes in cells undergoing mitosis (Cannon et al., 2000). Furthermore, when the infected proliferating spindle cells overgrew the entire culture, their aligned swirling morphology began to resemble that of the characteristic bundled fascicles of nodular KS lesion tissue itself (Fig. 19.3).

Spontaneous induction of early KSHV lytic cycle antigens such as replication activator protein (RAP or K8), VMIR2, and VGPCR was also documented in a highly focal pattern within a small percentage of the spindle cells. Furthermore, a subset of those cells, which were all expressing the late viral K8.1 lytic antigen, displayed a second morphological change resembling the typical cytopathic rounding or cytopathic effects (CPE) of productive herpesvirus infections. In the titration assays, after about 3 weeks, the oldest infected cells in the spindle colonies all became lytic, producing a plaque in the center of just the largest colonies (i.e., those over about 5 mm in diameter). Long-term passaging of these cultures required addition of new uninfected DMVECs, and there was no evidence that they were immortalized or transformed, but rather that the cultures were sustained by reinfection, because the size and number of colonies were greatly reduced when grown in the presence of the viral DNA synthesis inhibitors gancyclovir or phosphonoacetic acid (Ciufo et al., 2001).

Several other cell culture models for growth of KSHV have been developed, and especially when the input virus is concentrated to high multiplicity of infection (MOI, pfu/cell), some degree of infection and growth can be obtained in monocyte derived endothelial cells (MDEC), human umbilical endothelial cells (HUVEC), keratinocytes, and diploid human fibroblasts, as well as E6/E7-immortalized dermal microvascular endothelial cells (DMVEC) and telomerase-immortalized DMVEC (=TIME) and HUVEC (=TIVE) cell lines, and even in primary mouse embryonic fibroblasts (MEF) (Flore et al., 1998; Moses et al., 1999; Lagunoff et al., 2002; Liang and Ganem, 2003;

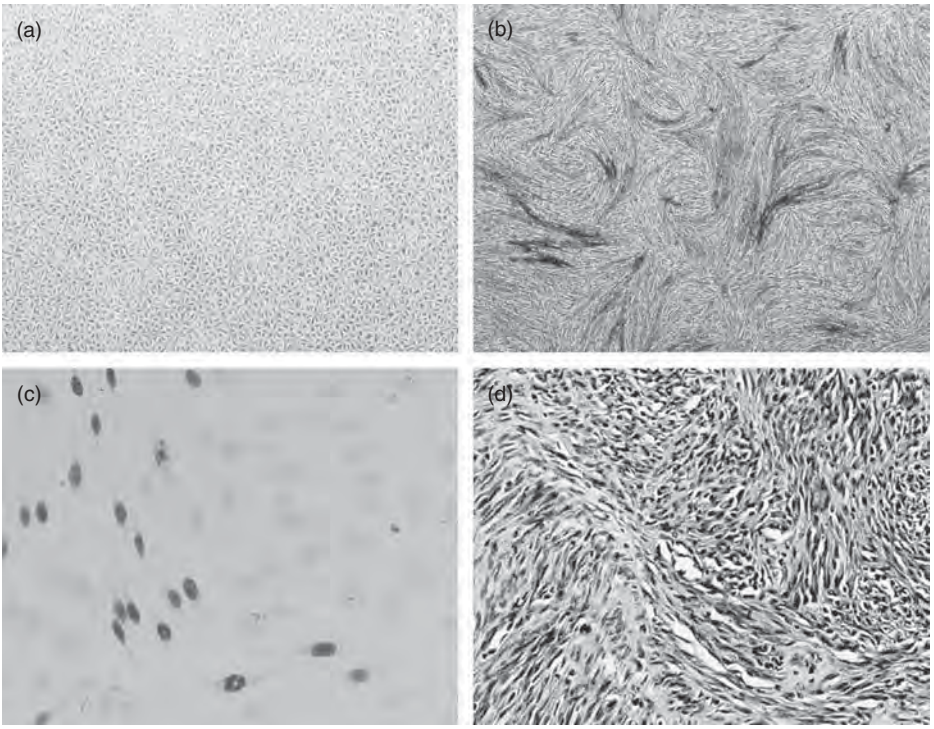

Figure 19.3. KSHV-infected DMVEC cultures as a model for KS. (a) Cobblestone monolayer of uninfected contact-inhibited DMVEC (stained with crystal violet). (b) Fully KSHV latently infected culture of spindle-converted DMVEC (second passage, stained with crystal violet). (c) High-power photomicrograph of a section of a partially KSHV latently infected culture showing a small spreading colony of LANA-positive proliferating spindle cells coexisting with uninfected DMVEC (brown nuclei = DAB-labeled IHC staining with rabbit polyclonal antibody directed against the KSHV LANA/ORF73 protein). More than 1% of the LANA-positive spindle cells were undergoing mitotic division and displayed LANA bound to the metaphase chromosomes. All LANA-positive spindle cells lose detectable expression of PECAM1 (CD31), VWF, and VE-CAD, but these were abundantly expressed in the adjacent uninfected cells (data not shown). (d) Typical thin sections of a nodular cutaneous KS lesion showing the characteristic fascicles of bundled spindle cells (stained with hematoxylin and eosin). Over 80% of the cells in this section proved positive for both LANA and PROX1 (not shown) (figure modified from Ciufo et al., 2001). See color insert.

Grundhoff and Ganem, 2004; McAllister and Moses, 2007). The Chandran group has shown that infection of HUVEC or DMVEC cultures with purified KSHV virions at high MOI involves envelope-mediated surface contacts that engage integrin and heparin sulfate-dependent receptor processes and produce PI3K and AKT signaling even without viral gene expression. Early activation of NFkB and NFkB-dependent expression of cytokines, growth, and angiogenic factors, including VEGF-A, VEGF-C, and COX2, also

occur under these conditions (Naranatt et al., 2003, 2004; Sharma-Walia et al., 2006; Sadagopan et al., 2007; Sivakumar et al., 2008). However, these viral gene expression-independent events are presumably just transient and also highly multiplicity dependent, and it is highly unlikely that infection occurs like that naturally *in vivo*. Nevertheless, this system clearly provides important insights, including evidence that both latent and lytic transcripts are synthesized simultaneously at initial stages of *de novo* EC infection, but this then leads to a default latent state (Krishnan et al., 2004). Two other KSHV-specific receptors, DC-SIGN (Rappocciolo et al., 2006) and cystine/glutamate transporter xCT (Kaleeba and Berger, 2006), that apparently enhance virus entry into cells have been described. Other particularly important experimental breakthroughs with doxycyclin-inducible PELs (Naranatt et al., 2003) or green fluorescent protein (GFP)-tagged (Vieira and O'Hearn, 2004) and Bacmid forms of KSHV (Zhou et al., 2002; Gao et al., 2003) have also recently revolutionized opportunities for KSHV genetic analysis.

REPROGRAMMING AND SHUTOFF OF EC FUNCTIONS IN SPINDLE CELLS

The primary DMVEC that we have used as a cell culture model for KSHV-induced spindle cell conversion forms contact-inhibited "cobblestone" monolayers and expresses high levels as detected by IFA of the functional metabolic EC proteins PECAM1, VWF, and VE-cadherin as well as of β-catenin in all cells, plus CD34 in small subset of cells and VCAM1 in about 1% of the cells. After tumor necrosis factor-α (TNF-α) treatment, the levels of VCAM1-positive DMVECs increased up to 85%, showing that they retain the expected ability to produce an inflammatory response. However, after several days of long-term latent KSHV infection initiated at low MOI, all IFA-detectable expression of these six proteins in the LANA-positive cells in the spindle colonies was abolished, whereas expression in the adjacent uninfected cells was unaffected (D.J. Alcendor, P. Desai, and G.S. Hayward, unpublished data). Manyfold reduction in the levels of these same proteins was also demonstrated by Western immunoblotting in fully infected DMVEC cultures. The same patterns of loss of metabolic EC protein expression occurred in uninfected DMVEC cultures by 48 h after TPA treatment and were also exhibited in the immortalized TIME cell cultures, which are often used as a more convenient but functionally less relevant model for KSHV infection than the primary cells. This is consistent with the concept that the virus is driving the target ECs away from their original metabolic state and into a proliferating preangiogenic state. Interestingly, in the initial gene arrays carried out in KSHV-infected versus uninfected DMVEC cultures, TPA also induced very similar overall changes in the uninfected cell gene transcription profiles to those that occurred in the latently infected cells in the absence of TPA (Poole et al., 2002). The secretable VWF in Weible–Palade bodies, which are necessary for clotting and thrombotic responses, and the immune tyrosine activation/inhibition motif (ITAM/ITIM)-containing PECAM1/CD31 adhesion protein, which forms a scaffold for numerous tyrosine kinase signaling interactions, are both well-known features of vascular EC. VE-cadherin interacts with β-catenin and F-actin in a process that is essential for the homeotypic cell–cell contacts and formation of intercellular adherens junctions that maintain the quiescent endothelial monolayer and are critical for the maintenance

of vascular permeability and blood pressure regulation. VCAM1 plays important roles in chemotaxis, survival responses and in adhesion contacts between vessel EC with platelets, monocytes, and lymphocytes. The isolated KSHV-encoded early lytic protein vMIR2 is capable of removing surface expression of PECAM1/CD31 from ECs (Tomescu et al., 2003; Mansouri et al., 2006), but whether this can explain the effects in long-term latently infected spindle cells remains unresolved.

Recent evidence strongly implies that the viral latent state vFLIP protein alone is capable of inducing spindle cell conversion and that inhibition of NFkB activity, which is enhanced by vFLIP via interaction with IKK complex, abolishes it (Grossmann et al., 2006; Matta et al., 2007; Efklidou et al., 2008). The cellular cKIT receptor protein, which is up-regulated in KSHV-infected primary and immortalized EC cultures may also be involved, because the spindle character of infected cells can also be abolished by treatment with Gleevec, an inhibitor of cKIT signaling (Moses et al., 2002). Two other cellular proteins, RDC1 and neuritin, which were both also identified to be transcriptionally up-regulated by gene profiling in KSHV-infected primary or immortalized DMVEC (Moses et al., 2002; Poole et al., 2002; Carroll et al., 2004), have similarly been shown to be required for KSHV-driven proliferation of spindle cells in culture (Raggo et al., 2005). Up-regulation of angiopoeitin-2 and STAT3 has also been observed (Punjabi et al., 2007; Vart et al., 2007). Two isolated viral genes, vGPCR and VIP, have each been implicated as being independently capable of transforming or immortalizing primary ECs when constitutively overexpressed (Bais et al., 2003; Wang et al., 2006), and vFLIP also provides survival properties (Efklidou et al., 2008). However, KSHV infection itself does not seem to do so, possibly because lytic infection eventually overwhelms the system, or instead the virus is lost because of an unstable latent state, both of which have been observed to occur in the various cell culture models.

The DMVEC cultures used in our studies above, represent a mixture of about 85% blood endothelial cells (BECs) to 15% lymphoid endothelial cells (LECs) as judged by IFA for nuclear PROX1, but both seemed to respond similarly. Interestingly, other studies with purified BECs versus LECs have revealed that KSHV infection tends to activate PROX1 protein levels in BECs but reduce them in LECs, suggesting that the virus can infect both equally well, but takes them to the same intermediate stage of differentiation partway between the two (Carroll et al., 2004; Hong et al., 2004; Wang et al., 2004a). Therefore, arguments that KS spindle cells are actually derived from LECs may not be valid, and in fact, they could equally well be derived from BECs or from some progenitor of either or both. Overall, the DMVEC and probably also HUVEC cell culture models for spindle cell conversion by KSHV latent state infection show remarkable parallels to what is observed *in vivo* for the spindle cells in KS lesions.

CONTRIBUTIONS OF KSHV LYTIC AS WELL AS LATENT STATE PATHWAYS TO PEL AND KS

In distinct contrast to EBV systems, where only latent state viral gene expression has been implicated in producing and maintaining the immortalized proliferating B cells in LCL cultures, as well as in the transformed epithelial cell phenotype in NPC tumors, there has been a growing acceptance of the probability that early lytic cycle genes may

contribute significantly to KSHV pathogenesis. There are two major reasons for this concept: First, probably all KS lesions and PEL cell lymphomas and established cell lines display at least some low-level fraction of cells that express lytic cycle viral proteins, and second, that many of the novel "captured" cellular cDNA genes of KSHV have been found to have properties that would be expected to produce important functional effects related to KS and PEL pathology (reviewed recently by Nicholas [2007] among others). Furthermore, the boundaries defining what are latent state versus lytic state gene expression patterns may also be becoming somewhat blurred with the idea and accumulating evidence that some of the very earliest expressed lytic proteins may sometimes also be expressed either latently or independently of the rest of the lytic program or at least in a delayed "abortive" lytic pattern.

In rigid simplistic terms, the only true latent state genes of KSHV are those encoded by multiple spliced transcripts driven from the well-defined LANA latency promoter, which include LANA, vCYC, and vFLIP. All three are now thought to be nuclear proteins that have dramatic pleotrophic roles in the maintenance of the latent state. For LANA, in addition to controlling the latent Ori-P plasmid mode viral DNA replication and partitioning of progeny viral genomes, these functions include stimulation of S-phase and proliferative cell growth via disruption of the GSK3β pathway, leading to up-regulation of β-catenin and cMYC (Fujimuro et al., 2003; Fakhari et al., 2006; Liu et al., 2007), transcriptional repression of cellular genes (Krithivas et al., 2000; Shamay et al., 2006), and interaction with p53-mediated DNA mismatch repair pathways (Koopal et al., 2007). There are also additional pro-S-phase proliferative effects produced by vCYC (Swanton et al., 1997; Verschuren et al., 2002; Sarek and Ojala, 2007). vFLIP induces powerful activation of NFkB via interaction with IKK (Matta et al., 2007; Zhao et al., 2007), leading to a multitude of important downstream pro-latency and antiapoptotic effects including up-regulation of hIL6 and VEGF, as well as antagonism of the lytic cycle pathway via inhibition of AP1 (An et al., 2003; Zhao et al., 2007; Efklidou et al., 2008; Ye et al., 2008). The roles of these and other likely key KSHV proteins deduced from their *in vitro* and biochemical functions are summarized in Table 19.1 and are described in greater detail in reviews such as that by Nicholas (2007). The LANA latency promoter is one of only two known latency promoters in the virus, which do not respond to either TPA or RNA transactivator protein (RAP) and give high constitutive expression when transfected into latently infected PEL cells. A variety of additional latent state spliced transcripts from this promoter have also recently been found to extend an extra 3–4 kb and give rise to a number of viral miRNA species as well as perhaps the T0.7 RNA and/or some kaposin-A/B transcripts in PEL cell lines (Samols et al., 2005; Grundhoff et al., 2006; Skalsky et al., 2007).

The two membrane tyrosine kinase signaling proteins VIP and TMP/LAMP encoded by the spliced K1 and K15 gene transcripts, respectively, would, by analogy with EBV LMP1 and LMP2, be expected to function in KSHV oncogenesis by promoting and maintaining the latent state in B cells at least. VIP in particular has antiapoptotic, angiogenic, and transforming properties via PI3K/AKT pathways and seems likely to play a major role in ECs (Lee et al., 1998, 2002, 2005; Wang et al., 2004b, 2006, 2007; Prakash et al., 2005). However, evidence that they are expressed at the protein level in the latent state has been hard to come by, and no latency promoters

TABLE 19.1. KSHV-Encoded Proteins Implicated or Potentially Involved in Tumor Pathogenesis: Expression Class

Protein/Gene Name	Known or Plausible Functions and Roles
True latent state	
LANA ORF73	Ori-P plasmid replication and chromosome tethering; promotes S-phase progression, transcription repression, and demethylation; interacts with GSK3/β-catenin, RING3, MAPK, pRB/E2F, p53/HDM2, and hTERT
vCYC ORF72	Transforming; promotes S-phase progression and hIL6, and CDK6/pRB interactions; insensitive to cdk inhibitors
vFLIP ORF71/K13	Up-regulation of NFkB via IKK interaction; antiapoptotic via FAS-R; contains DEDD domain
miRNAs intergenic 12x cluster	Derived from both latent and lytic mRNAs downstream from LANA/cCYC/vFLIP
Possibly conditionally latent	
vIRF3/LANA2 ORF-K10.5	Inhibits p53; interferes with cellular IRF function
TMP/LAMP ORF-K15	Blocks BCR signaling; interacts with SRC, TRAFs, and HAX-1
vIRF1 ORF-K9	Transforming; interferes with cellular IRFs
Very early lytic/possibly independent	
vIL6 ORF-K2	Growth stimulatory; anti-apoptotic and angiogenic; activates STAT1/STAT3/MAPK; can signal via both gp130 alone or via gp130/gp80 receptors; essential for growth of PELs
VIP ORF-K1	Transforming; constitutive ITAM-SH2 PT-signaling; antiapoptotic; inhibits FAS-R; blocks BCR signaling; binds SYK and LYN, induces PI3K, Ca ion influx, VEGF, and MMP9; immortalizes primary endothelial cells
vMIR2 ORF-K5	Immune evasion; UbE3L; targets HLA-I, ICAM1, B7.2, and CD31
Kaposin-A ORF-K12	Transforming; interacts with cytohesin-I; may be latent also
Immediate-early lytic	
RTA ORF50	Transcriptional trigger of reactivation; direct DNA binding; also targets CBF1/CIS plus C/EBPα and cFOS; intrinsic UbE3L; targets cellular IRF3/7; inhibits IFN-α, β mRNA
Early lytic	
RAP ORF-K8	Cell cycle arrest; promotes Ori-Lyt DNA replication; interacts with C/EBPα, p21, and CDK
MTA ORF57	Promotes nuclear transport of unspliced
vCCL1 ORF-K6	Angiogenic and antiapoptotic; binds CCR8; promotes TH2 responses; induces VGEF
vCCL2 ORF-K4	Angiogenic and antiapoptotic; induces eotaxin chemotaxis via binding to CCR8; broad-spectrum CCR antagonist
vCCL3 ORFK4.1	Binds CCR4; targets chemotaxin receptor; promotes TH2 responses
Kaposin-B K12/DR repeats	Stabilizes cytokine mRNAs via AREs
PAN/T1.1/NUT	Abundant nuclear nonpolyadenylated RNA

TABLE 19.1. *Continued*

Protein/Gene Name	Known or Plausible Functions and Roles
Delayed-early lytic	
vGPCR ORF74	Transforming; constitutive Gα-mediated signaling; induces MAPK signaling; stimulates NFkB and proinflammatory cytokines; angiogenic VEGF- and VEGF-related receptors and HO-1; generates KS-like lesions in transgenic mice
vMIR1 ORF-K3	Immune evasion; UbE3L; inhibits HLA-I
vOX2 ORF-K14	Macrophage stimulation
vBCL2 ORF-K16	Antiapoptotic; inhibits Bax
vSOX ORF37	Cellular shutoff; degrades U-box mRNAs

have yet been identified for them. But even if they are made at low levels in the latent state, both (like kaposin) are also highly induced at the mRNA level early during the lytic cycle. Two other enigmatic viral proteins/transcripts are vIRF3 (sometimes called LANA2) and vIRF1 encoded by the K10.5 and K9 ORFs, respectively. Current evidence suggests that nuclear vIRF3 is made in and essential for the latent state, but only in B cells (Rivas et al., 2001; Wies et al., 2008), whereas other studies suggest that vIRF1 although primarily a lytic cycle protein may be made in the latent state, but only in KS and not in B cells.

Other than the immediate-early reactivation triggering protein RTA encoded by ORF50, which is at least partially cycloheximide (CHX) resistant during reactivated infection, the earliest viral proteins produced in abundance are vIL6, an antiapoptotic and proangiogenic inflammatory cytokine, which is of critical importance in maintaining the PEL phenotype (Nicholas, 2007; Nicholas et al., 1997b), and vMIR2, a membrane ubiquitin ligase (UbE3L) involved in evading immune responses (Ishido et al., 2000; Coscoy et al., 2001; Coscoy, 2007) and perhaps also the proangiogenic vCCL1, vCCL2, and vCCL3 beta-chemokines (Table 19.1). Although clearly inducible in response to TPA, butyrate, and RTA, there is evidence that vIL6 (Chatterjee et al., 2002) and possibly also vMIR2 can be produced by transcriptional programs or alternative inducers, such as interferon-α (IFN-α) that may be partially independent of the bulk of the lytic cycle pathway, although, like VIP and TMP, the details of these possible novel classes of transcripts have yet to be resolved. Other exclusively lytic viral proteins with potential pathogenic roles that are produced later, but still relatively early, are the nuclear protein RAP involved in Ori-Lyt DNA replication and cell cycle arrest (Wu et al., 2002) and vMIR1, another UbE3L involved in evading immune responses (Nicholas et al., 1997a; Coscoy and Ganem, 2000; Ishido et al., 2000; Means et al., 2002), but vGPCR and vBCL2 are made even later, together with the core DNA replication proteins such as single-stranded DNA binding protein (SSB) and polymerase processivity factor (PPF or ORF59) at what might be considered classical delayed-early times. It is important to appreciate that these interpretations primarily apply to observations on protein expression in TPA-induced PELs, and they do not necessarily always correlate with defined transcription classes and patterns. Functionally, the viral-encoded vGPCR gene is of

great interest. This is a constitutively signaling member of the IL8-receptor family that has transforming properties via MAPK pathways that induce NFkB, AP1, HIF1α, and VEGF and can be enhanced by GROα or reduced by IP10 ligands (Bais et al., 1998, 2003). The expression of this single viral gene on its own, especially when targeted to vascular ECs, produces KS-like lesions in transgenic mice (Yang et al., 2000; Guo et al., 2003; Montaner et al., 2003). Therefore, despite being an exclusively lytic expressed gene, vGPCR has been suggested to contribute to KS *in vivo* through paracrine mechanisms, influencing adjacent uninfected or possibly also latently infected cells (Cesarman et al., 2000; Sodhi et al., 2000; Hayward, 2003; Montaner et al., 2006; Martin et al., 2007; Mutlu et al., 2007).

Early evidence of the potential extent of lytic expression in a PEL lymphoma was presented by Cannon et al. (1999) where up to 30% of the peripheral lymphoblasts in a sample examined directly without culturing displayed vIL6-positive cells by IFA with a specific antipeptide antibody. Similarly, Cannon et al. (2000) also reported on a newly established double KSHV+/EBV+ PEL cell line JSC1 that displayed up to 10% spontaneous vIL6- and vMIR2-positive cells and could be induced to show early lytic cycle antigen positivity in up to 60% of the cells by TPA or butyrate treatment (and produced commensurately higher levels of infectious virus particles). In comparison, two other popular PEL lines (BC1/HBL6 and BCBL1) have spontaneous uninduced vIL6 positivity levels of 0.1% and 0.5%, with inducibility in parallel experiments up to just 3% and 15%.

Photomicrographs showing detection of three early lytic cycle KSHV-encoded proteins (vGPCR, vMIR2, and vIL6), by single- or double-label IHC with LANA using specific antipeptide (PAb) or monoclonal antibodies (MAb) in a Ugandan cutaneous nodular KS sample are presented in Figure 19.4. At around 20% early lytic-positive compared with LANA-positive cells, this represents the highest levels of KS lytic cycle gene expression that we have seen. It should be stressed that this is an exceptional case, whereas levels and ratios of KS lytic antigen/LANA positivity were at least 10-fold lower than this from all United States KS samples tested, even in nodular cases, as well as in Ugandan patch or plaque samples. It is possible that the higher fraction of lytic expression that we have seen from some African samples represents late-stage untreated disease, but if so, it raises the question of whether in fact KS may really be a perhaps as yet imperfect attempt by this virus to develop the ability to produce infectious skin lesions, somewhat equivalent to the typical skin or mucosal lesions of many alpha herpesviruses.

Another important point to make is that in all of our observations on both spontaneous reactivating and induced PELs, there is a distinct hierarchy of lytic expression with the very early proteins such as vIL6, vMIR2, RTA, and RAP tending to be made in a significantly higher proportion of the cells than the delayed early proteins such as vGPCR, vMIR1, and SSB. These in turn are made in more cells than the true late proteins exemplified by gK8.1. In addition, double-label IFA in induced PELs shows that not all cells produce all lytic proteins at the same time—there are either temporal differences in expression including shutoff of the earliest proteins or at least two distinct separate expression pathways. The same kind of hierarchy also applies in KS lesions, with vIL6/vMIR2/RAP positivity always being greater than vGPCR/vMIR1/SSB

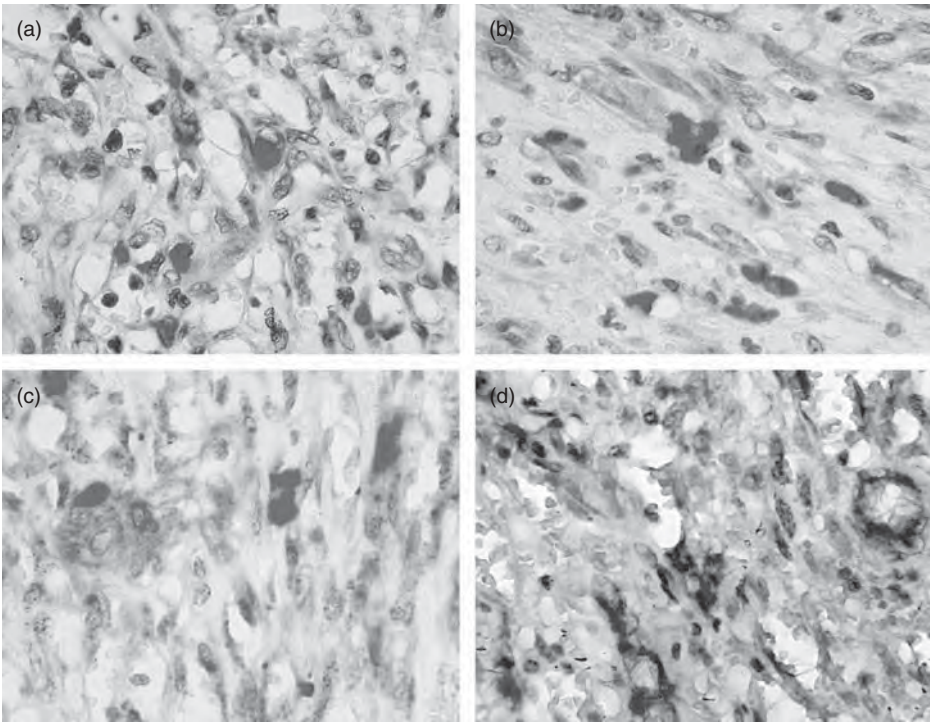

Figure 19.4. Latent and lytic cycle KSHV protein expression in an African KS skin lesion. Detection of various proteins by single- and double-label IHC in sections of a cutaneous nodular KS lesion from Uganda. (a) KSHV vGPCR (red cytoplasm, rabbit PAb). (b) KSHV vMIR2 (red cytoplasm, rabbit PAb). (c) KSHV LANA (brown punctuate nuclei, rat MAb) and vIL6 (red, cytoplasm, rabbit PAb). (d) KSHV LANA (brown punctate nuclei, rat MAb) and cellular PECAM1/CD31 (red cytoplasm, mouse MAb). Similar double-label IHC for VWF and VE-cadherin also showed complete discordance between LANA-positive spindle cells and CD31/VWF/VE-cadherin-positive neovascular ECs within the KS lesion (data not shown). All are counterstained with hematoxylin and eosin. See color insert.

positivity and gpK8.1 positivity being far less still. Furthermore, the lytic antigen-positive cells are always sporadic rather than focal, and this strongly supports the notion of abortive or multistage lytic expression, which could have the advantage of potentially allowing some pathogenic lytic protein expression to persist for long periods of time without the concomitant cell killing and lysis that is expected to occur once the lytic cycle has been initiated. Double-label IHC in the KS lesions also shows that the small proportion of spindle cells expressing lytic proteins lack expression of LANA, which is rarely the case in induced PEL cell lines, again suggesting likely long-term expression of vIL6 and vGPCR for example in some cells. This would also make sense of the fact that many of the captured very early lytic cycle cellular proteins have antiapoptotic prosurvival properties.

CURRENT CLINICAL TREATMENT AND FUTURE TARGETED THERAPY

The clinical spectrum of AIDS-related KS is highly variable, and treatments range from local to systemic therapy. Limited disease can be treated with excision or intralesional therapy and generally responds well to radiation therapy. Both advanced KS and PEL have traditionally been treated with chemotherapy, although there has been limited benefit in PEL patients. Pegylated liposomal doxorubicin, liposomal daunorubicin, and taxane paclitaxel are currently approved for the treatment of KS (Northfelt et al., 1998). Recombinant IFN-α was also tested and used but with mixed success. All AIDS-related KS patients should receive immune reconstitution HAART, because effective antiretroviral regimens result in regression in size and number of existing lesions. However, the response in KS is independent of whether the type of HAART regimen used includes RT inhibitors or PIs (Sgadari et al., 2002; Martinez et al., 2006). Undetectable HIV viral load was strongly associated with KS remission, while CD4 cell count was not. Nevertheless, there are escalating reports of recurrences and this may not represent a universal cure. Ganciclovir, foscarnet, and cidofovir inhibit the replication of KSHV *in vitro*, but both classical KS and PEL show minimal response to these drugs. However, MCD supports more lytic KSHV replication, and patients have responded to ganciclovir (Casper et al., 2004). These antiherpesvirus agents may also play a role in decreasing the risk of developing KS in HIV-infected patients, as shown by therapy for CMV retinitis that has reduced the risk of KS by 75% and 93% with oral and intravenous ganciclovir (Martin et al., 1999). Methods of reactivating the KSHV lytic cycle with valproic acid, for example, to increase susceptibility to cell killing by ganciclovir (GCV), are also being evaluated (Klass et al., 2005).

The PI3K/Akt/mTOR pathway is at a juncture of multiple critical signaling pathways involved in the pathogenesis of KS. Receptor tyrosine kinases activate Akt through the generation of PI3K, which leads to Akt phosphorylation. Akt is an activating kinase for mTOR and is among the most frequently activated kinases in human cancer. Rapamycin (sirolimus) is used as an immunosuppressive agent in the context of organ transplantation, because it inhibits IL2 translation and secretion and thus inhibits T-cell proliferation. Rapamycin (by inhibiting mTOR) also inhibits protein translation leading to G_1 arrest (Konstantinopoulos et al., 2007). Rapamycin proved to have efficacy against PEL in culture and in a murine xenograft model. Furthermore, cell lines expressing KSHV vGPCR as well as vascular tumors that developed in vGPCR transgenic mice showed up-regulation of the Akt/mTOR pathway and were susceptible to inhibition by rapamycin (Sodhi et al., 2006; Sin et al., 2007). An initial study of rapamycin in 15 renal transplant recipients revealed that switching from a cyclosporine A-based immunosuppressive regimen to single-agent rapamycin (sirolimus) resulted in regression of cutaneous KS lesions (Stallone et al., 2005). All KS lesions disappeared over a 3-month period, but graft function did not deteriorate, thus separating the immunosuppressive activity of rapamycin from its anticancer effects. Therefore, a shift from cyclosporine to rapamycin is now strongly advocated in iatrogenic KS (Lebbe et al., 2006) and is being tested in AIDS-KS.

KS provides a unique example of an infectious pathogen for which understanding its molecular pathogenesis has begun to trigger the development and clinical use of a

wide variety of novel specific, molecularly targeted therapies. In particular, the fact that KS involves a combination of inflammatory responses, angiogenic stimulation, and proliferative effects, as well as being, to some extent, more reversible and involving fewer genetic insults than most other tumors, creates numerous opportunities for intervention. The viral vFLIP induction of NFkB, latent state effects of LANA and vCYC on GSK3/β-catenin/pRB/cMYC, and p53/HDM2 pathways, as well as the angiogenic and inflammatory effects of viral-encoded vGPCR and vIL6, all present especially attractive targets for potential relatively specific therapies in the future (Montaner et al., 2006; Sodhi et al., 2006; Sarek and Ojala, 2007). Sorafenib, IL12, glycyrrhizic acid, TNP-470, thalidomide, bortezomib, bevacizumab, COL-3, Bay-11-7082, and imatinib mesylate (Gleevec) are among the promising antiangiogenic (Dezube et al., 2006), anti-NFkB (Keller et al., 2000), anti-cKIT (Koon et al., 2005), and other agents that have already been examined in PEL or KS or are currently undergoing clinical trials for AIDS-KS, and more are on the way (Raggo et al., 2005; Dittmer and Krown, 2007; Hansen et al., 2007).

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20

MOLECULAR PATHOBIOLOGY OF EBV INFECTION

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INTRODUCTION

The unraveling of the molecular pathogenesis of malignancy that had its beginnings in the last third of the twentieth century was, in many ways, sparked by the discovery of virus-associated tumors and oncogenic viruses. Epstein–Barr virus (EBV) and other viruses that soon followed were not only exciting stories in themselves, but provided models with starting points and outcomes, which, since they were specific, could be dissected and generalized. In these annals, EBV qualifies as a human tumor virus—the first identified. According to updated criteria (Carbone et al., 2004; Pagano et al., 2004), it has all the features of DNA tumor viruses including consistency of association with given tumors at the epidemiological and molecular level and oncogenic properties in cell culture and animal models. As with other tumor viruses, some EBV tumors are marked geographically by endemic patterns of incidence that imply that cofactors, largely unidentified, must be invoked in the genesis of several of the EBV tumor types. The diversity of tumors caused by or associated with EBV infection, which are both lymphoid and epithelial in origin, is certainly the greatest for any tumor virus and complicates questions of causality. EBV-associated tumors include lymphomas, carcinomas, and even rare myosarcomas. However, this seemingly paradoxical spectrum can be rationalized by the pathobiologic features of EBV infection, exemplified by the

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TABLE 20.1. EBV-Associated Malignancies

Malignancy	Features
Lymphomas	
Burkitt's	Endemic (>99%+), sporadic (~15%+)
B cell, immunoblastic	100%, AIDS, PTLD, congenital immunodeficiency
Hodgkin's	~40%–50%+
T cell	Rare
Carcinomas	
Nasopharyngeal	100%+
Parotid	Alaskan Inuits
Gastric	~15%+
Breast	Association?
Others	
Leiomyosarcoma	100%+, AIDS (children)

benign condition, infectious mononucleosis, in which both epithelial and lymphoid cells are infected.

The central molecular feature common to the established EBV tumors is persistence of latent EBV genomes in an intact monoclonal episomal form in the tumor cells. However, in some instances, such as Hodgkin's lymphoma (HL) and gastric carcinoma (GC), only a proportion of tumors is infected, which, while puzzling, may suggest histologically undefined subsets of these tumors. The earlier established EBV tumors, Burkitt's and B-cell lymphomas and nasopharyngeal carcinoma (NPC), are all essentially positive, and every cell contains the viral episomes usually in low copy numbers. In contrast, some breast cancers, which are EBV positive, the virus–cell relation is different in that not every cell is infected, and in some cells, the virus is not latent but replicating. The EBV-associated malignancies are listed in Table 20.1. This chapter will briefly review the aspects of the pathobiology of EBV infection in relation to the malignancies with which it is associated. It will also provide selected examples from this laboratory of mechanisms of control of latent EBV gene expression still under investigation.

EPSTEIN-BARR VIRUS

EBV is a linear, double-stranded, 184 kb DNA-containing virus that is a member of the γHerpesviridae. Almost all the world's population is infected beginning in infancy and extending into young adulthood. Congenital and neonatal infections are rare because of maternal EBV antibodies that prevent fetal infection. Infection and viral replication are primarily in epithelial cells in the oropharynx with infection of B lymphocytes closely linked (Niederman et al., 1976; Sixbey et al., 1984). Transmission is through virus shed in the saliva, but infection is also sexually transmitted (Sixbey et al., 1986). There are innumerable strains of EBV, and sometimes multiple infection leads to strain recombination in the oropharynx. In most cases, primary infection is asymptomatic or has nondescript symptoms earlier in life, but with pubescence, the classic syndrome of infectious mononucleosis comes to the forefront (Pagano, 2005).

The EBV episome is the basis for latent infection. In contrast to the linear genomes encapsidated in virions, episomes are circularized genomes of unit length formed by the joining of the termini, which have a complementary sequence. EBV's episomes are intracellular genomes found only in the nucleus, specifically in the chromatin, and packaged in nucleosomes (Shaw et al., 1979; Sexton and Pagano, 1989). Copies of episomes are restricted to small numbers in each latently infected cell, which remain the same in progeny cells. This mechanism for viral persistence is captured in EBV-immortalized cell lines, which facilitate molecular biologic dissection. Such cells do not ordinarily produce virus. In contrast to productive infection in which as many as 100 viral genes are expressed and viral replication is mediated by an EBV-encoded DNA polymerase, episomes are replicated by host DNA polymerase, initiated at a specific autonomous replication site, the existence of which was suspected (Henry et al., 1983) but not defined until the *trans*-acting function of EBNA1 was recognized (Yates et al., 1985) and *ori-P* was defined; up to 11 genes are expressed in latent infection. Thus, both episomal replication and, as brought out later, gene expression are tightly regulated and tied to the cell cycle. The only EBV gene that must be expressed is EBNA1, which is essential for the initiation of replication of the episomes and their maintenance in cells.

There are three main types of EBV latent infections. In type I latency, which is characteristic of Burkitt's lymphoma (BL), essentially only EBNA1, but not any of the transforming genes, is expressed. In type II latency, exemplified in NPC and HL, EBNA1, latent membrane protein-1 (LMP1), and LMP2A are expressed, whereas in type III, all nine of the canonical EBV latency genes are expressed: EBNA1, 2, 3, 4, 5, and 6, and LMP1, 2A, and 2B. In some cases, BART1 and BARF0 RNAs may also be expressed in type II latency; there is some evidence that BART1 may have transforming properties, but it is not certain whether BARF0 protein is expressed. In all latency types, EBERs, which are noncoding RNA polymerase II and III transcripts of uncertain function, are abundant and used as a marker for latent infection. In addition, latency type G0 has been described in circulating resting memory B cells, in which EBNA1 protein, which has a very long half-life, is expressed either periodically or not detected at all (Tierney et al., 1994; Chen et al., 1995). All latently infected cells are immortalized, as shown by their ability to grow indefinitely in cell culture.

However, only growth of type III cells is dependent on the expression of EBV transforming genes. Type I cells are BL in origin whose growth is driven by one of three chromosomal translocations that cause the overexpression of c-myc (Dalla-Favera et al., 1982), which alone is sufficient to maintain and probably to initiate this distinctive B-lymphoid malignancy, whether EBV positive or negative. Type II latency typically found in epithelial cells in NPC *in vivo* is rarely captured in cell lines. The EBV-infected pathological cell type of HL, the Reed–Sternberg (RS) cell, while it is of B-cell origin, has rarely been adapted to grow in culture. Normal B cells immortalized by infection *in vitro* or cultured from circulating B cells are not inherently tumorigenic in contrast to BL cells. EBNA2 is indispensable to immortalize B cells; virus deleted for EBNA2 is not immortalizing. EBNA2 is the master transcriptional activator of all the type III genes, in particular, the principal EBV oncogene, LMP1; except for EBNA1, the expression of the other EBNAs and LMP2 contributes directly to the

transformed state. *In vivo*, type III latency is manifested in immunoblastic B-cell lymphomas.

The expression of latency genes from the episome is regulated by a number of mechanisms that apply to specific genes. There is also evidence of global mechanisms that may govern the expression of all the EBV latency genes. Examples of each scenario, one for type I and the other for type III latency, follow. In type I latency, EBNA1 is transcribed from the Q promoter (Qp). Both viral and cellular factors regulate this promoter. The effects of binding of EBNA1 and E2F to Qp are well studied. This promoter is autoregulatory in that while Qp is responsible for the expression of EBNA1, the protein can also repress this promoter by binding to it downstream of the RNA start site. Adjacent to and partly overlapping these binding sites are E2F binding sites, which are postulated to relieve the repression presumably by protein binding that displaces EBNA1. The cell cycle appears to synchronize these events in S phase when E2F is synthesized and becomes available for binding. The result is that EBNA1 RNA levels in Raji cells fluctuate during the cell cycle, reaching their peak in S phase and their nadir in G1 (Davenport and Pagano, 1999). Thus, whether Qp is "off" or "on" depends on a balance between the binding of the viral and cellular proteins. However, the interpretation of how this mechanism functions is complicated by the long half-life of EBNA1 protein that makes it continuously available. Presumably small shifts in intranuclear concentrations of EBNA1 and E2F protein determine the net outcome. These are speculative interpretations but may be relevant to the apparent status of EBNA1 expression in resting memory B cells, where it has been reported that EBNA1 RNA is not detected. However, since memory B cells are only sporadically stimulated to enter mitosis by ambient mitogenic stimuli (Chen et al., 1995; Thorley-Lawson, 2005), the activation of Qp by E2F and the resulting intermittent presence of EBNA1 RNA would not have been detected under the experimental conditions used, nor would residual EBNA1 protein that could bind to Qp have been detected since so few cells are infected.

A cell cycle-triggered mechanism for the global regulation of the expression of type III latency genes has also been proposed. This quite different mechanism depends on the fact that EBNA2 is the master transcriptional activator of all the type III latency genes, since it activates the relevant promoters for the expression of LMP1, LMP2, and LMP2A, and the six EBNA proteins via an EBNA2-responsive enhancer element that is coactivated by EBNA2 binding in complex with RBJκ or PU.1; EBNA2 does not bind directly to these promoters. However, during mitosis, the expression of many cellular genes is down-regulated, accomplished by protein phosphorylation by cdc2/cyclin B1 kinase, and EBNA2 is affected similarly (Yue et al., 2004). Consequently, the LMP1 promoters as well as Cp used for EBNA1, 2, 3A, 3B, and 3C and EBNA-LP (leader proteins) are also down-regulated. Thus, cell cycle may modulate latent type III gene expression so that not only episomal replication but also gene expression may be regulated in this manner. However, depending on latency type, distinct mechanisms seem to be used.

The pathobiology of *infectious mononucleosis*, itself a benign disease, embodies basic elements of the array of malignancies associated with EBV infection (Pagano, 2005). The virus can infect both epithelial cells and B lymphocytes. Infection begins in epithelial cells where it is cytolytic with viral replication continuing presumably through serial infection persisting for months or years, but epithelial cells are

ordinarily not latently infected (however, see NPC). In contrast, the infection of B cells leads not to cytolysis but to cell proliferation. EBV is the first virus found to be able to immortalize human B cells, which it does efficiently. In the proliferating B cells, all the EBV latency genes are expressed in contrast to only EBNA1 in memory cells. The strong but transient atypical T-lymphocytosis characteristic of infectious mononucleosis (IM) is a manifestation of the cytolytic response to the wave of proliferating B cells induced by EBV infection. That cells circulating in the blood of patients with acute IM could destroy EBV-immortalized lymphocytes in culture was the early recognition of the origin and effect of the lymphocytosis of IM, which was then coming to be ascribed to T cells and demonstrated experimentally with paired autochthonous B- and T-cell lines (Hutt et al., 1975; Yue et al., 2004). This cytolytic response limits the proliferation of the viral antigen-expressing B cells in normal hosts so that ultimately, only a few EBV-infected B cells persist. These are memory B cells in which EBV episomes are perpetuated, but most of the EBV latency genes are no longer expressed.

The potential for these cells to renew growth *in vivo* is realized in persons with severe immunodeficient states—early in life with inborn, later with acquired deficiencies—in whom immunoblastic lymphomas arise. A link to another lymphoma, HL, is indirect and uncertain pathobiologically. The incidence of HL later in life is increased slightly in younger persons with a history of IM. This increased risk has been detected only following symptomatic EBV infection and applies only to EBV-positive HL (see HL).

BURKITT'S LYMPHOMA

BL was first described by Dennis Burkitt in Africa as an endemic childhood neoplasm with patterns of incidence that suggested an infectious etiology (Burkitt, 1962). After failed effects to recover a virus, eventually, the fifth human herpesvirus was detected by electron microscopy in cultured BL cells in 1964 (Epstein et al., 1964) and later named EBV; it was identified as a new virus by W. and G. Henle (Henle et al., 1968). The virus was not visualized in the original BL tissue, but EBV DNA was detected in an apparently virus-negative BL cell line (Raji) by DNA–DNA hybridization (zur Hausen and Schulte-Holthausen, 1970). Later, the association was quantified and found to be consistent by direct analyses of sets of BL tissues by more sophisticated cRNA–DNA hybridization analyses and DNA–DNA reassociation kinetics (Nonoyama and Pagano, 1971; Nonoyama et al., 1973; Pagano, 1975). It was soon clear that virtually all (>98%) BLs in the African endemic belt, while virus free, contained EBV genomes in low copy numbers consistent with latent infection. Interestingly, one or two BL specimens were EBV negative by both methods, and most (>80%) American BLs were also negative (Gravell et al., 1976).

Thus, even then, it could not be said that EBV was the causal agent for classic BL of the African variety as well as for sporadic BL in Western countries; the latter do differ somewhat clinically and pathologically from the African variety (Ferry, 2006). Unifying both endemic and sporadic forms of BL are the (8;14), (8;22), and (8;2) chromosomal translocations that dysregulate the c-myc locus and reactivate the expression of

this proto-oncogene, which is the primary molecular lesion common to all BL (Dalla-Favera et al., 1982). EBV, while apparently a potent contributor to this B-cell lymphoma, is thus relegated to cofactor status. Holoendemic malaria has also been suspected of contributing to BL of the endemic variety (Moormann et al., 2007). A few EBV-negative as well as many latently EBV-infected BL cell lines are routinely cultivated today, and the infection of negative lines seems to enhance their growth.

The relatively low copy numbers of EBV genomes detected in BL, the constancy of their numbers in Raji cells, and the observation that all the genomes were associated with the chromatin provoked a search for the mechanism of their maintenance (Henry et al., 1983). The discovery of the EBV episome, the first found in eukaryotic cells save for certain yeast, soon followed (Nonoyama and Pagano, 1972).

IMMUNOBLASTIC LYMPHOMA

The connection of such B-cell lymphomas to EBV infection was recognized years ago in children with congenital immunodeficiency well before their appearance in larger numbers in adults with the two leading acquired immunodeficiency states in contemporary medicine: posttransplant lymphoproliferative diseases (PTLDs) and B-cell lymphomas in AIDS. Lymphomas in X-linked primary immunodeficiency and combined immunodeficiency diseases were all found to contain latent EBV genomes (Sixbey and Pagano, 1984; Purtillo et al., 1993), and the virus was presumed then to be responsible for their genesis, based in part on the early recognition that EBV could be detected in B, not T, cells (Pagano, 1975). However, the EBV proteins expressed were largely unknown then and not identified in the tumor tissues. We now understand all these lymphomas to be similar or identical pathologically, although they may differ clinically, most strikingly in the unique solitary central nervous system (CNS) B-cell lymphomas that arise in patients with AIDS.

It is easier to trace the course of evolution of such lymphomas in transplant recipients since the onset of the immunodeficiency begins with the immunosuppressive therapy. The neoplasm begins as a diffuse polyclonal proliferation of B cells, all of which are EBV positive and presumably represent pathological expansion of EBV-infected memory B cells. Such proliferation can be halted and the proliferation can be reversed by stopping therapy. However, with continued immunosuppression, lymphoproliferation evolves into a biclonal and then a monoclonal state, becoming autonomous in the process, presumably through selection among proliferating cells (Snow and Martinez, 2007). Initially, lymphoproliferation is driven by reexpression of type III latency genes triggered by the expansion of type G0 cells. Whether the expression of the oncoproteins continues to be necessary once proliferation has entered its autonomous monoclonal phase is unclear, but these antigens are uniformly expressed and recognized during virus-specific immunotherapy, to which the lymphomas are responsive. Thus, the EBV oncoproteins are needed for the initiation of the tumors and can serve to target the tumors for therapy. Unlike BL, no stereotypic translocations or oncogene activations have been recognized in EBV-immortalized cells (Klein and Raab-Traub, 1987) or in immunoblastic lymphomas,

although presumably random acquired genetic changes do occur, enhanced by genetic instability.

Recently unexpected insight into the mechanism of the EBV-driven proliferation has come from the finding that β-catenin is stabilized and functional as a transcriptional coactivator with transcription factor 4 (TCF4) in type III but not type I latently infected cells. The implication of this finding is that the β-catenin transcriptional complex can then drive the expression of activators of cell proliferation such as *c-myc*. In type I cells, *c-myc* is already activated by the BL translocations. In other words, a viral oncoprotein, probably EBNA2, can substitute for *wnt* in the proximal portion of this signaling pathway to activate β-catenin. The stabilization of the protein is accomplished by novel means, namely, through up-regulation of a deubiquitinating enzyme (Shackelford et al., 2003).

The incidence of PTLD varies widely depending on the intensity and type of immunosuppressive therapy (Snow and Martinez, 2007). In patients with AIDS, the incidence of immunoblastic lymphomas has declined with the use of highly active retroviral therapy (HAART). However, since T-cell levels are not entirely restored, some risk of lymphoma may persist as life span is prolonged. Once such lymphomas do become established, they are notoriously difficult to treat by conventional means and usually rapidly fatal. New means of therapy based on the expansion of isogenic cloned T cells that recognize EBV antigens expressed in the B-lymphoma cells have been used for the treatment of individual patients with notable successes (Dotti et al., 2005).

HODGKIN LYMPHOMA

These lymphomas had been tentatively linked to EBV for years by epidemiological observations, suggesting that persons with a history of IM were somewhat more likely to develop HL than similar populations without such a history. Subsequently, this association has been placed on a firm basis by detection of EBV genomes in HL (Jarrett et al., 2005; Hjalgrim et al., 2007). However, as many HL tissues are EBV negative as are positive, with age, geography and histopathologic type seeming to combine as determinants of tumor-cell infection status. In the interim, it has become clear that the experience of IM itself and not subclinical infection determine whether HL is one of the EBV-positive varieties. HL is often diagnosed in young adulthood, thus following the peak incidence of IM in Western countries; however, there is little insight into the role of EBV in this common lymphoma.

The lymphoma first appears as enlarged lymph nodes, often accompanied by fever and other systemic symptoms. The extent and distribution of these nodes and whether organs such as spleen are involved determine the stage of the disease (Jarrett et al., 2005). Most (80%–90%) younger patients respond well to chemotherapy and radiation, but relapses occur in 10%–30% of patients (Ansell and Armitage, 2006). The pathology of HL is quite varied, reflected in histological subtypes such as nodular lymphocyte-predominant and classical forms, nodular sclerotic, lymphocyte depleted, and mixed cellularity (Harris, 2006). The histopathology of HL is characterized generally by a florid mixture of cell types, mainly lymphocytes, histiocytes, and

other blood cells including eosinophils. Distinctive in this mixture are rare RS cells, thought for years to be pathognomonic of HL, and finally identified as the clonal malignant cell of HL relatively recently. The many other cells in the lymphoma are believed to represent a reactive response in the malignant process. Determining that RS cells are of germinal B-cell origin was a major step in rationalizing the involvement of EBV in HL. In contrast to type III immunoblastic lymphomas, HL is a type II latency disease, and indeed, clonal EBV episomes as well as the expression of oncogenic EBV proteins such as the antiapoptotic LMP2A on the plasma membrane of RS cells together with the expression of LMP1, which activates NF-κB, point to an intimate and significant relation between EBV and the neoplastic cell of HL (Ambinder, 2003).

OTHER LYMPHOMAS

Rarely EBV can be detected in T cells and natural killer (NK) cells in an unusual non-HL termed natural killer T-cell lymphoma (NKTL). These tumors are often EBV positive, especially where they are more common, such as Korea and Japan, and they arise in extranodal sites, distinctively in the nasopharynx (Jones, 2006).

NASOPHARYNGEAL CARCINOMA

The tumor cells of most carcinomas of the head and neck are relatively differentiated. In contrast, carcinoma of the posterior nasopharynx (NPC) is distinctive not only because it almost always is anaplastic, but through its unique intimate association with EBV infection, the episomes of which are consistently found in the tumor cells. Viewed worldwide, it is the most common EBV malignancy. The only other virus-associated head-and-neck tumors are found in the oropharynx and are infected with human papillomavirus (HPV) in about 20% of cases. NPC occurs sporadically with low incidence in Western countries, but it has much higher incidence in regions of Southern China where the tumor is endemic, generally in males in midlife. This high incidence is maintained in Chinese émigrés who live in Taiwan, Singapore, Hong Kong, and California. There is also a high incidence of NPC in North Africa and Alaskan Inuits (Raab-Traub, 2005).

This distinctive tumor arises in a cryptic site, the fossa of Rosenmüller in Waldeyer's ring, but most usually, it is diagnosed only after it has spread to the lymph nodes in the neck. This proclivity to metastasize early is probably due to the expression of LMP1, the principal EBV oncoprotein, which can induce a wide range of cellular invasion, metastasis, and angiogenesis factors that cause disaggregation of cells, disruption of extracellular matrix, and direct metastasis (Yoshizaki et al., 2005). Low copy numbers of episomes are in each tumor cell, which exhibits type II latency expression, and are detected in NPC worldwide regardless of whether of sporadic or endemic origin. Monoclonal episomes can be detected in the earliest hyperplastic and dysplastic lesions (Pathmanathon et al., 1995).

A hallmark of NPC is that it is heralded or accompanied by an unusual circulating immunoglobulin A (IgA) response to viral replicative antigens, which suggests that a wave of viral reactivation precedes the onset of NPC (Fachiroh et al., 2004). These antibody responses signal incipient malignancy and are used to screen for NPC in endemic regions. The sites of viral replication that generate these responses and how it leads to NPC are unknown. In any case, it is thought that the infection of epithelial cells destined to transform into NPC occurs in selected cells with acquired mutations, such as loss of p16, a tumor suppressor gene, that culminate relatively late in life in susceptibility of these epithelial cells to viral infection and transformation (Raab-Traub, 2005; Cho, 2007). Virus enters these cells, but does not cause cytolysis, and triggers the final oncogenic steps relatively rapidly. Together with environmental cofactors and some degree of genetic susceptibility, the endemic varieties of the malignancy are the result. Almost all NPC cells are latently infected, but spotted in a field of tumor cells is an occasional virus-producing cell, which could be simply the result of viral reactivation in an apoptotic cell. However, NPC histopathology reveals T-cell infiltration, which may be evoked by a cytokine, IL6. In the pathogenesis of B-cell lymphomas, it has been hypothesized that a lytic cycle cytokine may contribute to the oncogenic process (Hong et al., 2005), but a similar mechanism has not been linked to NPC.

LMP1 is an integral membrane protein that is a member of the tumor necrosis factor receptor (TNFR)-associated superfamily and shares signaling pathways with CD40 through the carboxy-terminal cytoplasmic domain of the protein. Unlike TNFR activation, LMP1 does not require a ligand but is constitutively activated. An important consequence of LMP1 signaling is up-regulation of epithelial growth factor receptor (EGFR), which becomes constitutively expressed. The key to LMP1's oncogenic effects is its ability to activate NF-κB, which in turn activates many of the LMP1-inducible genes and is a central factor in triggering oncogenic pathways. Others include those mediated through jun N-terminal kinase (JNK) and PI3K. LMP also inhibits apoptosis, which thus may circumvent any need for p53 mutations in NPC, and is rarely found except in some metastasic tissue perhaps as a result of late secondary mutations (Raab-Traub, 2005).

A quite different target protein for LMP1 is interferon regulatory factor 7 (IRF7), which is essential for activating many interferon-stimulated genes, in particular, type 1 interferons. However, IRF7 can also up-regulate the expression of EBV genes such as LMP1, the promoter for which contains a functional interferon stimulated response element (ISRE) (Ning et al., 2003). IRF7 itself has oncogenic properties, and moreover, it is overexpressed in LMP1-positive CNS B-cell lymphomas. The positive regulatory circuit between LMP1 and IRF7 thus may contribute to the viral protein's oncogenic effects (Zhang et al., 2004). Dissection of how IRF7 is activated provides clues to LMP1 intracellular signaling since IRF7 can be activated by regulatory (ub lysine 63) ubiquitination through interaction with receptor-interacting protein (RIP) and TNF-receptor associated factor (TRAF) 6, the latter protein acting as the E3 ligase for the ubiquitination of IRF7. RIP and TRAF6 have long been known to participate in signaling by LMP directed to other targets, and these recent findings indicate how these two necessary factors function in such signaling.

The circuitry between IRF7 and LMP1 expression may help to explain a paradox in the type II gene expression profile noted in NPC, that is, how is LMP1 expressed in the absence of EBNA2. In cultured type III lymphoblastoid cells in which appreciable

LMP1 is expressed, endogenous levels of IRF7 are generally high. An exception is one of the original lymphoblastoid lines, p3HR1, in which the EBNA2 gene is disabled by a deletion acquired in passage. Consequently, LMP1 protein is barely detectable, and the IRF7 level is also low. However, transfection and overexpression of IRF7 in these cells increase the expression of LMP1, although not to the level usual in EBNA2-expressing cells. The implication is that IRF7 may contribute to IRF7 levels in NPC although inefficiently, and in fact, LMP1 protein levels in NPC are generally relatively low compared with type III infection states (Ning et al., 2003).

Interaction between genetic and environmental factors may help to explain NPC's endemic patterns of incidence. For years, a diet of salted fish has been suspected to contribute to high incidence of NPC. Subsequent findings of mutation of the cyclin-dependent kinase inhibitor p16 and of the tumor suppressor RASSF1A at 3p21.3, caused by loss of heterozygosity, may predispose to the transformation of epithelial cells that become infected by EBV in the nasopharynx (Chow et al., 2004).

Parotid tumors are of special interest because of their incidence in Alaskan Inuits and because only they are EBV positive among salivary gland malignancies. Interestingly, parotid tumors have some histopathologic similarities to NPC in terms of their lymphoid infiltration (Scott et al., 2005). EBV was detected years ago in what appeared to be parotid ductal cells by *in situ* cytohybridization after cannulation of the duct in patients with IM, but the source of EBV-infected epithelial cells in the oropharynx has remained obscure (Lemon et al., 1977).

GASTRIC CARCINOMA

GCs are sometimes (2%–16%) infected with EBV, findings made significant because again monoclonal episomes are detected in the transformed epithelial cells, not in reactive cells that infiltrate the tumor. Two histological types are associated with EBV: a poorly differentiated lymphoepithelioma-like tumor, which recalls NPC and parotid tumors and is rare, and typical adenocarcinomas. Again p53 mutations are rare, but chromosomal aberrations have been described that may predispose to the malignancy. EBNA1 is expressed in the tumor cells along with LMP2A in half the cases plus BARF0, LMP2A, and EBER RNAs. The histopathology of these tumors is quite distinct from the chronic gastritis produced by *Helicobacter pylori* infection. Whether there is a pathogenetic interaction between these two infectious agents is unknown. Interestingly, *H. pylori* GC is more often located in the distal portions of the stomach, whereas EBV-positive carcinoma is more frequent in the region of the cardia (Iwakiri and Takada, 2005).

BREAST CANCER

EBV is detected in breast milk of normal women (Junker et al., 1991) and in some breast cancers, but the relation of the virus to the tumor cells differs from that of the other EBV-associated tumors in that the genome copy number is lower than in the other

tumors probably because only some of the tumor cells are infected (Bonnet et al., 1999). Moreover, some cells are not latently infected but produce virus (Huang et al., 2003). These inconsistencies in virus–cell relations may be explained by postulating that the virus does not have an etiologic role but infects existing tumor cells and modifies malignant progression. Interestingly, the detection of EBV was first reported in tumors of more aggressive phenotype with prognostic markers such as estrogen receptor (ER) and progesterone receptor (PR) being negative and HER2/HER3 being positive (Arbach et al., 2006). LMP1, which has been detected in some of these tumors, induces the expression of a wide range of invasiveness and metastatic factors and so could contribute secondarily to oncogenesis (Yoshizaki et al., 2005).

LEIOMYOSARCOMA

EBV departs from its established lymphocytic and epithelial cell tropisms in rare cases of leiomyosarcomas, all of which are EBV positive mainly in children with AIDS, although cases in adult and pediatric organ-transplant recipients are also reported (Jenson, 2006). The virus is not detected in sarcomas in nonimmunodeficient persons, although it is detected in normal smooth muscle cells in patients with AIDS. The EBV receptor CD21 is present not only in these cells but also in other cell types and tissues not infected by EBV. The predisposition to EBV infection of the smooth muscle cells in AIDS may suggest that human immunodeficiency virus (HIV) up-regulates CD21 receptor levels.

CONCLUSIONS

In contrast to other tumor viruses, such as HPV, hepatitis B virus (HBV), hepatitis C virus (HCV), and human T-cell leukemia virus-type 1 (HTLV-1), all of which are associated with a single malignancy, which they cause, Kaposi's sarcoma herpesvirus (KSHV) is an exception in that it is accepted as causing three malignancies. The situation with EBV with its diverse associations is more complex. The virus is accepted as a causative agent for a single type of malignancy, immunoblastic lymphomas, that arise in immunodeficient persons. The case for an etiologic relation with NPC is strong, even compelling, but not conclusive in part because the identification of the cofactors that seem to be needed to close the circle of causality is fragmentary and incomplete.

An etiologic role for the other EBV-associated malignancies is problematic for various reasons. There is a strong molecular evidence of a significant relation because of the finding of monoclonal episomes in the malignant cell type in all except breast cancer. The implications of this demonstration are that since the replication and perpetuation of EBV episomes depends on cell division and proliferation, the viral genome must have been present at the outset and triggered the emergence of a primordial clonal malignant cell.

An alternate scenario is that infection occurs in a subset of clonal cells after they had emerged and conferred on the infected cells' selective growth advantage—a hallmark of EBV infection, at least for B-lymphocytic cells. This scenario seems attractive in HL where RS cells in only some HL are EBV positive. That EBV is associated in

less than half of HL is in any case problematic in that the identification of HL subsets that might explain this disparity uses varied criteria, and subsets are ill defined. Moreover, the strong epidemiological evidence for consistent linkage, as exemplified for HPV in cervical cancer and HTLV-1 in subacute T-cell leukemia, is quite inconclusive for EBV and HL. It seems likely, however, that EBV contributes in some way, if not as a prime etiologic factor then as a contributor early in oncogenesis.

As to EBV and gastric cancers, the case is again different. Gastric cancers are clearly caused by inflammation induced and made chronic through *H. pylori* infection. Whether the small subset of GCs associated with EBV have a similar chronic inflammatory basis is far from clear. There is a suggestion from IgA responses that active infection precedes the development of the cancer. However, the gene expression profile of GC is one of latent infection that includes EBNA1, and may include LMP2A, BARF0, and BART1, but neither LMP1 nor EBNA2. The prevalence of coinfection with *H. pylori* and EBV is unknown.

EBV's weakest association is with breast cancer. The majority are not infected but, more importantly, tumor cells in the same specimen can be either positive or negative so that the virus–cell relation is quite inconsistent. EBV can inhabit normal breast in a cell type that has not been identified, and virus is excreted in milk. Do all these mean that EBV is a trivial secondary infectious agent for this carcinoma? However, another possibility is that while the virus is not a primary pathogen, it may contribute to later stages of breast oncogenesis. This scenario is given weight by the gathering evidence that LMP1 is a potent inducer of multiple cellular factors well established to contribute to invasion, metastasis, and neoangiogenesis. Breast cancers are heterogeneous, and thus the virus could propel progression of some tumor cells. Indeed, EBV is purported to be detected in more aggressive forms of the carcinoma.

Thus, the principal EBV oncoprotein LMP1 may drive oncogenesis in distinct phases, one primarily causal and the other driving tumor progression in the different tumor types (or even in the same tumor). Stages of neoplasia are not simply demarcated, and disentangling EBV's possible roles is complicated by the fact that the same oncoprotein can drive both proliferation and modification of tumor-cell phenotype. Possibly, however, EBV's ability to trigger such late-stage phenotypic changes may provide clues to the virus's role in malignancies with which it is inconsistently associated as well as provide a fuller picture of viral oncogenesis in highly invasive malignancies such as NPC and immunoblastic lymphomas.

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21

EPSTEIN–BARR VIRUS AS A PATHOGEN

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INTRODUCTION

Herpesviruses are large, enveloped viruses containing a linear, double-stranded DNA as their genome. Human cytomegalovirus (CMV), for example, has the largest yet identified genome, 235 kbp, of any human virus (Dolan et al., 2004). Epstein–Barr virus' (EBV) genome is approximately 165 kbp in length (Bloss and Sugden, 1994). It contains about 80 open reading frames that encode proteins, two genes for small noncoding RNAs, (EBERs), and approximately 30 genes for its miRNAs (Fig. 21.1). Only subsets of these viral genes are expressed in different, latently infected cells.

While herpes simplex virus type 1 (HSV-1) latently infects neurons and can persist in these nondividing cells without having to replicate their own genome, EBV infects B lymphocytes and can both induce and maintain its proliferation *in vivo* and in cell culture. EBV needs to encode machinery to replicate its genome as an extrachromosomal element, a plasmid, and to encode the controls to drive the proliferation of its host cell. What EBV expresses in and contributes to infected epithelial cells is not well understood largely because we lack tractable cell culture models for infection of these cell types.

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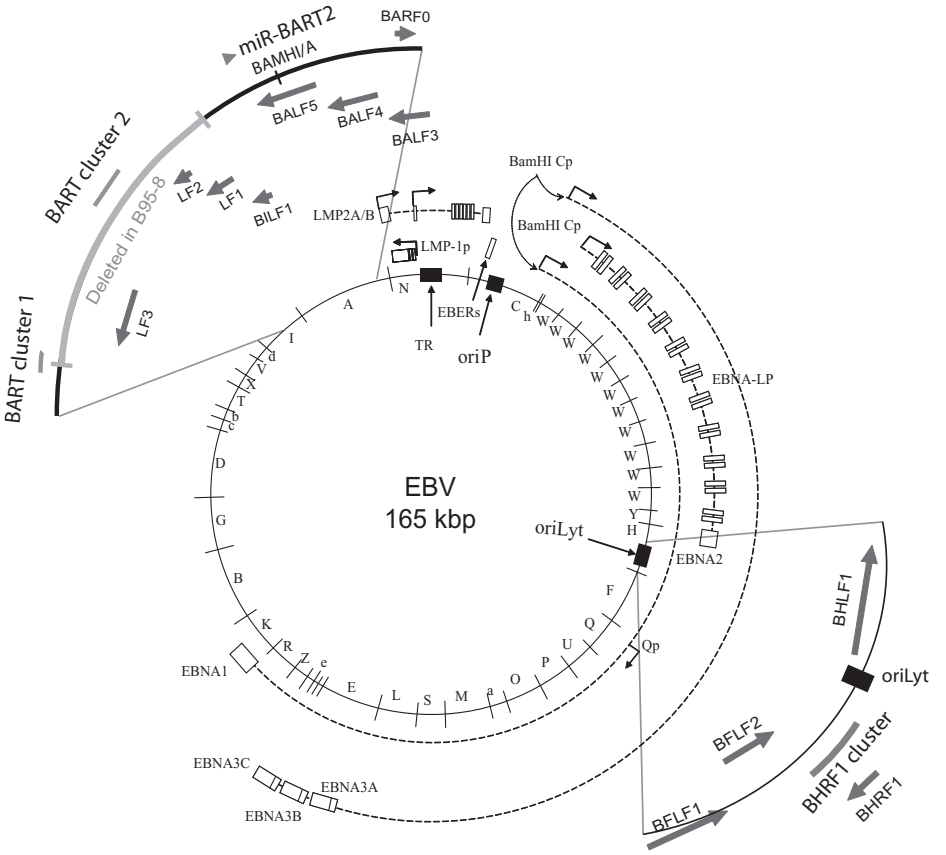

Figure 21.1. The figure shows a reconstructed, ideal map of the circularized genome of EBV. The inner circle represents the sequence of the B95-8 strain of EBV with its fragments resulting from cleavage, with the BamHI endonuclease denoted by the letters on its inner face (Baer et al., 1984). The dashed lines represent primary transcripts of genes expressed during latent infections with the open boxes representing their exons. The black boxes represent three *cis*-acting elements: TRs are the terminal repeats located at the ends of the linear virion DNA, which fuse upon infection to form EBV's plasmid replicon; oriP, which is EBV's origin of plasmid replication; and oriLyt, which is EBV's origin of lytic replication. Promoters used during latent infection are shown as filled arrows pointing in the direction of their encoded transcripts. The two expanded sectors are the regions of EBV DNA encoding its miRNAs. The BHRF1 cluster near the shown copy of oriLyt encodes three miRNAs. The two BART clusters encode 28 miRNAs, most of which are deleted in the B95-8 strain of EBV, a commonly used laboratory strain. The gray arrows represent the open reading frames in the vicinities of the miRNA clusters. Not shown is a second copy of oriLyt located in the ~12 kbp of DNA marked in light gray, which is deleted in the B95-8 strain.

Some of the genes encoding proteins that EBV expresses during latent infections have been characterized by whether they localize to membranes (latent membrane protein-1 and -2A [LMP1 and 2A]) or to nuclei (Epstein–Barr viral nuclear antigens 1, 2, 3A, 3B, and 3C (EBNA1, 2, 3A, 3B, and 3C). EBV's EBERs are in the nucleus (Howe and Steitz, 1986), while its processed miRNAs are in the cytoplasm. These properties permit one kind of cataloging for these genes. More importantly, the functions of many of these viral genes have been elucidated, at least in part, and allow an appreciation of their contributions to EBV's pathogenesis (Kieff and Rickinson, 2007).

LMP1 and 2A are multifunctional mimics of cellular genes. LMP1 drives the proliferation of infected B cells by, in part, mimicking the cellular receptor CD40 (Lam and Sugden, 2003). LMP2A can substitute for B-cell receptor (BCR) signaling, allowing the survival of B cells lacking functional BCRs (Mancao and Hammerschmidt, 2007). EBNA1 can mediate the initiation of DNA synthesis of viral genomes by recruiting origin recognition complex (ORC) to a viral origin (Chaudhuri et al., 2001; Dhar et al., 2001; Schepers et al., 2001); it is essential for the partitioning of viral genomes to proliferating daughter cells (Nanbo et al., 2007), and it regulates the transcription of at least one viral promoter (Altmann et al., 2006). EBNA2 and, apparently to a lesser extent, EBNA 3A, B, and C mimic Notch signaling to regulate the expression both of some viral and some cellular genes (Hayward, 2004). The EBERs have been assigned multiple functions. They can induce IL-10 and interferons (IFNs) and confer resistance to apoptosis induced by IFN-α (Kitagawa et al., 2000; Nanbo et al., 2002; Ruf et al., 2005; Samanta et al., 2006). EBV's miRNAs have only recently been identified, so their functions are largely yet to be discovered, although it is clear that they regulate some genes posttranscriptionally (Pfeffer et al., 2004; Cai et al., 2006; Grundhoff et al., 2006). Additional details of some of EBV's genes expressed in latently infected cells are presented in Table 21.1. This cursory listing underscores one essential finding: EBV encodes multiple genes that can profoundly affect the infected host cell's physiology.

Are these profound, virally induced changes sufficient to contribute causally to the diseases with which EBV is associated? Yes; prospective, epidemiological surveys have identified infection with EBV as a risk factor for developing acute infectious mononucleosis (AIM) (Evans and Niederman, 1997), Burkitt's lymphoma (BL) (de-The et al., 1978), and nasopharyngeal carcinoma (NPC) (Zeng et al., 1985). In addition AIM has been found to be a risk factor for developing EBV-positive Hodgkin's lymphoma (HL) (Hjalgrim et al., 2007). Another kind of evidence documenting EBV's necessary role in maintaining its associated malignancies has come from the analysis of its plasmid genome in proliferating cells (Nanbo et al., 2007). This study showed that approximately one-sixth of EBV plasmids in a population of proliferating cells fail to be synthesized each cell cycle. Thus, a portion of EBV DNA will necessarily be lost from dividing cells each cell cycle. Only if those cells that retain EBV survive and/or grow better, than those that have lost it, will EBV be found in proliferating cells. EBV genomes are found in most malignant cells of the individual cancers with which it is associated. We can conclude, therefore, that EBV contributes significant selective advantages to its various malignancies.

Here we shall describe the benign lymphoproliferative disease (AIM), three lymphomas (posttransplant lymphoproliferative disease [PTLD], HL, and BL), one

TABLE 21.1. EBV's Transforming Genes

Gene	Properties of the Gene's Product	Disease Association
LMP1	LMP1 mimics the cellular CD40 receptor to independently drive the proliferation of infected B cells of a ligand through its activation of NF-κB, AP1, and Jak/Stat signaling (reviewed by Brinkmann and Schulz [2006] and Soni et al. [2007]).	AIM, PTLD, and Hodgkin's lymphoma
LMP2A	LMP2A substitutes for functional BCRs in infected B cells to rescue those lacking this signaling from apoptotic death (reviewed by Brinkmann and Schulz [2006]).	AIM, PTLD, and Hodgkin's lymphoma
EBNA1	EBNA1 binds site specifically to oriP of EBV to support its initiation of plasmid DNA synthesis, to maintain EBV genomes in proliferating cells, and to activate viral transcription from one or more viral promoters (reviewed by Hammerschmidt and Sugden [2006]).	AIM, PTLD, and Hodgkin's and Burkitt's lymphomas
EBNA2	EBNA2 mimics activated Notch signaling to affect cellular gene expression and that of LMP1 (reviewed by Hayward [2004]).	AIM and PTLD
EBNA3C	EBNA3C affects Notch signaling and likely fosters proliferation and survival of infected B cells (Maruo et al., 2006; Anderton et al., 2008).	AIM and PTLD
EBERs	EBERs elicit multiple phenotypes in infected B cells in part by binding RIG I and inducing the release of several cytokines (Kitagawa et al., 2000; Samanta et al., 2006)	AIM, PTLD, and Hodgkin's and Burkitt's lymphomas

carcinoma (NPC), and one lytic infection (oral hairy leukoplakia [OHL]) to which EBV contributes. EBV is causally associated with additional diseases including natural killer/T cell lymphomas and gastric carcinoma, but we know less about EBV's contributions to these malignancies (Kutok and Wang, 2006). We shall describe the pathogenesis of these diseases and, where possible, EBV's roles in these processes. Finally, we shall outline the current therapies for these diseases.

AIM

EBV acutely infects nearly 90% of the world's population. In most people, infection with EBV occurs prior to adolescence, is usually mild and self-limited, or is entirely asymptomatic. However, in a small percentage of people, primary infection with EBV produces significant symptoms (Henle and Henle, 1966). These symptomatic infections largely reflect the latent phase of EBV's life cycle in infected B lymphocytes

and are termed AIM (CDC, 2007; Henke et al., 1973). AIM predominantly occurs in adolescents who have not been infected with EBV in early childhood. EBV infection in adolescents leads to appreciable symptoms in 30%–40% of cases. These symptoms, which include fever, sore throat, fatigue, enlarged lymph nodes (lymphadenopathy), enlarged spleen (splenomegaly), and enlarged liver (hepatomegaly), constitute some of the most common clinical manifestations of AIM in affected populations (CDC, 2007). These symptoms develop 21–28 days following successful exposure to EBV (Hallee et al., 1974).

One of the most impressive symptoms in AIM is the rapid, pronounced enlargement of the lymph nodes and enlargement of the spleen resulting from the proliferation of lymphoid or "white blood" cells. This proliferation of lymphoid cells is benign in contrast to other diseases in which long-term infection with EBV induces the proliferation of lymphoid cells ultimately leading to the development of hematopoeitic malignancies such as BL and Hodgkin's disease (Vetsika and Callan, 2004). Thus, although AIM represents a benign disease process, it has the possibility to offer insights into the mechanisms by which infection with EBV alters the life cycle of lymphoid cells prior to any steps that lead to malignant transformation.

The study of the natural course of AIM following its initial diagnosis has yielded a number of findings about the nature of infection by EBV in humans. The diagnosis of AIM is often based on the detection of antibodies of the IgM class to viral capsid antigens (VCAs). These antigens are synthesized during the productive cycle of EBV's infection, and thus, antibodies to them indicate that by the time AIM is diagnosed, the virus has replicated, killed host cells, and generated progeny somewhere in the patient's body. This progeny virus can be detected in the saliva and blood immediately following diagnosis (Balfour et al., 2005). EBV can infect oral epithelial cells to cause OHL, but there is currently no evidence that this cell type is infected during AIM (Karajannis et al., 1997). There is evidence, though, that EBV may be replicating in B cells in the blood (Prang et al., 1997). The number of viral copies of EBV DNA in the peripheral blood peaks around 3–5 days after diagnosis and then declines rapidly while that in the saliva persists for months (Balfour et al., 2005). The levels of viremia as measured by detection of viral DNA in the blood and the rate at which the virus is cleared correspond to the extent of symptoms and resolution of symptoms, respectively (Balfour et al., 2005).

One unexpected finding is that multiple distinct infections may often contribute to AIM (Sitki-Green et al., 2004). Raab-Traub and colleagues identified patients with AIM carrying more than one strain of EBV. Some patients had even five to six strains of the virus (Sitki-Green et al., 2004). While some of this complexity may reflect strain evolution *in vivo*, some of it likely reflects initial infection by multiple strains of EBV because the authors sampled patients immediately following diagnosis of AIM when possible. The role that infection with multiple strains of EBV plays in the pathogenesis of AIM is not known. No correlation between the number of strains present with the severity, length, or type of symptoms manifested could be identified (Sitki-Green et al., 2004).

The classic symptoms of lymphadenopathy and splenomegaly associated with AIM are manifestations of the latent infection of B cells by EBV and not its productive infection (Vetsika and Callan, 2004). It seems likely that the high viral loads in the

peripheral blood constitute a pool from which EBV infects B cells to foster their proliferation. As EBV is cleared from the peripheral blood and the levels of virus drop, opportunities for B cells to become latently infected would decrease and thereby symptoms of latent infection such as lymphadenopathy would slowly resolve.

The groups of Rickinson and Kuppers have documented that EBV is replicating in a latent fashion in B cells in patients who have AIM through polymerase chain reaction (PCR) and immunohistochemical (IHC) methods, respectively. Rickinson's group found that B cells from individuals who were diagnosed with AIM expressed the latent genes EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, LMP1, and LMP2A. After acute infection had resolved, B cells from the same patients now had a restricted pattern of gene expression (still consistent with latency); namely, they expressed only EBNA1, EBER, and/or LMP2A (Tierney et al., 1994). Interestingly, this latter pattern of latent expression of the genes of EBV in B cells is associated with malignancies such as BL and Hodgkin's disease (Cohen, 2000). This latter finding indicates that AIM might represent an entryway for the virus to ultimately cause the malignant transformation of B cells.

Kuppers' group found similar patterns of expression of the latent genes of EBV through immunohistochemistry of tonsils from patients diagnosed with AIM. They observed that B cells infected with EBV (EBV-positive B cells) had proliferated in the germinal centers of the tonsils well in excess of those B cells that were not infected with EBV (EBV-negative B cells). These EBV-positive B cells were proliferating in the germinal centers without acquiring additional mutations in the complementarity-determining regions (CDRs) of their encoded antibody genes (Kurth et al., 2000, 2003). It is thus reasonable to argue that EBV is driving these cells to proliferate independently of the environment provided by the germinal center.

Memory B cells are found to be infected with EBV in blood soon after diagnosis of AIM (Hochberg et al., 2004; Souza et al., 2005). These cells are not proliferating, do express functional BCRs, and are thought to be the long-term reservoir in which EBV resides in its human host. They decline in number dramatically following recovery from AIM (Prang et al., 1997). People who have not had AIM but are seropositive for EBV (indicating prior asymptomatic infection with the virus) have significantly lower levels of EBV-positive B cells in their memory compartment than do asymptomatic individuals who have a prior history of AIM (Hochberg et al., 2004). This higher load of EBV-infected B cells in people who have recovered from AIM may contribute to their increased risk of developing EBV-positive Hodgkin's disease (Hjalgrim et al., 2007).

Initial reports suggested that lymphadenopathy and splenomegaly in patients with AIM resulted from the proliferation of B cells driven by infection with EBV (Henke et al., 1973; Vetsika and Callan, 2004). Recent data indicate that white blood cells derived from the thymus or T cells also proliferate in lymphoid organs in response to acute infection with EBV and constitute the bulk of the lymphoid expansion (Vetsika and Callan, 2004). Rickinson's group demonstrated that cytotoxic T cells (CD8+ T cells) specific for antigens derived from EBV proliferate extensively during AIM (Callan et al., 1996, 1998; Goldsby et al., 2000; Maini et al., 2000). This proliferation of CD8+ T cells represents a prominent mechanism by which the virus is cleared from the host (Cohen, 2000; Kurth et al., 2000; Kurth et al., 2003). CD4+ T cells also proliferate in response to

antigens derived from EBV during AIM. These CD4+ T cells can contribute to the death of B cells infected with the virus and can induce the production of cytokines such as IFN-γ(Munz et al., 2000; Marshall et al., 2007). The extent of the contribution of CD4+ T cells to resolve AIM through the clearance of EBV, though, is unclear (Vetsika and Callan, 2004).

Independent evidence for the role of a T-cell-mediated immune response in limiting AIM has come from the retrospective analyses of polymorphisms in human leukocyte antigen (HLA) class I genes of patients and controls (McAulay et al., 2007). Certain polymorphisms tightly linked to HLA-A1 and HLA-A3 alleles are found at significantly different frequencies in AIM-positive and -negative, EBV-positive subjects as well as in AIM-positive and EBV-negative subjects. HLA class I molecules present antigens to CD8+ T cells, and their different alleles will differ in their capacities to do so. That certain alleles are associated with AIM patients supports the notion that an optimal T-cell response limits the development of AIM.

Another component of the immune response contributes little to the clearance of the virus but does help in diagnosing AIM. Acute infection with EBV induces the production of a number of antibodies by the host during AIM. These antibodies, which are not specific for EBV, form the basis of the two common tests used to diagnose the disease: the monospot antibody test and the Paul–Bunnell test (CDC, 2007; Vetsika and Callan, 2004). These antibodies, although not specific for EBV, can agglutinate red blood cells from other species, namely red blood cells from sheep (Paul–Bunell) or from horses (monospot). The monospot test, which is the more commonly used test, is rapid and qualitative. It has a sensitivity of 65%–85% and a specificity of 90% (CDC, 2007). If the monospot test is negative and the clinical suspicion for AIM caused by EBV is high, further testing of serum for antibodies specifically against EBV-encoded antigens can be undertaken to confirm the diagnosis. Such confirmation is particularly important in light of the fact that the other AIM-like diseases are attributable to treatable causes such as CMV or *Toxoplasma gondii* (CDC, 2007).

Two large population-based studies have provided a solid basis for AIM being a risk factor for developing EBV-positive Hodgkin's disease. Studies done in England and Scandinavia have found that those with a history of AIM have an increased risk for the development of EBV-positive HL (Alexander et al., 2003; Hjalgrim et al., 2003). The Scandinavian data are particularly compelling. A total of 38,555 subjects with clinical and serological evidence of AIM from Denmark and Sweden were prospectively followed to determine the incidence of EBV-positive and -negative HL as compared with a control population. Those subjects who had AIM had a fourfold higher risk of developing HL than those who had no history of the disease. The incidence of EBV-negative HL was essentially the same in the two populations. These data indicate that there can be a contribution of the seemingly benign proliferation of B cells caused by EBV during an acute, latent infection to the subsequent development of an infected, B-cell malignancy.

Current treatment of AIM is generally limited to supportive care (CDC, 2007; Gershburg and Pagano, 2005). One recent trial with valacyclovir demonstrated a significant decrease in circulating EBV in treated patients and possibly some clinical

benefit for them (Balfour et al., 2007). The demands on some patients, the cost to society for caring for them, and their increased risk of developing EBV-positive malignancies justify additional research effort to develop effective therapies for AIM.

PTLD

PTLD is a diverse group of diseases characterized by the proliferation of cells of lymphoid origin ("white blood cells") after solid organ or bone marrow transplantation/hematopoeitic stem cell transplantation (BMT/HSCT). In most cases, the proliferating cells in PTLD are B cells latently infected with EBV. The proliferation of B cells in this setting can range from nonmalignant hyperplasia to B-cell lymphomas that are clonal in nature and pose serious risks to the health of the affected patients. Indeed, the mortality rate of patients with PTLD after organ transplantation is between 35% and 50%; mortality from PTLD after BMT/HSCT is a dismal 80% (Paya et al., 1999; Gottschalk et al., 2005). These mortality rates have driven attempts to understand the pathogenesis of PTLD in order to treat it successfully.

PTLD was first identified as a proliferation of lymphoid cells in patients who had undergone solid organ transplant and were receiving immunosuppression (Murray et al., 1968; Penn et al., 1969; Geis et al., 1978). Ninety percent of these tumors consisted of EBV-infected B cells (Penn et al., 1969; Paya et al., 1999; Gottschalk et al., 2005). This initial finding implicated EBV as having a causal role in PTLD. We now know that the incidence of PTLD varies widely, ranging from 0.8% to 20%, depending on the organ transplanted. These incidences are approximately 1%, 1.8%, 2.2%, and 9.4% following kidney, liver, heart, and combined heart and lung transplantation, respectively (Nalesnik et al., 1988; Yousem et al., 1989; Curtis et al., 1999; Jain et al., 2005). The incidence of PTLD after small bowel transplantation can be up to 20% and may reflect the large amount of lymphoid tissue in the gut, particularly B lymphocytes, that could contribute to the PTLD (Goldsby et al., 2000; Kato et al., 2006). The incidence of PTLD after BMT/HSCT is around 1%–2% (Curtis et al., 1999). These tumors arise form the donor's cells because the recipient's bone marrow has been ablated prior to the transplantation.

The active replication of other herpesviruses, specifically CMV, in transplant patients increases their risk for the development of PTLD. Manez et al. found that active CMV disease such as CMV pneumonitis was the single highest predictor for the development of PTLD after liver transplantation. Patients with active CMV infections had a sevenfold increased risk of developing PTLD (Manez et al., 1997). It seems likely that active viral infections in transplant recipients reflect their degree of immunosuppression: The more acute these infections, the more immunosuppressed the patients are, and the higher their risk of developing PTLD.

Patients receiving transplants are often treated both prior to receiving the tissue and early after transplantation with agents that deplete recipient T cells. This depletion of T cells helps to reduce the risk of acute rejection of transplanted organs or bone marrow (Wilkes and Burlingham, 2004). Unfortunately, such strategies also increase

the risk of developing PTLD. In particular, therapies which specifically target T cells such as the antibody OKT3 can result in a 10-fold increase in PTLD after organ transplantation (Swinnen et al., 1990). On the other hand, depletion strategies that target both T cells and B cells prior to and just after transplantation do not appear to result in an increased incidence of PTLD. Hale and Waldmann found that patients who had been given CAMPATH-1-H, an antibody against the protein CD52, which is expressed ubiquitously on the surface of cells originating from the lymphoid compartment, as a depletion strategy prior to BMT/HSCT, had an incidence of PTLD of 1.3%, a level consistent with treatments having no T-cell or B-cell depletion (Hale and Waldmann, 1998).

The combination of EBV-driven lymphoproliferation and iatrogenic immunosuppression underlies PTLD. Multiple observations support this etiology. People initially infected with EBV normally mount a robust cytotoxic T-cell response against EBV-encoded antigens, which eliminate the majority of infected cells (Hislop et al., 2007). Children uninfected with EBV who receive BMT/HSCT from EBV-positive donors are at a 10- to 75-fold increased risk for PTLD relative to EBV-positive recipients (Cockfield, 2001). The absence of educated T cells in these recipients coupled with immunosuppression to limit rejection of the graft allows the EBV-infected donor cells to evolve efficiently into PTLD. The already noted specific targeting of T-cells prophylactically to prevent graft rejection for solid organs increases the risk for PTLD up to 10-fold, presumably by limiting the immune response needed to control EBV-infected B cells. This proposed requirement for a cytotoxic response to control EBV-infected B-cells in order to prevent PTLD has been substantiated by insightful, clinical efforts. PTLD patients receiving BMT/HSCTs have been treated with their donor's autologous cytotoxic T cells educated against EBV-encoded antigens, and their tumors abrogated (Burns and Crawford, 2004; Gottschalk et al., 2005).

The EBV-infected tumor cells in PTLD are not equivalent to the infected B cells that proliferate in AIM. For example, in some cases of PTLD, the proliferating B cells have defectively rearranged immunoglobulin (Ig) genes (Kanzler et al., 1996; Brauninger et al., 2003; Capello et al., 2003; Timms et al., 2003). The unsuccessful rearrangement of these genes normally leads to cell death (Lam et al., 1997). Multiple groups have now shown that B cells that have undergone unsuccessful rearrangements of their Ig genes can be rescued from apoptosis and transformed both *in vivo* and *ex vivo* by infection with EBV (Bechtel et al., 2005; Chaganti et al., 2005; Mancao et al., 2005). This rescue is mediated by EBV's LMP2A gene and is necessary for the survival not only of some PTLD tumors but also for HLs (Brauninger et al., 2006). EBV-infected B cells that lack functional BCRs survive and proliferate. If their LMP2A gene is deleted from these proliferating cells, they die (Mancao and Hammerschmidt, 2007).

Levels of EBV can increase in the peripheral blood after immunosuppression for transplantation (Muti et al., 2005). This increase has fueled interest in the idea that the viral load could be used as a measure of the risk for the development of PTLD, and assays with quantitative PCR to measure EBV DNA in peripheral blood have confirmed it (Kogan-Liberman et al., 2001; van Esser et al., 2002; Lee et al., 2005, 2006; Weinstock et al., 2006). This insight has led to a shift in the paradigms for the treatment of PTLD. If the viral loads of EBV increase, indicating an increased risk of

PTLD, patients are now treated prophylactically and successfully with a number of therapies including simple reduction in immunosuppression and treatment with monoclonal antibodies directed against B cells such as the anti-CD20 antibody, rituxamab. These treatments have decreased the incidence of PTLD and improved survival in those patients in whom PTLD occurs after being treated prophylactically as compared with patients treated after clinical manifestations of PTLD became apparent (Kogan-Liberman et al., 2001; Tsai et al., 2001; van Esser et al., 2002; Blaes et al., 2005; Lee et al., 2005, 2006).

Rooney and colleagues have used the tools of molecular virology and immunology to create PTLD patient-specific and EBV-specific T cells cytotoxic to their EBV-infected B cells. Their findings demonstrate an excellent response of PTLD to infusions of cytotoxic T cells directed against antigens derived from EBV and dramatic improvements in survival of patients with PTLD, particularly in those patients with PTLD refractory to other treatments (Rooney et al., 1998; Gottschalk et al., 2005; Savoldo et al., 2006).

The advances in the treatment of PTLD have been made through understanding the biology of EBV, both its mechanisms of inducing B-cell proliferation and the infected host's immune responses to it. This understanding has then been coupled with clinical insights into PTLD, allowing the development of novel effective therapies for this iatrogenic tumor.

HL

HL is a disease with multiple, distinctive features. First, its tumor cell, Hodgkin's Reed–Sternberg (HRS) cell, comprises only a small minority of the cells in the lymphoma mass, the bulk being infiltrating lymphocytes, histiocytes, and granulocytes (Brauninger et al., 2006). This HRS cell remained enigmatic for years because its origin could not be identified. However, microdissection of single HRS cells followed by a PCR-based analysis identified these cells as having rearranged Ig genes and thus being of a B-cell origin (Kuppers et al., 1994). Some HRS cells are often multinucleated, providing them also with a distinctive appearance in histological sections. Second, HLs present as multiple, clinically recognized subtypes with two of them, nodular sclerosing and mixed cellularity, comprising 90% of the cases (Tzankov and Dirnhofer, 2006). Third, the incidence of HL is a bimodal function of age with an early peak of incidence in people aged 25–30 and a second after the age of 55 (Rodriguez-Abreu et al., 2007). Finally, about 40% of cases of this tumor are infected with EBV, and its viral genes are essential for phenotypes of the HRS tumor cell.

HL occurs at rates of two to three cases per 100,000 people per year, a rate that has remained constant in Europe and the United States for 25 years (Rodriguez-Abreu et al., 2007). EBV is found in all subtypes of HL with approximately 30% of the nodular sclerosing and 65% of mixed cellularity types being positive (Tzankov and Dirnhofer, 2006). It is found at similar frequencies in patients diagnosed at all ages (Flavell et al., 2000). EBV-positive cases have slightly lower mortality levels among young patients and higher levels among older patients (Keegan et al., 2005).

At least three findings indicate that EBV contributes causally to EBV-positive HL. First, a large prospective, epidemiological study has found previous EBV-positive AIM to increase the risk of developing EBV-positive HL by fourfold while not affecting the risk for the EBV-negative form of the disease (Hjalgrim et al., 2007). Second, a retrospective survey typed the HLA-A alleles of a large number of patients with HL and controls. Significantly fewer patients with the EBV-positive disease carried an HLA-A*2 allele than did the patients with the virus-negative tumor or a control population (Niens et al., 2007). The HLA-A*2 allele is known to present peptides for EBV's antigens expressed in HLs (Niens et al., 2007). Third, most cases of HLs in which the Ig genes are "crippled," that is, they cannot be functionally expressed, are EBV positive (Brauninger et al., 2006). B cells expressing crippled Ig genes die *in vivo* because these cells require signaling through their Ig-alpha/Ig-beta signaling complex to insure their survival. Mice in which signaling by this complex is blocked by its conditional deletion lose their affected B cells with a half-life of 3–6 days (Kraus et al., 2004). The EBV-positive, crippled HRS cells require the expression of EBV's LMP2A gene to survive in the absence of functional BCR signaling. This conclusion is based on the finding that crippled B cells newly isolated from the lymph nodes can be infected with EBV in culture, survive, and proliferate. However, when LMP2A flanked by LoxP sites is deleted from the EBV resident in these cells by conditional expression of the Cre recombinase, the cells die (Mancao and Hammerschmidt, 2007).

It is likely that EBV contributes more to HL than just LMP2A allowing the survival of B cells with crippled Ig genes. First, LMP2A may have additional functions that affect the phenotypes of this tumor. Analyses of mouse B cells derived from animals transgenic for LMP2A, human B cells infected with derivatives of EBV that do and do not express LMP2A, and HRS cells indicate that the expression of LMP2A confers on B cells a profile of cellular gene expression similar to that in HRS cells (Kuppers et al., 2003; Portis and Longnecker, 2003; Portis et al., 2003). Thus, it is likely that LMP2A affects the expression of cellular genes in a manner characteristic of HRS cells. Additional viral genes are generally expressed in EBV-positive HRS cells including EBNA1, LMP1, and the EBERs. LMP1 likely fosters their proliferation. It is required for B cells infected in culture to proliferate (Kilger et al., 1998), and its signaling *per se* is required for their proliferation, too (Dirmeier et al., 2005). Much of LMP signaling is mediated through its induction of NF-κB (Soni et al., 2007), and HRS cells both express atypically high levels of NF-κB constituents and require their signaling to proliferate (Bargou et al., 1997). Therefore, LMP1's activation of the NF-κB signaling pathway probably mediates the proliferation of EBV-positive HRS cells. Finally, neither LMP2A nor LMP1 can be maintained in these proliferating cells in the absence of EBNA1; its expression therefore is pivotal to both the survival and proliferation of these tumor cells.

HL is usually diagnosed following a lymph node biopsy and identification of HRS cells. The tumors are subsequently staged as a function of the number of lymph nodes involved and their location in the patient (Fuchs et al., 2006). The presence of the EBERs in the HRS cells is usually assayed by *in situ* hybridization as a means of identifying those tumors that are EBV positive (Ambinder and Mann, 1994). Standard treatments include chemotherapy with a combination of adriamycin, bleomycin,

vinblastine, and decarbazine (ABVD) with or without radiotherapy, depending on the stage of the tumor (Fuchs et al., 2006). Such treatments are remarkably successful with 95% of patients initially 15–44 years of age alive and disease free for 16 years in one study (Keegan et al., 2005). Additional immunotherapies are being developed and proving effective for cases of relapsed disease. The infusion of autologous, cytotoxic T cells educated in culture against a patient's EBV-infected B cells to recognize LMP1 and LMP2A induced remission in one-third of the patients (Bollard et al., 2004). This latter treatment is impressive not only for its success but also for its testament to the role EBV plays in maintaining EBV-positive HLs.

BL

BL is a childhood lymphoma endemic to equatorial Africa. It is common in Africa where malaria is holoendemic, reaching rates of 2.3 cases per 100,000 children per year in parts of Kenya (Rainey et al., 2007). Infection with EBV is a risk factor for developing endemic BL (de-The et al., 1978) and, as with all cancers, multiple events are required for this tumor to form. One striking, additional event needed for BL is a chromosomal translocation of one of three Ig loci to that of the c-myc proto-oncogene, which leads to the constitutive expression of c-myc's translocated allele (Taub et al., 1982; Wilda et al., 2004). BLs that arise elsewhere in the world are not as frequently associated with EBV as in equatorial Africa. Approximately 95% of endemic BL is EBV positive, while about 30% of "sporadic" BLs are EBV positive in the United States and 70% are virus positive in Brazil (Blum et al., 2004; Klumb et al., 2004). Sporadic cases have a much lower incidence than endemic ones with BL in the United States occurring only 2% as frequently as in equatorial Africa (Blum et al., 2004). Sporadic cases do have translocations involving c-myc whether EBV positive or not.

BL was identified largely by the remarkable efforts of Denis Burkitt who worked as a surgeon in Uganda in the 1940s and 1950s. He described this common childhood tumor, mapped its regional occurrence, collaborated with Anthony Epstein to aid in the discovery of EBV, and treated the tumor successfully with methotrexate and cyclophosphamide (Coakley, 2006). The endemic tumor is characterized by its rapid growth with survival times measured in months after diagnosis without treatment. The tumor is sensitive to chemotherapy so that intensive therapies in the United States can achieve event-free survivals of 2 years and beyond of 90% (Magrath et al., 1996). A lack of advanced medical support in equatorial Africa, however, means that the mortality resulting from endemic BL is now as high as when Denis Burkitt first described it (Coakley, 2006).

We know much about endemic BL and can trace its pathogenesis quite well (Brady et al., 2007). This lymphoma appears to be the result of two interacting, infectious agents: EBV and *Plasmodium falciparum*. EBV infects B cells and induces and maintains their proliferation. Infected people normally develop robust, immune-mediated, cytotoxic responses that recognize multiple, latent, viral antigens including, in particular, the EBNA3s (Rickinson and Moss, 1997). Endemic BL occurs where malaria is

holoendemic (Rainey et al., 2007), and chronic malarial infection suppresses a patient's immune responses. Children with holoendemic malaria ranging in age from 5 to 9 have suppressed cytotoxic T-cell responses specifically related to EBV (Moormann et al., 2007). Children in the Gambia with acute malaria have fivefold more cells productively infected with EBV in their blood than do convalescing children (Lam et al., 1991). Gabonese children with severe malaria under 5 years of age have higher levels of EBV DNA in their blood than do those with mild disease throughout all phases of the disease cycle (Yone et al., 2006). Immunosuppression resulting from severe infection with *P. falciparum* likely contributes to the increased levels of EBV-positive cells and of viral DNA in the bloodstream of children. It is also possible that the parasite directly induces EBV's productive cycle. The CIDR1-alpha moiety of falciparum's membrane protein 1 stimulates the lytic cycle of EBV-infected cells in culture and may do so *in vivo* (Chene et al., 2007).

The increase in EBV-infected cells in the blood of children having holoendemic malaria means that more such cells are at risk of acquiring a chromosomal translocation juxtaposing the c-myc and Ig loci. These translocations probably occur in the germinal centers of lymph nodes where the Ig CDR undergo somatic hypermutation (SHM) and antigen selection. Both endemic BL and those occurring in acquired immune deficiency syndrome (AIDS) patients express mutated Ig genes consistent with their having undergone SHM and antigen selection (Bellan et al., 2005). These translocations require the enzyme, activation-induced cytidine deaminase or AID, which is expressed specifically in the germinal centers and is also necessary for SHM (Ramiro et al., 2006; Dorsett et al., 2007). The translocations involving c-myc and the Ig loci that are recombinations between different chromosome pairs may be favored at least for the IgH allele because it and c-myc colocalize in the same transcription factories in activated B cells (Osborne et al., 2007).

The Ig/c-*myc* translocations lead to the translocated c-myc allele being expressed constitutively (Nishikura and Murray, 1988; Wilda et al., 2004). The c-*myc* is a transcription factor that regulates multiple cellular pathways including ones involved in proliferation and apoptosis (Eisenman, 2001). The constitutive expression of c-*myc*, when its apoptotic functions are blocked, can drive proliferation and foster tumorigenesis (Arvanitis and Felsher, 2006). The c-myc's driving proliferation in EBV-infected cells would be redundant with LMP1's regulating proliferation, too (Kilger et al., 1998; Dirmeier et al., 2005). However, LMP1 and other latently expressed viral genes are recognized by cytotoxic T cells, leading to the elimination of these infected cells (Rickinson and Moss, 1997; Duraiswamy et al., 2003). Cells that evolve into endemic BLs turn off most of their viral genes and often express only EBNA1, the EBERs, and viral miRNAs (Rowe et al., 1987). EBNA1 is the only protein among these viral products, and it is poorly recognized by the host's cytotoxic immune response (Levitskaya et al., 1995; Heller et al., 2007). Thus, infected cells that express only the viral genes common to endemic BL are not efficiently recognized by cytotoxic immune responses. The viral genes that are expressed in them do block apoptosis (Kennedy et al., 2003) so that any proapoptotic functions of the expressed c-myc would be inhibited. This combination of viral antiapoptotic and cellular proliferative signals in the absence of an effective immune response drives tumorigenesis for endemic BL.

The pathogenesis of sporadic, EBV-positive BL must differ from that of the endemic disease. Cofactors other than holoendemic malaria or distinct cellular mutations in addition to Ig/c-myc translocations likely contribute to its genesis. Clearly the immunosuppression resulting from AIDS is one such cofactor. BLs, which are approximately 30% EBV positive in these patients, can arise at frequencies much higher than does endemic BL in Africa (Kelley and Rickinson, 2007). Recent phase II trials indicate that treatment with chemotherapy and anti-CD20 antibodies can yield complete remissions for limited times in up to 75% of these patients. Multiple new therapies using anti-CD20 antibodies and cytotoxic T cells to treat EBV-positive lymphomas in general have been envisioned and are being tested (Davis and Moss, 2004). Additional, less expensive therapies requiring less advanced medical care must also be developed to treat BL endemic to equatorial Africa, however.

OHL

OHL is perhaps the only disease in humans caused by acute lytic infection with EBV. OHL is described as a white, plaque-like appearance of the oral epithelium that manifests itself primarily in patients who are immunosuppressed (Slots et al., 2006). OHL was first identified by Greenspan and coworkers in patients who had AIDS secondary to infection with the human immunodeficiency virus (HIV). Of the 80 patients who had presented to them with OHL, 79 patients were HIV positive (Leads from the MMWR, 1985). Subsequent work established that the incidence of OHL in those patients with AIDS or those at high risk for AIDS was approximately 10%. These findings led to OHL being described as an AIDS-defining illness or an illness in the absence of any other immunosuppression as being a risk factor for HIV-positive patients to progress to full-blown AIDS (Silverman et al., 1986).

Initial pathology of OHL lesions described them as containing vacuolated epithelial cells of the oropharynx without evidence of any underlying cellular inflammation (Slots et al., 2006). The epithelial cells involved in OHL lesions were found to contain herpesvirus particles. Using *in situ* hybridization, Greenspan's group found the herpesvirus present in OHL lesions to be EBV (Greenspan et al., 1985). In fact, the whole EBV genome was present in 95% of the OHL lesions that were studied (19/20) and was absent in biopsies of oral mucosa from negative controls (Greenspan et al., 1985). Based on these studies, EBV was presumed to have a causal role in OHL, and use of *in situ* hybridization to detect the EBV genome in oral epithelial lesions has become the gold standard by which OHL is diagnosed (Slots et al., 2006).

Further molecular studies have established that EBV replicates persistently in the oral epithelial cells to produce OHL lesions and that this replication is lytic in nature. A number of genes of EBV characteristic of its productive infection are expressed in OHL including BZLF-1, BHRF1, and gp220 (Walling et al., 2001). However, the latent EBV gene EBNA1 has also been found to be expressed in OHL (Cruchley et al., 1997). EBNA1 is expressed in epithelial cells involved in OHL but not in the basal progenitor cells. EBNA1 is expressed only in cells that have already started to differentiate present in the middle layers of the epithelium (Niedobitek et al., 1991;

Murray et al., 1996). This expression of EBNA1 may represent the cycle of reinfection of EBV-negative cells that quickly gives rise to productive infection in fully differentiated keratinocytes.

The identity of the source of EBV for this cycle of reinfection is not yet fully clear. It may come from EBV in the blood or simply from low-grade lytic infection that persists in the oropharynx. In some HIV-positive patients treated with valacyclovir for OHL, resolution of infection can lead to the disappearance of one genotype of EBV from the oral epithelium and the appearance of a new genotype during reactivation of the disease, a genotype that had initially been found in the blood of the same patient (Walling et al., 2004). Other patients in the same study had reactivation of their disease, and the same genotype of EBV that was originally discovered in the OHL lesions was present again in their new bout of disease. This latter observation indicates that replication of EBV in the oral epithelium can persist despite treatment and that it can reinfect cells there.

Despite its association with a state of profound immunosuppression, the treatment of OHL is largely supportive. OHL rarely causes discomfort, and its appearance will likely resolve with reversal of the immunosuppressed state (Slots et al., 2006). There is some datum to suggest that topical treatments with podophyllum and systemic treatment with antiviral agents can lead to resolution of the disease; this datum is limited in that much of it is not from randomized or robustly founded trials (Baccaglini et al., 2007). OHL is benign and represents a disease that is likely to help us understand the pathophysiology of EBV in epithelial lesions, an as of yet set of unsolved mysteries.

NPC

EBV contributes causally to the undifferentiated form of NPC. Early prospective epidemiological surveys showed that high titers of antibodies of the IgA class to EBV's capsid antigens were a 30-fold risk factor for the development of EBV-positive NPC in a region of China in which the cancer is endemic (Zeng et al., 1985). Both genetic and environmental risk factors have also been identified that contribute to NPC (Lu et al., 1990; Hildesheim et al., 2002; Yang et al., 2005; Tao and Chan, 2007). These diverse risk factors may explain NPC's surprising geographic distribution. It is common in some parts of Southeast Asia, occurring at rates of 30 per 100,000 males per year in Guangdong, China, where it is endemic and dropping to levels of less than 1 per 100,000 males per year in Japan or the United States (Tao and Chan, 2007). The high incidence of NPC in some regions of the world means that it is probably the most prevalent EBV-associated tumor, having a worldwide incidence in the tens of thousands up to, perhaps, one hundred thousand new cases per year. NPC is also the second malignancy to be associated with EBV (de Schryver et al., 1969; Henle et al., 1970). However, we still lack mechanistic insights into EBV's contributions to this malignancy in part because we lack cell culture or animal models with which to study EBV in epithelial cells.

EBV can readily be detected in NPC tumor cells by assaying them for the expression of the EBERs by *in situ* hybridization (Wu et al., 1991). In addition to the EBERs,

EBV in NPC cells consistently expresses EBNA1, the BART miRNAs, and a small secreted protein, BARF1 (Seto et al., 2005, 2008; Sengupta et al., 2006; Tao and Chan, 2007). The EBERs induce the expression of various cytokines that likely affect the proliferation or survival of these cells (Wong et al., 2005). The transcript encoding the BART miRNAs is peculiarly efficiently expressed in NPC biopsies, making it likely that these viral products contribute to the tumor's phenotypes (Raab-Traub, 2002; Sengupta et al., 2006). BARF1 is functionally related to the cellular receptor for colony stimulating factor 1 (Strockbine et al., 1998), and its forced expression in epithelial cell lines via a recombinant EBV fosters the proliferation of the infected cells in culture (Seto et al., 2008). EBNA1, as in all cases of EBV-infected proliferating cells, is minimally required in NPC for the synthesis and maintenance of the viral DNA. The two viral membrane proteins, LMP1 and LMP2a, are expressed detectably only in a fraction of NPC cases, indicating that they are not generally necessary for this tumor's maintenance (Sengupta et al., 2006; Tao and Chan, 2007). The consistent expression of a subset of viral genes in NPC could form a foundation for investigating their roles in maintaining NPC tumors in cell culture but for the observation that EBV DNA is lost from these cells when placed in culture. Many cells derived from NPC biopsies have been described as maintaining EBV, only one of which (C666-1) maintains EBV today (Cheung et al., 1999). It thus appears that while EBV provides growth and/or survival advantages to NPC *in vivo* such that effectively all malignant cells in an NPC tumor are EBV positive, these advantages are not expressed in cell culture and the virus is lost. It is quite possible, for example, that EBV inhibits the expression of cellular genes involved in the host's immunologic recognition of the infected tumor cell, providing the tumor cell a selective advantage recognized *in vivo* but not in cell culture (Sengupta et al., 2006). This scenario is also consistent with the finding that early lesions in people where NPC is endemic display somatic mutations present in frank NPC prior to these cells being infected by EBV (Chan et al., 2000). Thus, EBV may contribute not to the initiation of this tumor but to its progression.

NPC is sensitive to radiotherapy and, if it is diagnosed early enough, responds well to this treatment (Tao and Chan, 2007). However, many patients are identified after their tumor has metastasized, and the metastases respond poorly to current chemotherapy regimens (Tao and Chan, 2007). New approaches to identify patients at risk are needed. It has been found that the levels of EBV DNA in plasma or serum are a strong predictor of survival for NPC patients (Leung et al., 2006); thus, it is appealing to envision early detection assays for NPC based on the measurement of blood levels of EBV DNA in locales where the tumor is endemic. NPC patients with advanced disease have been treated with immunotherapies in which autologous cytotoxic T cells raised against a patient's EBV-infected B cells are infused into the patient. This experimental therapy has proven strikingly successful (Comoli et al., 2005; Straathof et al., 2005). It is thus possible that successful treatments for EBV-associated NPC will be developed before we understand the virus's contributions to this tumor. It is to be hoped, though, that the many efforts to generate models of epithelial infection by EBV will illuminate its role in NPC and aid in additional rational designs for its therapies (Yoshiyama et al., 1997; Shannon-Lowe et al., 2006; Turk et al., 2006).

SUMMARY

EBV was the first human tumor virus identified and serves as a paradigm for all such viruses. It infects many people and rarely causes disease. Multiple events in addition to infection are thus required for the development of its associated diseases. These include genetic backgrounds that predispose a particular person to disease, somatic mutations that explicitly affect cells that evolve to be malignant, and additional microbial infections that affect a patient's response to infections. EBV-associated malignancies arise at different times following an initial infection, but no associated cancer results from the primary infection. This delay in time reflects again a requirement for infected cells to accumulate somatic mutations and/or viral epigenetic changes in order to develop into a tumor. EBV contributes not only to the development of its associated tumors but also to their maintenance. It does not act as a "hit-and-run" agent as do chemical carcinogens. All of these properties are shared by other human tumor viruses and their associated cancers.

A great hope for human tumor viruses is that the tumors they cause can be prevented by preventing infections with them. This hope is being realized with the vaccines for hepatitis B virus and recently with that for human papillomaviruses. No such hope can be foreseen for EBV in the near future. However, as its mechanisms of tumorigenesis are being elucidated, it is reasonable to hope that therapies that target its tumor-maintenance functions can be developed rationally.

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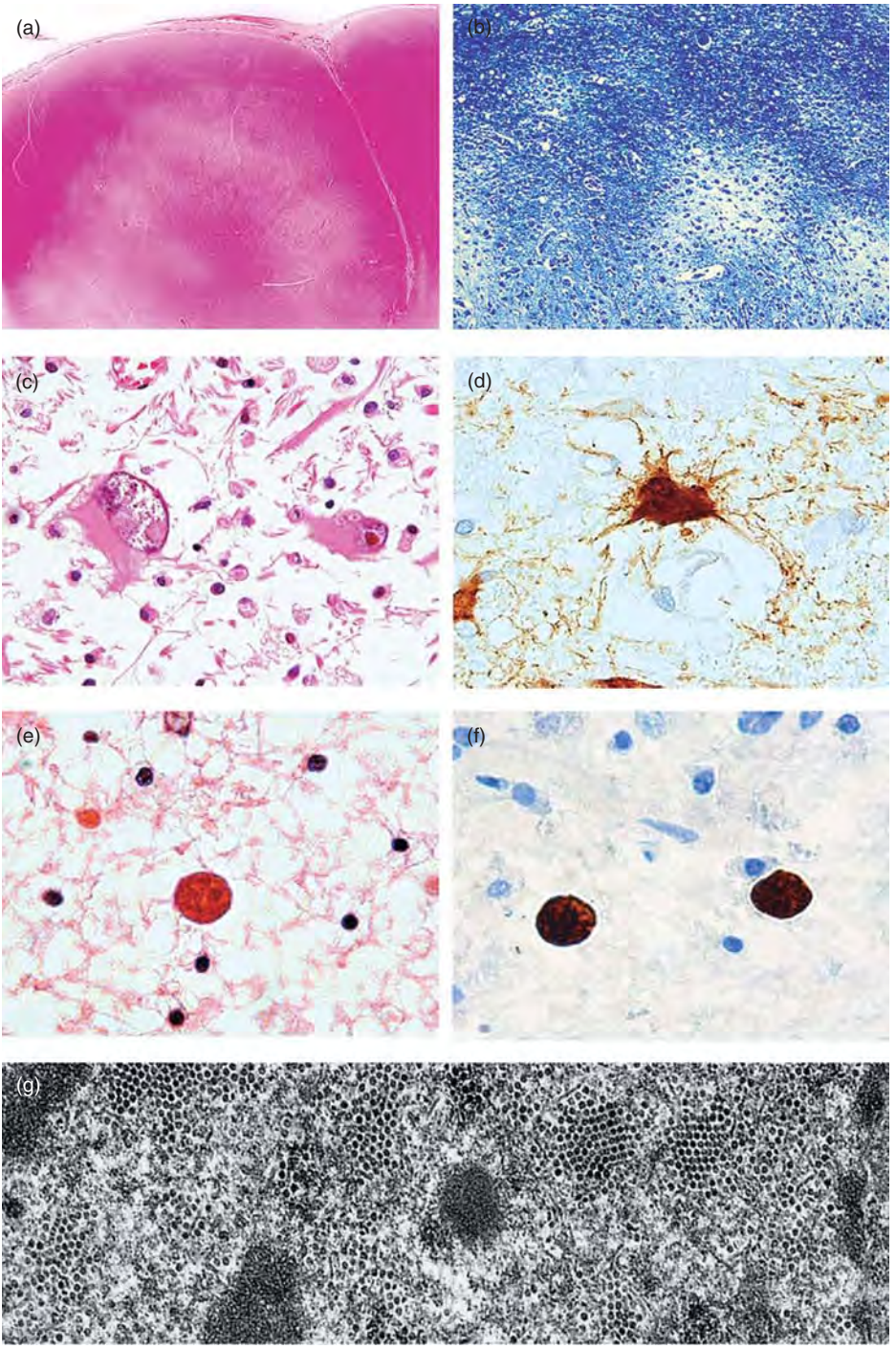

Figure 4.1. Histopathology of progressive multifocal leukoencephalopathy (PML). (See text for full caption)

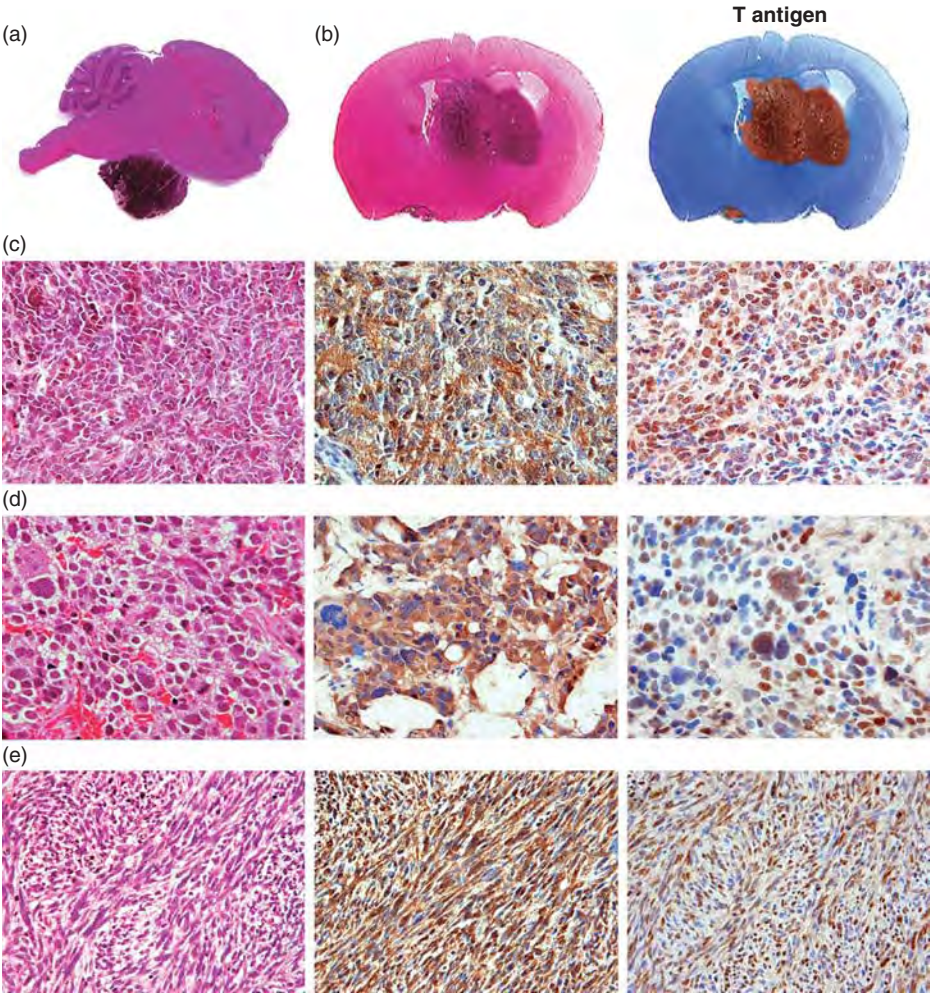

Figure 4.2. JCV-induced tumors in transgenic animals. Transgenic mice containing the early, T antigen-coding region of JCV driven by its own promoter develop several varieties of neural tube origin tumors, including PNETs, pituitary tumors, GBMs, and MPNSTs. (a) Montage of a mouse brain with a large pituitary tumor (H&E). (b) Montage of a transgenic mouse brain with a PNET located in the area of the basal ganglia, infiltrating the septum and protruding into the lateral ventricles (H&E). Immunohistochemistry for T antigen in a consecutive section demonstrates extensive expression of T antigen in the tumor but not in the normal adjacent brain. (c) PNETs are composed by numerous small cells with slightly elongated nuclei and scant cytoplasm (H&E). Immunohistochemical studies demonstrated the expression of class III β-tubulin and T antigen. (d) Pituitary tumors are composed of large pleomorphic cells with abundant cytoplasm and frequent giant nuclei (H&E). These tumors express prolactin. T antigen was detected in the nuclei of neoplastic cells. (e) MPNSTs are characterized by spindle cells arranged in interdigitating fascicles (H&E). Neoplastic cells express S100. Immunohistochemistry for T antigen demonstrates the activity of the transgene.

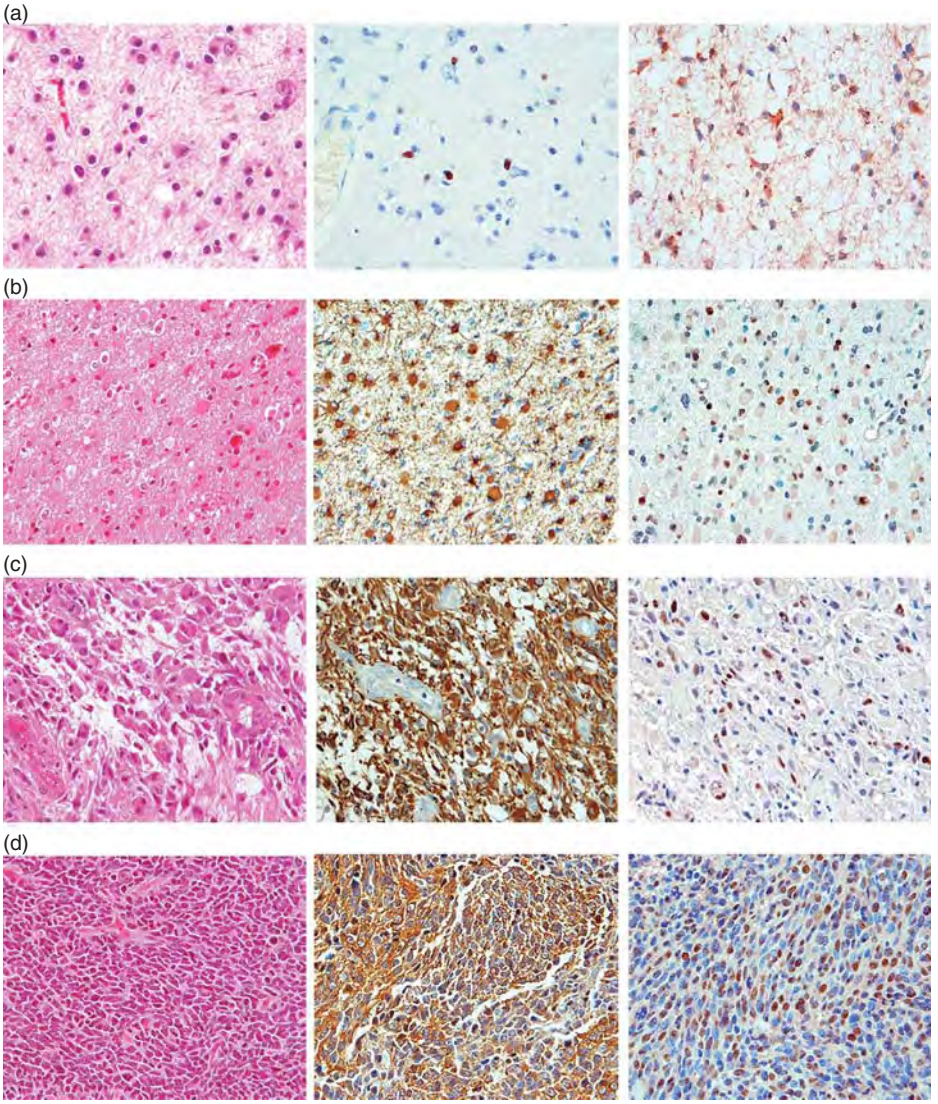

Figure 4.3. Detection of JCV T antigen in human brain tumors. JCV genomic sequences have been amplified in a variety of human brain tumors. In addition, expression of T antigen has been demonstrated in a high percentage of such tumors. (a) Oligodendrogliomas, characterized by round neoplastic cells with a clear halo (H&E) show nuclear expression of T antigen and the cytoplasmic presence of agnoprotein. (b) Low-grade diffuse astrocytomas, with small neoplastic cells, some of them gemistocytic (H&E) , demonstrating cyoplasmic glial fibrillary acidic protein (GFAP), show nuclear positivity for T antigen. (c) Immunohistochemical detection of T antigen has also been found in cases of glioblastoma multiforme, a highly aggressive tumor characterized by very pleomorphic and atypical cells, which still retain the ability to express GFAP. (d) Medulloblastomas, characterized by sheaths of numerous small neoplastic cells with a slightly elongated nucleus. Although poorly differentiated, these cells show early neuronal markers such as synaptophysin. Expression of T antigen has been detected in the nuclei of neoplastic cells in approximately half of the cases studied.

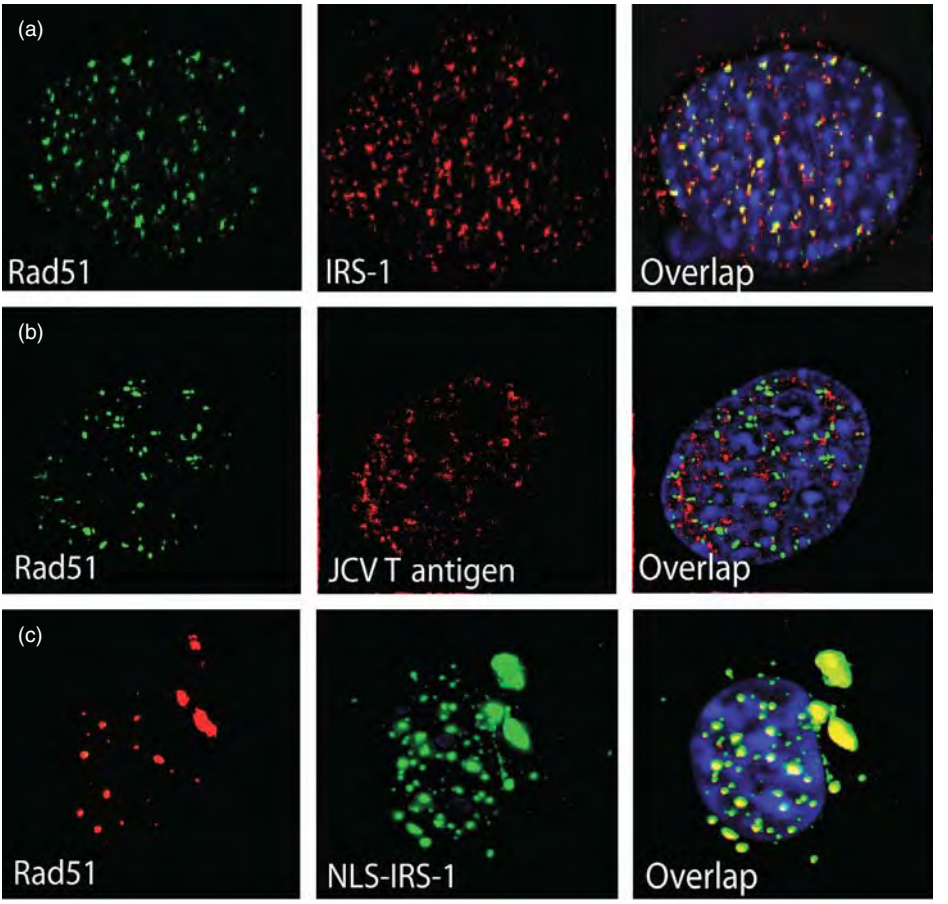

Figure 4.4. Interaction between IRS-1, Rad51, and JCV T antigen after DNA damage. (a) Double immunolabeling with anti-IRS-1 (rhodamine) and anti-Rad51 (fluorescein) was performed in JCV T antigen expressing R503 fibroblasts (R503/T clone #10) 6 h after the treatment with cisplatin (1 μg/ml). Digital images of 2 μg/m were collected with an inverted fluorescent microscope equipped with motorized *z*-axis and a deconvolution software (SlideBook4). The image marked "Overlap" represent a superimposition of two images depicted in the same row and demonstrates the nuclear colocalization between IRS-1 and Rad51 in JCV T antigen-positive cells (yellow fluorescence). Blue fluorescence represents 4′-6′-diamidino-2-phenylindole (DAPI) nuclear counterstaining. (b) Double labeling with T antigen (rhodamine) and anti-Rad51 (fluorescein). In contrast to the interaction between IRS-1 and Rad51 depicted in (a), in the same experimental setting, the interaction between JCV T antigen and Rad 51 is absent (lack of yellow fluorescence). (c) Detection of NLS-IRS-1 was carried out in T antigen-negative thymidine kinase negative (TK^-) cells at 48 h following transient transfection with the pCMV/myc/nuc/ IRS-1 expression system. The cells transiently expressing NLS–IRS-1 were treated with cisplatin for 6 h and analyzed for the possible interaction between Rad51 and NLS–IRS-1 at the sides of DNA damage (nuclear foci). Double labeling of IRS-1 (fluorescein) and Rad51 (rhodamine) demonstrates a strong nuclear colocalization between both proteins, shown as yellow fluorescence after overlapping corresponding images. The nuclei are counterstained with DAPI.

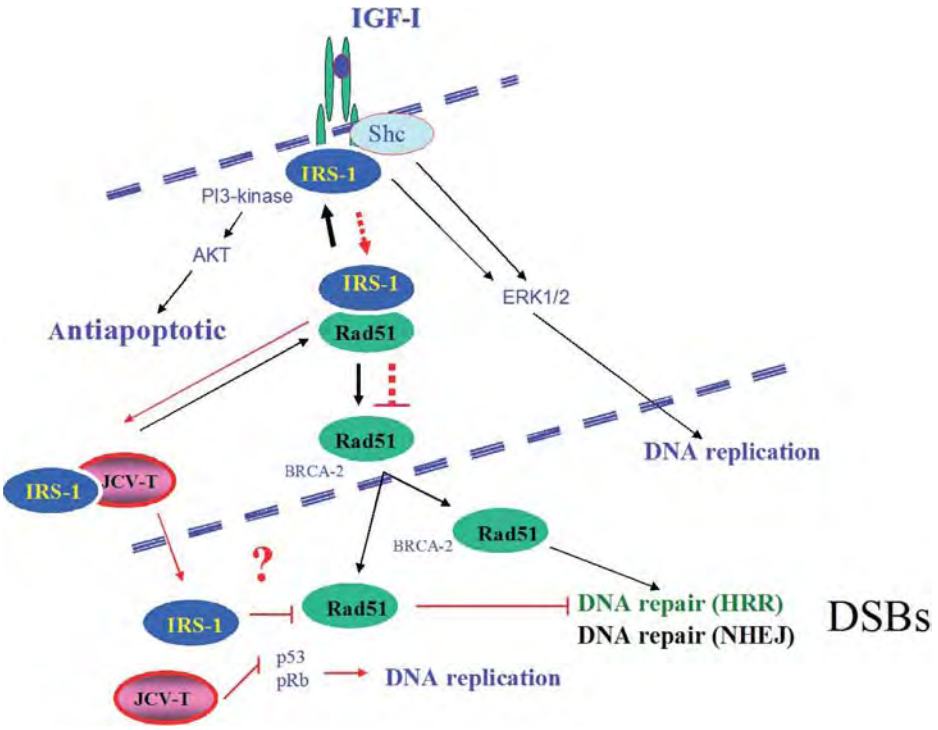

Figure 4.5. IGF-IR-JCV T antigen interplay and DNA repair of DSBs. In normal circumstances, the signal from activated IGF-IR supports cell proliferation and faithful DNA repair, which requires both homologous recombination directed DNA repair (HRR) and nonhomologous end joining (NHEJ). In contrast, factors capable of translocating IRS-1 to the nucleus, such as JCV T antigen, are strongly suspected to shift the balance from HRR to NHEJ by the inhibitory interaction between IRS-1 and the major enzymatic component of HRR, Rat51. This, in turn, may compromise the fidelity of DNA repair in cells replicating DNA. The rate of DNA damage and unfaithful DNA repair could be amplified in cells surviving genotoxic treatment (γ-irradiation, cisplatin, mitomycin). In such a scenario, accumulated mutations may lead to new cellular adaptations and the selection of cells capable of disseminating central nervous system.

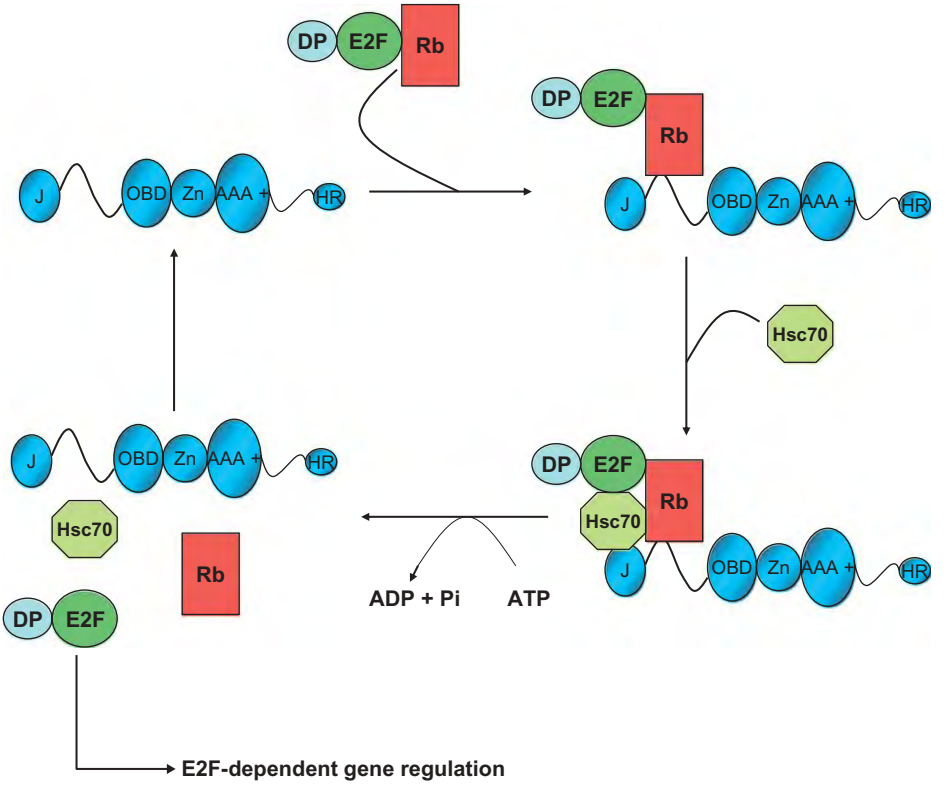

Figure 7.1. The chaperone model for disruption of Rb– E2F complexes. The large T antigen J domain recruits hsc70 and stimulates its ATPase activity, which in turn releases Rb from E2F. Free E2Fs are now able to transactivate genes required for cell cycle progression.

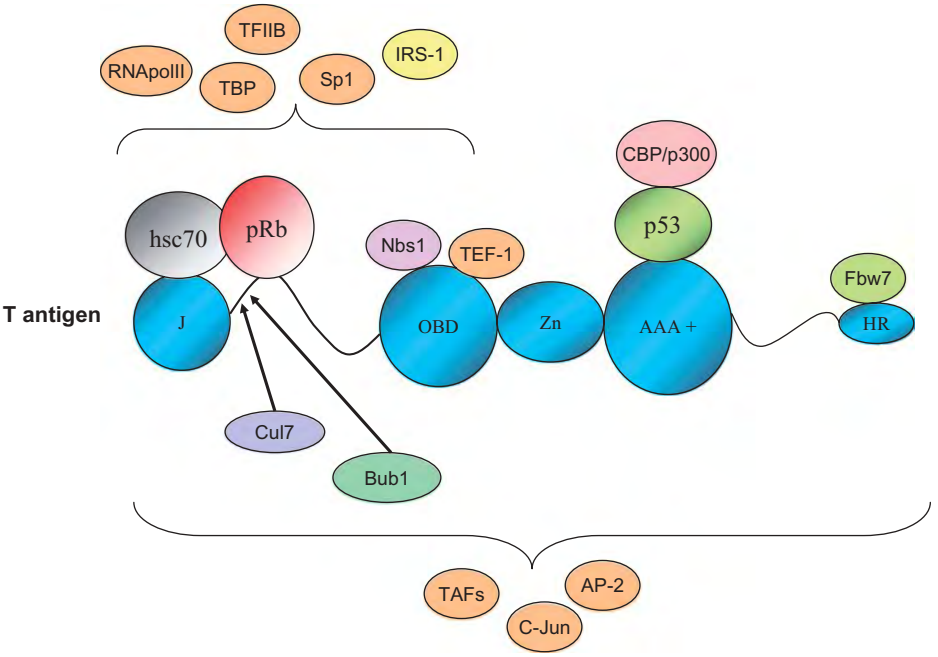

Figure 7.2. Interaction of cellular proteins with different domains/regions of SV40 large T antigen. T antigen binds to Rb-family proteins via an LXCXE motif, Hsc70 via the J domain, p53 via the p53 binding site, CBP/p300 via p53, Nbs1 and TEF-1 via the origin binding domain (OBD), and Fbw7 via the host range (HR) domain. In addition, the amino-terminus of T antigen interacts with TBP, TFIIB, RNApolII, Sp1, and IRS-1 . T antigen was shown to be coimmuno-precipitated with AP-2, c-Jun, and TAFs.

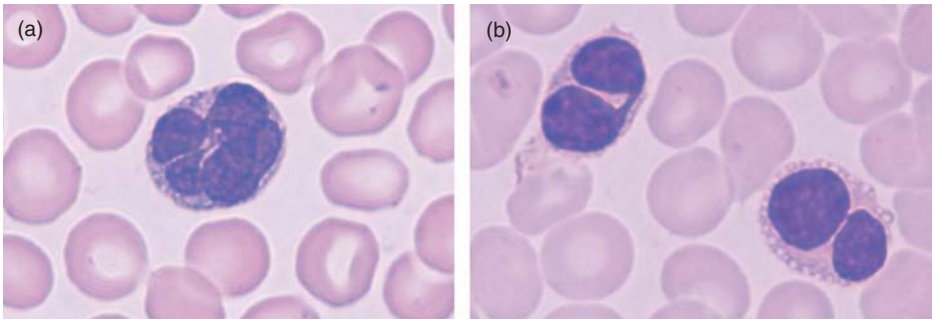

Figure 14.1. Leukemic cells in the peripheral blood of ATL patients. (a) An ATL cell in an acute ATL patient showed lobulated nuclei. (b) ATL cells in a chronic ATL patient.

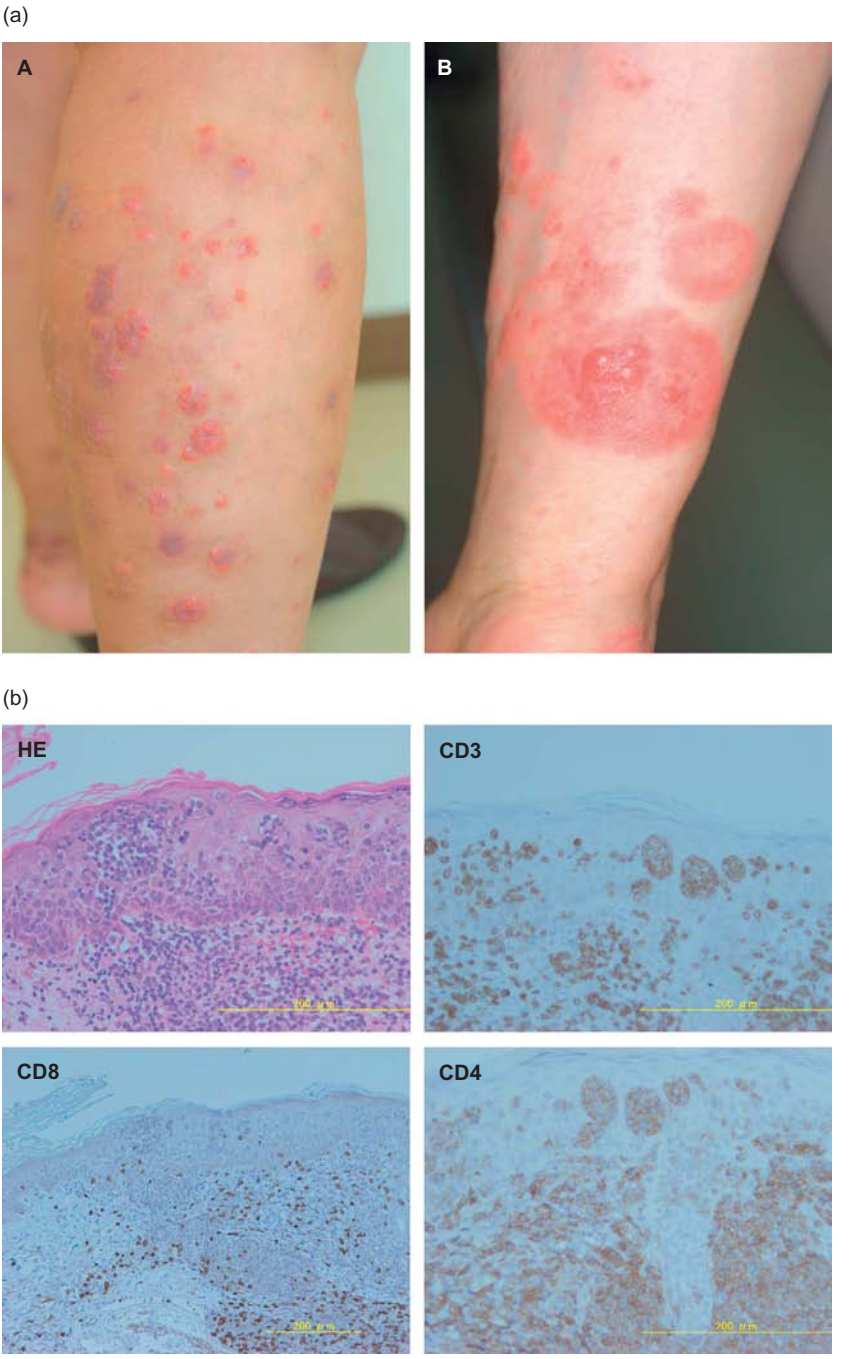

Figure 14.2. Skin involvement in ATL patients. (a) Skin lesions of ATL patients. (b) Immunohistochemical analyses of skin lesions. ATL cells infiltrated into the epidermis and formed Pautorier's microabscess. ATL cells were CD3+, CD4+, and CD8–. (Courtesy of Dr. Wakasugi, Kumamoto University School of Medicine.)

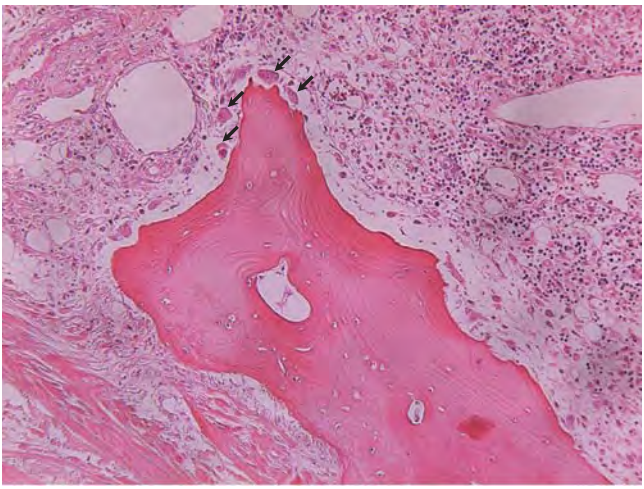

Figure 14.3. Increased osteoclasts in the bone of hypercalcemic ATL patients. Increased osteoclasts are indicated by arrows.

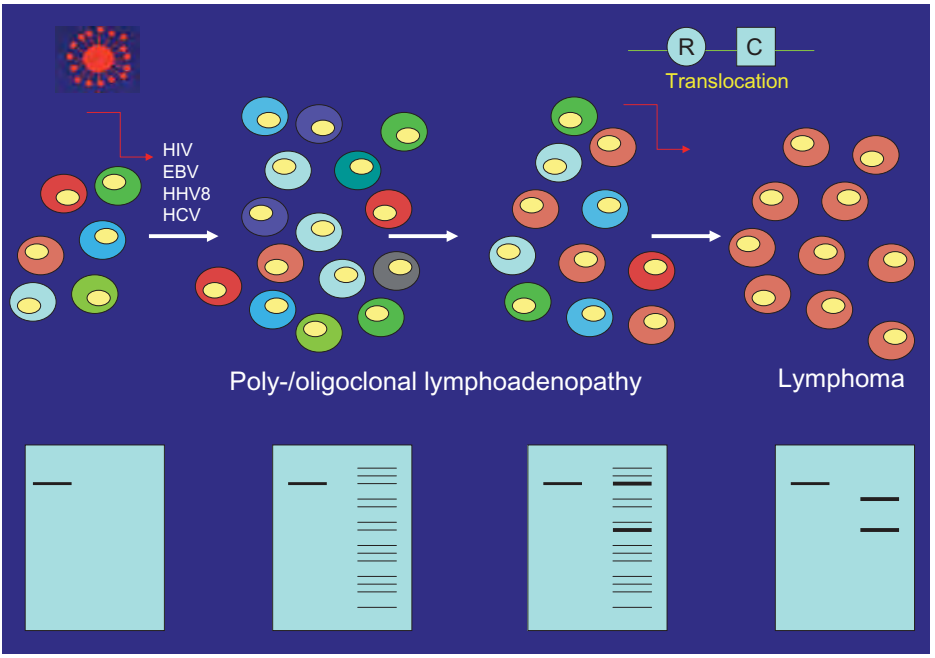

Figure 17.1. A model for virus-linked lymphomagenesis (see text).

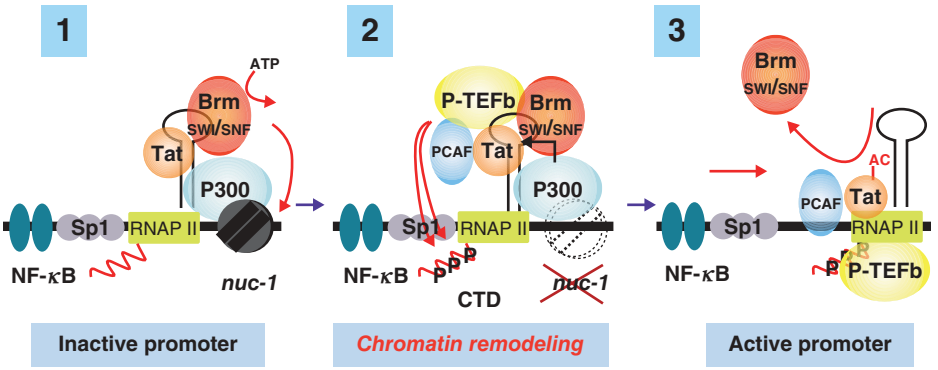

Figure 17.2. Role of Tat in chromatin remodeling (see text). Courtesy of Dr. Stephane Emiliani, Institut Cochin Department of Infectious diseases, Paris, France.

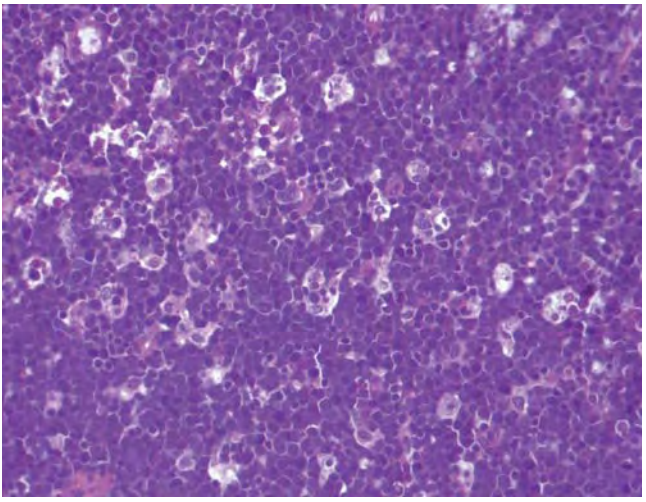

Figure 17.3. Burkitt's lymphoma. The cells are uniform in size and shape with multiple small nucleoli. The nuclei of the tumor cells approximate in size those of the admixed starry-sky histiocytes (20×).

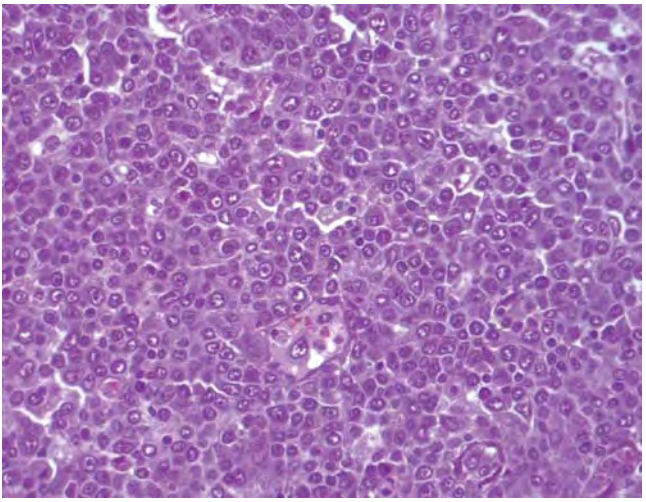

Figure 17.4. Diffuse large B-cell lymphoma. The medium-to large-sized tumor cells have a polymorphic and polylobulated appearance with vesicular nuclei, fine chromatin, and two to four membrane-bound nucleoli (20×).

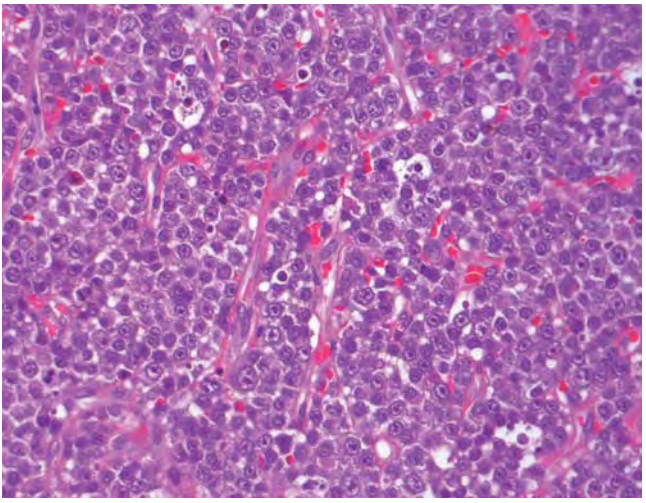

Figure 17.5. Plasmablastic lymphoma of the oral cavity. The tumor cells are large blasts with eccentrically located nuclei and usually single, centrally located, prominent nucleoli. The cytoplasm is deeply basophilic with para nuclear hof (20×).

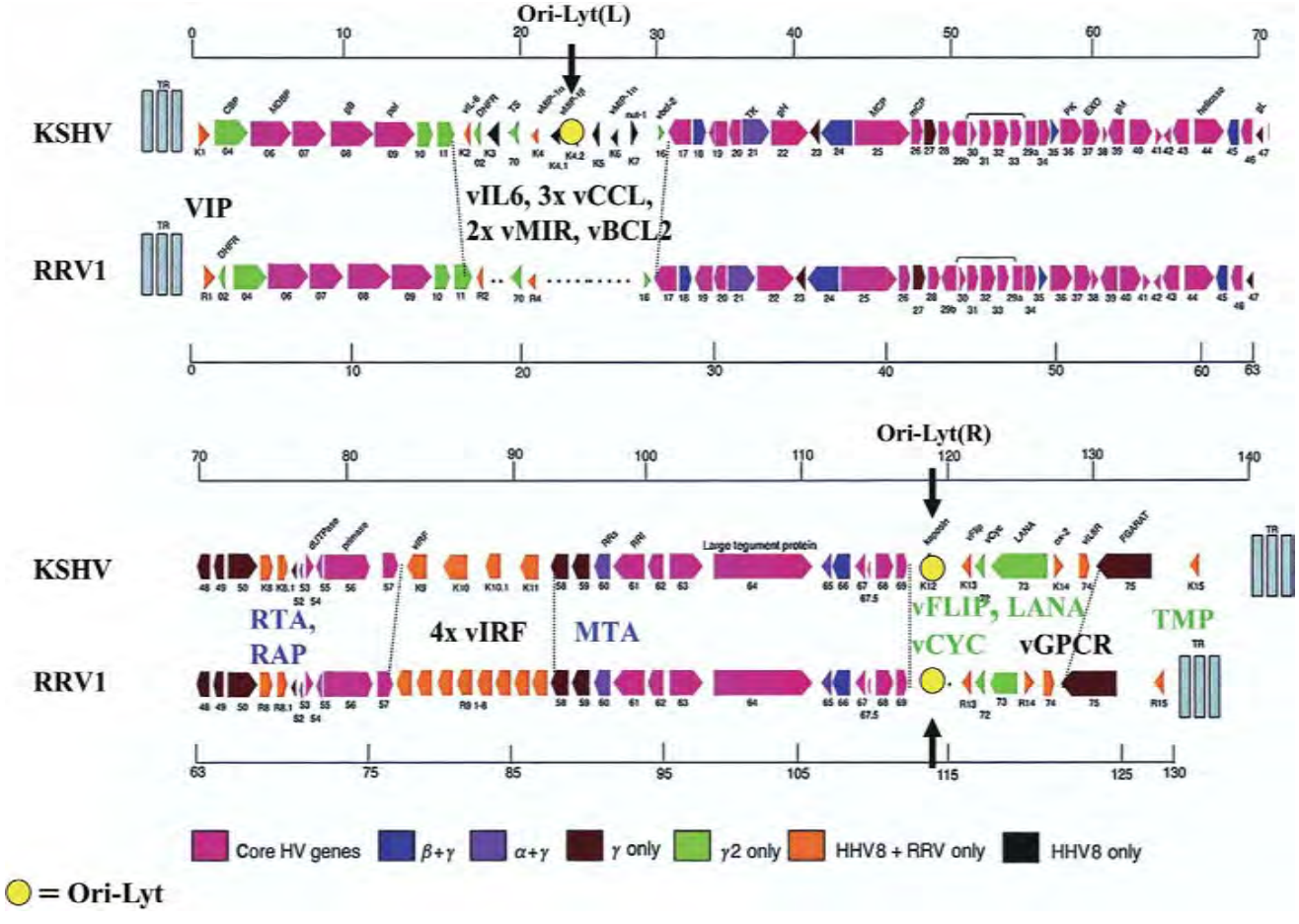

Figure 19.1. Genome map of KSHV and RRV1. The genomic organization of human KSHV compared with that of the related rhesus rhadinovirus RRV1 is depicted. All ORFs are shown as left-or right-oriented arrows of various colors representing gene classes. The duplicated Ori-Lyt domains (yellow circles) are indicated as well as the map locations of the selected critical latent and early lytic genes that are referred to in the text. The *cis*-acting, latent state Ori-P domains lie within the terminal tandem repeats.

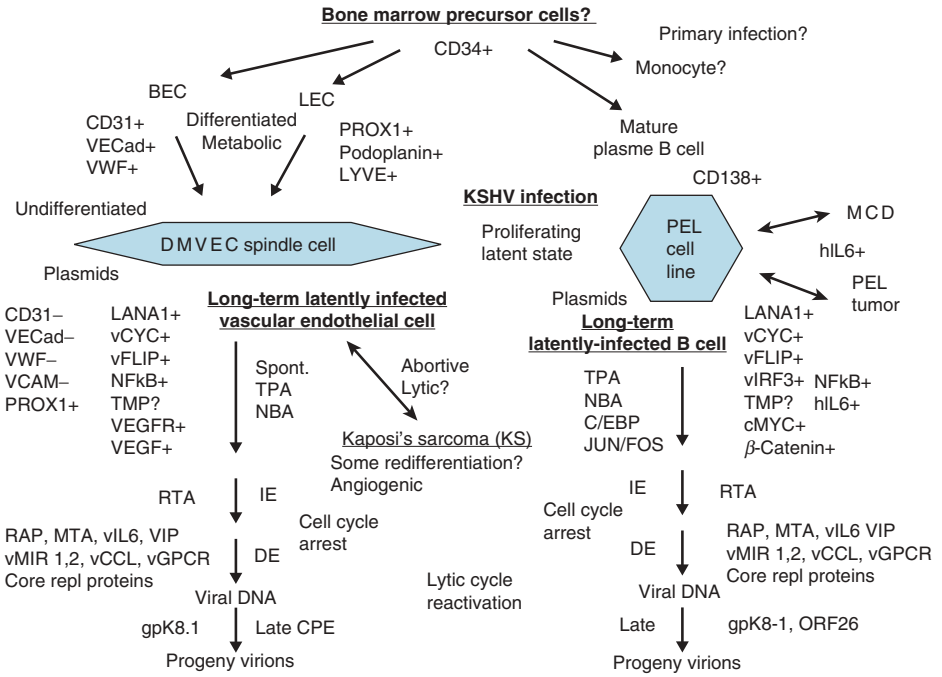

Figure 19.2. Summary of KSHV infection pathways in endothelial cells (ECs) and B cells. The primary target cells for *in vivo* infection by KSHV are unknown, but both ECs and B cells are expected to be involved is sustaining long-term latent infection (upper section of the diagram). The two models available for studying KSHV pathogenesis in cell culture involve KSHV-infected primary or immortalized vascular ECs (lower left-hand side of the diagram) or established B-lymphoblast cell lines derived from PEL tumors that carry multicopy KSHV plasmid genomes (lower right-hand side of the diagram). Major features of the latent and lytic cycle programs in both models are indicated, and manipulations that can induce partial switch into the productive lytic cycle in PELS are depicted. Older KS cell lines derived from KS lesions have lost their KS genomes and are unlikely to be valid models. The essential KSHV-mediated events that lead to KS lesions or PEL are not yet known but are assumed to be complex and multifactorial. Similarly, whether or not additional cellular gene mutational events are required for KS to produce aggressive disseminated visceral disease is not yet resolved.

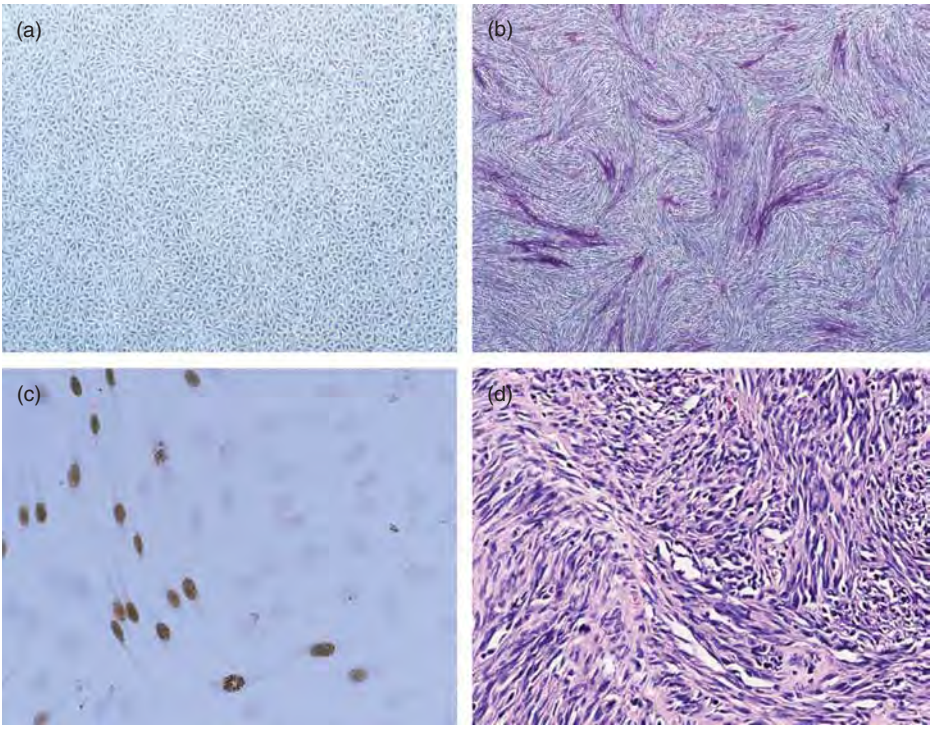

Figure 19.3. KSHV-infected DMVEC cultures as a model for KS. (a) Cobblestone monolayer of uninfected contact-inhibited DMVEC (stained with crystal violet). (b) Fully KSHV latently infected culture of spindle-converted DMVEC (second passage, stained with crystal violet). (c) High-power photomicrograph of a section of a partially KSHV latently infected culture showing a small spreading colony of LANA-positive proliferating spindle cells coexisting with uninfected DMVEC (brown nuclei = DAB-labeled IHC staining with rabbit polyclonal antibody directed against the KSHV LANA/ORF73 protein). More than 1% of the LANA-positive spindle cells were undergoing mitotic division and displayed LANA bound to the metaphase chromosomes. All LANA-positive spindle cells lose detectable expression of PECAM1 (CD31), VWF, and VE-CAD, but these were abundantly expressed in the adjacent uninfected cells (data not shown). (d) Typical thin sections of a nodular cutaneous KS lesion showing the characteristic fascicles of bundled spindle cells (stained with hematoxylin and eosin). Over 80% of the cells in this section proved positive for both LANA and PROX1 (not shown) (figure modified from Ciufo et al., 2001).

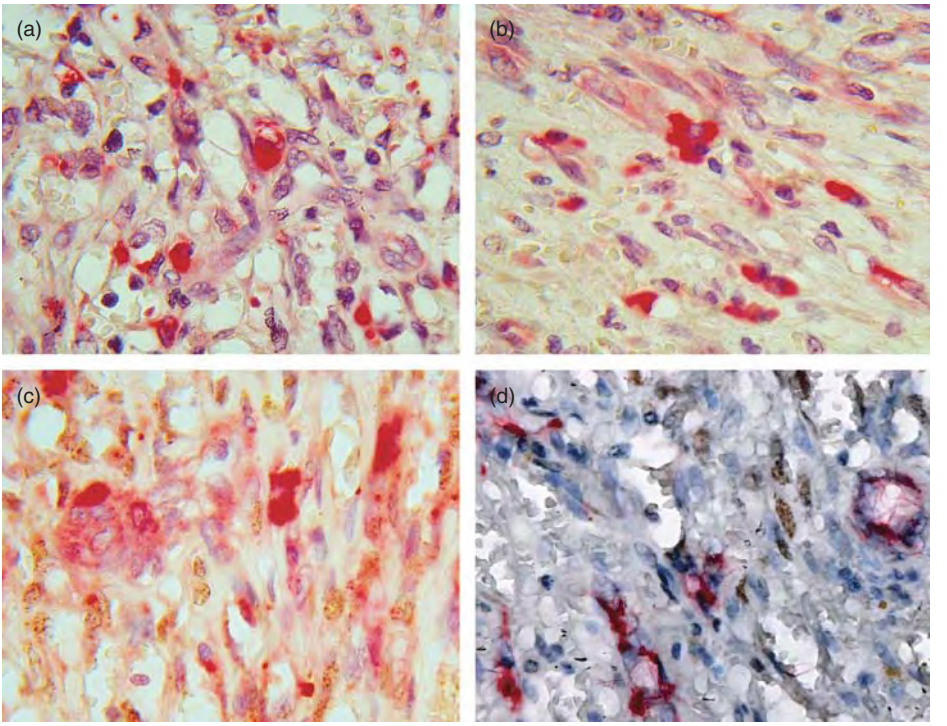

Figure 19.4. Latent and lytic cycle KSHV protein expression in an African KS skin lesion. Detection of various proteins by single-and double-label IHC in sections of a cutaneous nodular KS lesion from Uganda. (a) KSHV vGPCR (red cytoplasm, rabbit PAb). (b) KSHV vMIR2 (red cytoplasm, rabbit PAb). (c) KSHV LANA (brown punctuate nuclei, rat MAb) and vIL6 (red, cytoplasm, rabbit PAb). (d) KSHV LANA (brown punctate nuclei, rat MAb) and cellular PECAM1/CD31 (red cytoplasm, mouse MAb). Similar double-label IHC for VWF and VE-cadherin also showed complete discordance between LANA-positive spindle cells and CD31/VWF/VE-cadherin-positive neovascular ECs within the KS lesion (data not shown). All are counter-stained with hematoxylin and eosin.