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Molecular Basis for Therapy of AIDS-Defining Cancers

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We dedicate this book to the memory of our friend and colleague William J. Harrington Jr.

Foreword

More than 25 years into the AIDS pandemic, cancers that develop in individuals infected with human immunodeficiency virus (HIV) continue to challenge epidemiologists, virologists, molecular biologists, immunologists, and clinicians treating affected patients in both developed and resource-limited environments. The recognition that Kaposi's sarcoma (KS) and later, aggressive, B-cell, non-Hodgkin's lymphomas (NHLs) were developing at alarmingly high rates in young adults with an acquired form of immune deficiency predated the discovery of HIV by several years and a diagnosis of either of these tumors became part of the early case definitions of AIDS. These malignancies, both of which are associated with human herpesviruses, and invasive cervical cancer, which is linked to infection with certain human papillomaviruses (HPVs), remain the only cancers considered as "AIDS defining" according to the CDC definition.

The relatively recent widespread availability in developed countries of drug regimens that can effectively suppress HIV infection and maintain or restore cell-mediated immune function has modified the occurrence of at least some HIV-associated cancers. In particular, the incidence of KS has decreased markedly, NHL less so, but neither has been eliminated even among individuals receiving apparently effective antiretroviral therapy. At the same time, as HIV-infected individuals live longer – often with incompletely reconstituted immune function – the population at risk for both AIDS-associated cancers and other cancers not specifically associated with HIV infection (but often affecting older individuals or associated with other, known risk factors) has increased. These so-called non-AIDS-defining cancers (NADCs) include those associated with viruses (e.g., anal cancer, penile cancer, Hodgkin's disease, hepatocellular cancer, Merkel cell cancers) and those associated with environmental factors (e.g., lung cancer, head and neck cancers). In resource-limited settings where antiretroviral therapy has reached only a small fraction of the HIV-infected population, and where infection with the KS herpesvirus (KSHV/HHV-8) and oncogenic HPV types is far more common than in the developed world, KS and invasive cervical cancer continue to be leading causes of morbidity and mortality among HIV-infected individuals. Thus, there is a need to understand the mechanisms involved in the development of these varied tumor types so that we can improve the options available for their prevention and treatment.

In this volume, we have assembled expert reviews on the epidemiology of cancer in HIV-infected individuals, the particular challenges of HIV-associated cancer management in resource-limited settings and opportunities for collaborations that can advance both science and clinical care in those settings, and a series of articles that consider the biology of cancers in HIV and suggest ways in which improved insights into tumor pathogenesis may lead to innovative therapeutic strategies. Although these latter articles by no means exhaustively cover the entire spectrum of tumor types or potential mechanism-driven approaches to therapy of HIV-associated malignancies, they give a sampling of the rich possibilities that currently exist for novel therapeutic approaches and should inspire further investigations in this important and challenging area.

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The Epidemiology of Cancer in People with HIV

Andrew E. Grulich, Diego Serraino, and Denise Whitby

Abstract Three types of cancer, namely Kaposi's sarcoma (KS), non-Hodgkin's lymphoma (NHL), and cervical cancer, are formally designated as AIDS-defining cancers. KS occurs many thousandfold more commonly in people with HIV than in the general population and is causally associated with infection with human herpesvirus-8. Incidence of KS has greatly decreased in recent years in those populations of people with HIV who have access to highly active antiretroviral therapy (HAART). NHL occurs 50- to 100-fold more commonly in people with HIV than in the general population and in a proportion of cases is related to Epstein–Barr virus (EBV) infection. Use of HAART has also resulted in substantial declines in incidence of NHL. Cervical cancer occurs up to 20 times more commonly in people with HIV than in the general population, and rates have been little affected by HAART use in recent years.

In addition to the AIDS-defining cancers, it has recently become clear that a wider range of mostly viral-associated cancers occur at increased rates in people with HIV. These include Hodgkin's disease, the range of anogenital and oropharyngeal human papillomavirus associated cancers, liver cancer, and conjunctival cancers. Whether or not other cancers – including lung cancer and non-melanoma skin cancer – are associated with HIV infection is the subject of ongoing study.

Only three types of cancer, namely Kaposi's sarcoma, non-Hodgkin lymphoma, and cervical cancer, are termed AIDS defining, in that they constitute an AIDS diagnosis in a person who is infected with HIV. However, over the past decade it has become apparent that a wide range of cancers occurs in people with AIDS (Goedert et al., 1998) and HIV before an AIDS diagnosis (Grulich et al., 2007; Engels et al., 2008). Recently, the International Agency for Research on Cancer determined that there was sufficient evidence for a causal role for HIV in the three AIDS-defining cancers and for anal cancer, Hodgkin lymphoma, and conjunctival cancer. In addition,

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it determined that there were limited data that HIV may be causally associated with cancer of the vulva, vagina, and penis; non-melanoma skin cancer; and hepatocellular carcinoma (Bouvard et al., 2009). Thus the focus of interest in HIV-related cancer, as in the rest of HIV medicine, has moved from a consideration of the AIDS-defining cancers that occur at profound immune deficiency to a consideration of the cancers that occur in the milder immune deficiency that occurs in HIV infection prior to the development of AIDS.

In this chapter, we will consider the three AIDS-defining cancers, but also broaden the consideration to include those cancers that occur at increased rates earlier in the stages of HIV infection.

Kaposi's Sarcoma

In the early 1980s the frequent occurrence of Kaposi's sarcoma (KS) in otherwise healthy young men who have sex with men (MSM) was one of the first signs of the emerging AIDS epidemic. KS was almost synonymous with AIDS early in the epidemic and because it was so visible was often the first indication of the onset of disease and one of the most feared. Prior to the AIDS epidemic, KS was rare in the USA and Northern Europe. KS was reported in elderly men of Mediterranean or Jewish origin (classic KS), in parts of sub-Saharan Africa (African endemic KS), and in organ transplant recipients (iatrogenic KS). The commonality of immune suppression between transplant recipients and AIDS patients was suggestive of an infectious cause. In 1994, sequences of a novel human herpesvirus were discovered in a lesion from an AIDS-KS patient (Chang et al., 1994). The discovery of the Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus-8 (HHV-8), was rapidly followed by studies confirming that it was the causative agent of all forms of KS and greatly facilitated research on the pathology and epidemiology of KS.

Kaposi's Sarcoma in the Pre-HAART Era

In a landmark analysis of AIDS surveillance data from the CDC based on around 90,000 subjects, it was reported that the risk of KS in AIDS patients was 20,000 times greater than in the US general population and 300 times greater than in other immunosuppressed populations (Beral et al., 1990). The authors reported dramatic differences in KS risk according to HIV transmission risk group with the lowest risk observed in haemophilia patients (1%) and the highest risk in men who have sex with men (MSM) (21%). Similar differences in KS risk according to HIV risk group were reported from Europe (Brettle et al., 1996; Casabona et al., 1991; Hermans et al., 1996) and Australia (Elford et al., 1993).

The explanation for these differences became apparent with the discovery of a new human gamma herpesvirus, now termed KSHV, in KS tissue (Chang et al.,

1994). Serological assays for KSHV were quickly developed, and several groups reported that the prevalence of KSHV in HIV risk groups mirrored the disparities in KS risk. Prevalence of KSHV was reported to be 0–3% in HIV-infected haemophilia patients; 5% in HIV-positive blood recipients, and 30–35% in HIV-infected MSM (Gao et al., 1996; Kedes et al., 1996; Simpson et al., 1996). It is unclear why MSM have a higher prevalence of KSHV than other HIV risk groups, but the limited evidence available suggests that this pattern preceded the AIDS epidemic (Osmond et al., 2002). There is consistent evidence that KSHV is sexually transmitted among MSM, although sexual transmission is not the main mode of transmission in heterosexuals. The virus is predominantly excreted in saliva and is horizontally transmitted in childhood in Africa (Whitby, 2009). In longitudinal studies, KS risk has been shown to be predicted by detection of KSHV DNA in PBMC (Whitby et al., 1995) and in a case–control study with increased KSHV antibody titre (Newton et al., 2006). The most important risk factor for KS among KSHV-infected subjects appears to be a low CD4 count (Martro et al., 2007).

Reports of changes in KS incidence in AIDS patients over time prior to the introduction of HAART are somewhat inconsistent. Large studies based on AIDS registry data or linkage studies between AIDS and cancer registries in the USA, Europe, and Australia reported a decrease in KS risk in AIDS patients and in the percentage of AIDS patients presenting with KS (Dal Maso et al., 2001; Elford et al., 1993; Engels et al., 2006). Cohort studies in the USA and Europe, however, reported that KS incidence remained steady prior to the introduction of HAART (Franceschi et al., 2008; Jacobson et al., 1990; Serraino et al., 2005). A pooled analysis of 20 cohorts of HIV-infected subjects with known time of seroconversion showed no decrease in the incidence of KS prior to 1996 (Babiker et al., 2002). An international pooled study of prospective studies from the USA, Europe, and Australia including registry linkage studies also reported a steady incidence of KS before 1996 (International Collaboration on HIV and Cancer, 2000). There are few studies on possible changes in prevalence of KSHV in the pre-HAART era, with one study suggesting that prevalence in MSM changed little (Osmond et al., 2002).

Kaposi's Sarcoma in the HAART Era

The introduction of HAART therapy resulted in a rapid decrease in the incidence of KS in HIV-infected subjects. Incidence of KS decreased in the MACS cohort from 25 per 1,000 patient years (py) to 7.5 per 1,000 py in 1997 (Jacobson et al., 1999). KS risk was reported to decrease by over 80% between 1990–1995 and 1996–2002 in data from US cancer and AIDS registries (Engels et al., 2006). In Europe, a large multicentric study reported the incidence of KS decreased by 60% from 1994 to 1997 and then a further 80% between 1998 and 2003 (Mocroft et al., 2004). In the Swiss cohort study, KS incidence was 33.3 per 1,000 py between 1984–1986 and 1996–1998 when incidence decreased to 5.1 per 1,000 py. A further decrease to 1.4 per 1,000 py was observed in 1999–2001 after which the incidence

has remained steady (Franceschi et al., 2008). Similar changes were reported in Australia (Grulich et al., 2001) and Canada (Bahl et al., 2008). The International Collaboration on HIV and AIDS reported cancer incidence data from 23 prospective studies that included 47,936 HIV-infected subjects from the USA, Europe, and Australia. The incidence of KS decreased from 15.2 per 1,000 py in 1992 to 4.9 per 1,000 py in 1997–1999 (International Collaboration on HIV and Cancer, 2000). There are few studies reporting KSHV prevalence over time during the HAART era; one study from Europe and one from the US reported no changes in KSHV prevalence (Osmond et al., 2002; Rezza et al., 2000).

The risk of KS in HIV-infected subjects in the HAART era, although less than in the early years of the AIDS epidemic, is still high compared to the general population (Engels et al., 2008). The incidence has remained stable after the initial decrease (Franceschi et al., 2008). Risk of KS in HAART users is reported to be associated with low CD4 count at HAART initiation and decreases with length of time on HAART (Franceschi et al., 2008; Mocroft et al., 2004). Recently, KS has been reported in HIV-infected subjects with high CD4 counts and low HIV viral load (Krown et al., 2008; Maurer et al., 2007). The disease reported in such patients resembles classic KS (Maurer et al., 2007) and raises the possibility that KS risk may increase in HIV-infected subjects with age, despite effective HIV therapy. Understanding the underlying biological and molecular mechanisms that result in KS in HIV and KSHV co-infected subjects will be of paramount importance in formulating therapeutic interventions.

Non-Hodgkin Lymphoma

High-grade B-cell NHL was added to the AIDS definition in 1985 (CDC, 1985). The classification system for NHL has changed substantially since 1985, and describing trends in HIV-related lymphoma and the range of HIV-associated NHLs, is made more complex by these changes. In the 1980s initial reports of lymphoma in people with HIV used the Working Formulation (Dorfman et al., 1982). In the 1990s, the accepted classification system became the REAL (Revised European-American Lymphoma) classification system (Harris et al., 1994) and then the closely related World Health Organization system (Jaffe et al., 2001). More recently, a form of the WHO system that has been adapted for epidemiological study of lymphoma has been proposed (Morton et al., 2007). Overall, it has been estimated that NHL occurs about 75 times more commonly in people with HIV than in the general population of western countries (Grulich et al., 2007). NHL also occurs at markedly increased rates in other immune-deficient states, such as in solid organ transplant recipients (Grulich et al., 2007) and in people with primary immune deficiency (Beral and Newton, 1998).

In the 1980s and early 1990s, NHL was a frequent outcome of advanced HIV disease. NHL was described as the first AIDS-defining condition in about 3% of cases in western countries (Beral et al., 1991), but was found in up to 20% of people in

autopsy studies of people who died from AIDS (Wilkes et al., 1988). The incidence was around 4% per year in those with advanced immune deficiency (Polesel et al., 2008).

Initially, three types of NHL were described as being related to HIV infection. These are described individually:

1. *Systemic large cell immunoblastic lymphoma/diffuse large B-cell lymphoma*: Initially called large cell immunoblastic lymphoma, this is a neoplasm which now falls within the diffuse large B-cell lymphoma (DLBL) classification in the WHO system. DLBL comprises about 20% of lymphomas in the general population in the USA (Morton et al., 2006). In people with HIV, DLBL usually presents at advanced immune deficiency, with a median CD4 cell count of around 50–100 (Robotin et al., 2004). It is the most common form of AIDS lymphoma. It occurs frequently at extranodal sites and at sites of chronic inflammation, including sites that are rare in other settings, such as in the anus in homosexual men (Burkes et al., 1986).
2. *Primary central nervous system lymphoma*: Primary central nervous system lymphoma is most frequently of DLBL histology. It is very rare in the general population and occurs around 1,000 times more frequently in people with HIV than in the general population (Cote et al., 1996). In people with HIV, it typically occurs when immune deficiency is profound, often at CD4 counts of 10 or less (Robotin et al., 2004). Prior to the widespread availability of HAART, primary CNS NHL was a common condition in people with advanced HIV disease and was almost always fatal, with a median survival of 2–3 months (Levine et al., 1991). This tumour appears to behave as an opportunistic infection driven by Epstein–Barr virus (EBV) (Morris et al., 1995) and in that respect is similar to lymphoproliferative disease in transplant recipients receiving iatrogenic immune suppression. EBV can always be found in tumour DNA and usually in the cerebrospinal fluid. This tumour can be regarded as being a productive EBV-driven lymphoproliferation that is due to loss of cytotoxic T-cell control of EBV-infected B lymphocytes.
3. *Burkitt lymphoma*: Burkitt lymphoma is very rare in the general population in developed countries and occurs around 1,000-fold more frequently in people with HIV infection. In parts of sub-Saharan Africa and in Papua New Guinea, Burkitt lymphoma is the most common cancer in children, and tumour tissue is invariably EBV positive. These are areas with holoendemic malaria, and children with BL have higher levels of antibody to EBV and to malaria than age-matched controls (Carpenter et al., 2008; Mutalima et al., 2008).

   In people with AIDS, BL occurs at relatively preserved levels of immune function. Large case series reported at various times in the HIV epidemic have suggested that the median CD4 count at diagnosis is around 300 (Robotin et al., 2004). Unlike the situation in African BL, EBV is found in only 30–40% of cases (Kelly and Rickinson, 2007). Case–control studies in Uganda and Malawi have reported a substantially increased risk of BL among HIV-infected children (Mutalima et al., 2008; Newton et al., 2001).

4. *Primary effusion lymphoma*: Primary effusion lymphoma is a rare KSHV-associated neoplasm of large B cells that presents as a serous effusion in the pleural, peritoneal, and/or pericardial cavities without detectable tumour masses (Banks and Warnke, 2001)

HIV-Associated NHL Since the Introduction of HAART

After the introduction of highly active anti-retroviral therapy (HAART) in the mid-1990s, there was initially some debate about whether the incidence of NHL was decreased or not (Grulich, 1999). However, within a few years, a consensus emerged, based on large registry-based studies and cohort studies of people with HIV, that the incidence did indeed decrease markedly. A large-scale cohort study in Switzerland recently reported that the incidence of NHL had declined from around 1.4% per year in the early 1990s to around 0.18% a year in 2002–2006 (Polesel et al., 2008). In a US registry-based study, the incidence of NHL in people with AIDS declined from almost 30 per 1,000 person years before HAART to 6.5 per 1,000 after HAART (Diamond et al., 2006). A large cohort of HIV-infected people in the USA found that SIRs for NHL declined from 79 in 1992–1995 to 17 in 2000–2003 (Patel et al., 2008). In a review of registry-based studies and prospective cohorts, the incidence of NHL in people with HIV in developed countries fell from 6.6 per 1,000 person years in 1992–1996 to 3.2 in 1997–1999 (International Collaboration on HIV and Cancer, 2000). In people with a known date of seroconversion, the rate fell by 75% between before 1997 and 1999–2002 (Bhaskaran et al., 2004).The decline has occurred equally in those receiving HAART regimens containing protease inhibitors and those containing non-nucleoside reverse transcriptase inhibitors (Stebbing et al., 2004). It has now become apparent that the incidence of NHL drops rapidly after commencement of HAART, and risk declines by 80% or more within the first year of commencement of therapy (Polesel et al., 2008).

The decline in NHL incidence has been most marked for primary CNS NHL. In the Swiss HIV cohort study, primary CNS NHL accounted for 32% of NHL diagnosed before 1996, but only 13% of NHL diagnosed in 1999–2006. Over the same period, the annual incidence dropped to less than 0.3 per 1,000 person years (Polesel et al., 2008). In a UK clinic-based cohort, the incidence dropped from 3 per 1,000 to 1.2 per 1,000 person years (Bower et al., 2006). In US registry-based data, the SIR in people with HIV dropped from 490 to 170 (Engels et al., 2008). In contrast, the rate of BL appears to have declined little in the era of HAART and rates remain greatly increased compared to the general population (Osmond et al., 2002; Serraino et al., 2005; Mocroft et al., 2004).

Cervical Cancer

Compared to the other AIDS-defining cancers, NHL and KS, rates of cervical cancer are raised only moderately. A recent meta-analysis of studies in people with HIV/AIDS reported a summary standardized incidence ratio of 5.8 (Grulich

et al., 2007). As cervical cancer is caused by sexually transmitted HPV (Cancer IARC, 2007), it is not surprising that rates are increased in people with HIV. The incidence of both HPV infection and squamous intra-epithelial lesions is higher in HIV-positive than HIV-negative women (Ellerbrock et al., 2000). However, the finding that cervical cancer rates are also increased in transplant recipients (Grulich et al., 2007; Vajdic et al., 2006) does provide support for the view that immune deficiency also plays a role.

The relative risk of cervical cancer in women with HIV varies greatly between countries, depending in particular on the effectiveness of screening programmes which, if properly instituted, will prevent most cases of cervical cancer progressing from in situ to invasive lesions. Estimates of relative risk have varied from about 1 in the UK (Newnham et al., 2005) to 20- to 40-fold in Italy (Dal Maso et al., 2003) and Spain (Galceran et al., 2007).

In contrast to KS and NHL, there is no evidence that the incidence of cervical cancer has decreased since the availability of HAART. In a US registry-based study of cancer rates in the 5 years after HIV diagnosis, the SIR was raised about 3-fold and did not decline after HAART use became widespread (Engels et al., 2008). In a large US-based prospective study, incidence was raised about 10-fold both before and after HAART (Patel et al., 2008). A recent Italian study also concluded SIRs had not changed (Dal Maso et al., 2009). An international review of studies found incidence was little changed pre- and post-HAART (International Collaboration on HIV and Cancer, 2000).

Anal Cancer

Anal cancer rates are increased about 30-fold in people with HIV (Grulich et al., 2007). Most cases of anal cancer are causally related to anal infection with high-risk subtypes of HPV (Cogliano et al., 2005). The increased risk of anal cancer is most pronounced in homosexual men, who also have markedly increased rates of anal cancer even if they are HIV negative (Daling et al., 1987). Nevertheless, there are several strands of evidence which suggest that HIV confers an additional risk of anal cancer in people with HIV. First, anal cancer occurs at a much younger than usual age in people with HIV (D'Souza et al., 2008; Chiao et al., 2008). Second, among HIV-infected people, lower CD4 counts are associated with a higher probability of infection with multiple HPV types (Orlando et al., 2008). Third, anal cancer occurs at increased rates after organ transplant, and that population should not be at increased risk of sexual exposure to HPV (Grulich et al., 2007). Lastly, cohort data do demonstrate higher rates of anal cancer in HIV-positive as compared to HIV-negative homosexual men (D'Souza et al., 2008).

Like cervical cancer, there is no evidence that HAART leads to a decline in anal cancer risk, and in fact many studies have reported increasing incidence of anal cancer in the HAART era (Patel et al., 2008; D'Souza et al., 2008; Bower et al., 2004; Diamond et al., 2005; Hessol et al., 2007; Piketty et al., 2008). In the US

AIDS and HIV cancer matches, incidence of anal cancer was approximately stable (Engels et al., 2008, 2006).

Hodgkin Lymphoma

Hodgkin lymphoma (HL) occurs approximately 10 times more commonly in people with HIV than in the general population (Grulich et al., 2007). The increased risk is confined to the mixed cellularity and lymphocyte-depleted subtypes (Serraino et al., 1993; Frisch et al., 2001). The nodular sclerosis subtype, which is most common in young people in the general population, does not appear to occur at increased rates (Frisch et al., 2001). HL is more commonly EBV associated in people with HIV than in the general population (Frisch et al., 2001).

Interpretation of time trends in HL incidence is complicated by the unusual age-specific incidence pattern of this cancer. In the general population, there is a bimodal pattern, with peaks around the age of 30 and after the age of 50. Now that HIV disease is no longer usually fatal after 10–15 years, cohorts with HIV are tending to age. The expected pattern of occurrence of HL is complex and depends on the distribution of the population across these two peaks. Most studies which have looked at time trends have reported increasing incidence. In a large US cohort of people with HIV, the SIR increased from 11.7 in 1992–1995 to 17.9 in 2000–2003 (Patel et al., 2008). In a US registry-based study of people with HIV, the estimated SIR increased from 2.8 pre-HAART to 6.7 post-HAART availability (Engels et al., 2006), and increases have also been reported in France (Herida et al., 2003).

HPV-Related Anogenital and Head and Neck Cancers

In addition to cervical and anal cancer, infection with high-risk HPV types is also causally related to a proportion of cancers of the vulva, vagina, penis, and oropharynx (Cogliano et al., 2005). Rates of all of these cancers are increased in people with HIV infection. The fact that they also occur at increased rates in transplant recipients (Grulich et al., 2007) suggests that immune deficiency may be the underlying reason.

Conjunctival Cancer

In contrast to the other HIV-related cancers, most of the data on squamous cell carcinoma of the conjunctiva arise from African studies. Several African case–control studies have documented an approximately 10-fold increase in risk of conjunctival cancer in people with HIV (Kestelyn et al., 1990; Waddell et al., 1996; Newton et al., 2002). Increased risk in people with HIV/AIDS has also been reported in US-based registry linkage cohort studies (Frisch et al., 2000; Guech-Ongey et al.,

2008) and also in kidney transplant recipients in Australia (Vajdic et al., 2007). Based on the largest African study, this cancer appears to be associated with moderate to advanced immune deficiency, with a median CD4 count at occurrence of 111 (Waddell et al., 2006). A role for cutaneous HPV types has been suggested, but remains uncertain (de Koning et al., 2008).

Liver Cancer

HIV-positive persons, in particular injecting drug users, have a greatly increased prevalence of hepatitis B virus (HBV) and hepatitis C virus (HCV) infections than the general population and hence are at a high risk for hepatocellular carcinoma independent of any effect of HIV (McGinnis et al., 2006; Thio et al., 2002). HIV-related immune deficiency worsens the risk of cirrhosis and of liver disease-related death (Thio et al., 2002; Graham et al., 2001; Di Martino et al., 2001). Following the widespread use of HAART, liver disease has become a proportionately more important cause of morbidity and mortality among HIV-infected persons (Weber et al., 2006; Louie et al., 2002). Findings from cohort investigations, record linkage of HIV/AIDS registries with population-based cancer registries, and case–control studies have documented 2- to 20-fold excess risks for hepatocellular carcinoma. A 5.2-fold elevated risk (95% CI: 3.3–8.2) was reported in a meta-analysis of seven population-based studies of people with HIV (Grulich et al., 2007). Excess risks are more pronounced among HIV-infected IDU (SIR = 50.5, 95% CI: 15.9–11) (Clifford et al., 2005) (SIR = 24.3, 95% CI: 2.3–89.3) (Serraino et al., 2000). A recent cohort-based analysis has suggested that liver cancer risk among HIV-infected persons is higher among people with lower CD4+ cell counts (Clifford et al., 2008). Relative risks for hepatocellular carcinoma appear to be higher in the HAART era than previously (Hessol et al., 2007). However, studies based on individual data on HAART use reported either lack of association (Serraino et al., 2007) or a significantly reduced risk of hepatocellular carcinoma in people treated with HAART (RR = 0.3, 95% CI: 0.1–0.9) (Hessol et al., 2007).

Lung Cancer

Interpretation of lung cancer patterns in people with HIV is made complex by the fact that in most populations, HIV-positive people are more likely to report a history of tobacco smoking (Giordano and Kramer, 2005). Overall, it has been estimated that people with HIV are at approximately 3-fold increased risk of lung cancer (Grulich et al., 2007). Several studies have documented increasing rates of lung cancer in the era of HAART (Dal Maso et al., 2009; Bower et al., 2003; Dal Maso et al., 2003). Ideally, studies are required which control for lifetime history of tobacco smoking. Several studies have attempted to control for smoking and have reported an independent effect of HIV infection (Engels et al.,

2006; Kirk et al., 2007). Nevertheless, the control for tobacco intake has not been comprehensive in these studies, and residual confounding remains possible. In an African case–control study in a population where HIV status was not related to smoking status, lung cancer was not related to HIV infection (Stein et al., 2008). Whether or not there is an independent association of HIV with lung cancer remains uncertain.

Non-melanoma Skin Cancer

As non-melanoma skin cancer (NMSC) is not a registrable cancer in most locations, there are relatively few data on rates of this cancer in people with HIV. Overall, rates are raised about 4-fold (Grulich et al., 2007). In immune-suppressed transplant recipients a different pattern is seen, with a far greater excess in risk and an over-representation of squamous cell carcinoma (Grulich et al., 2007; Birkeland et al., 2000; Kyllonen et al., 2000; Adami et al., 2003; Kasiske et al., 2004). In people with HIV, as in the general population, basal cell carcinoma predominates (Bedimo et al., 2004).

Recently, increased rates of the rare Merkel-cell carcinoma have been described in people with HIV and in transplant recipients (Engels et al., 2002; Lanoy et al., 2009), and a newly described polyomavirus has been hypothesized to be the cause of this cancer (Feng et al., 2008).

Conclusion

HIV-infected people are at high risk of a wider range of mainly infection-related cancers (Grulich et al., 2007). The AIDS-related cancers KS and NHL, which are most closely related to severe immune deficiency, decrease in incidence dramatically after HAART initiation, but for both of these cancers, it is clear that incidence remains substantially raised, even in those taking HAART. In the era of HAART, when treatment can avert severe immune deficiency in most people, the term "AIDS-related cancer" has outlived its usefulness, and a comprehensive picture of cancer risk in this population can only be gained by including those cancers which occur earlier in the course of HIV disease.

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Epidemiology and Clinical Characteristics of Non-AIDS-Defining Malignancies

Elizabeth Y. Chiao

Abstract Since the widespread introduction of HAART over 10 years ago, the epidemiology of HIV-related malignancies has been evolving. While the incidence and the mortality of AIDS-defining cancers (Kaposi sarcoma and non-Hodgkin lymphoma, in particular) have been decreasing, the incidence of and the mortality from non-AIDS-defining malignancies appear to be increasing. This chapter reviews the recent literature published on the epidemiology and clinical manifestations, including outcomes of non-AIDS-defining malignancies. Although HAART has improved survival from opportunistic infections and AIDS-defining malignancies; it appears that HIV-infected individuals on HAART still have an elevated incidence of certain non-AIDS malignancies (especially those that are mediated by viral oncogenesis, such as EBV and HPV). In addition, it is unclear if utilization of HAART during treatment results in equivalent survival compared to HIV-negative individuals. The focus of the chapter will be on the more common non-AIDS malignancies such as Hodgkin disease, SCCA, and lung cancer. However, the epidemiology of other non-AIDS-defining malignancies will also be discussed including skin cancer, head and neck cancer, conjunctival cancer, testicular cancer, leiomyosarcoma, hepatocellular carcinoma, breast cancer, multiple myeloma, leukemia, colon cancer, and prostate cancer.

Introduction

Since the advent of widely available highly active antiretroviral therapy (HAART), individuals with human immunodeficiency virus (HIV) infection have had significantly improved survival and decreased mortality from AIDS-related infections and AIDS-defining malignancies, including Kaposi sarcoma, non-Hodgkin lymphoma, and cervical cancer (Goedert et al., 1998; Grulich et al., 1999). However, with longer

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survival, it has become evident that individuals with HIV disease are now at higher risk for other non-AIDS-defining malignancies. In addition to the three AIDS-defining malignancies, multiple recent cohort studies and linked AIDS–cancer registry studies have found that HIV-infected people are also at significantly higher risk for malignancies not included in the AIDS definition. These non-AIDS-defining cancers include squamous cell cancer of the anus (SCCA), Hodgkin lymphoma, lung cancer, head and neck cancers, testicular cancer, basal cell cancer of the skin, squamous cell cancer of the skin, and melanoma (Grulich et al., 1999; Petruckevitch et al., 1999; Serraino et al., 2000; Frisch et al., 2001; Mbulaiteye et al., 2003; Patel et al., 2004).

The non-AIDS-defining malignancies in HIV-infected patients present an evolving clinical picture. It is likely that non-AIDS-defining malignancies will become more prevalent as the HIV-positive cohort ages. However, there are many unresolved questions regarding the epidemiology, natural history, and outcomes for non-AIDS-defining malignancies. Although HIV-infected individuals are at higher risk for cancers such as squamous cell cancer of the anus (SCCA), Hodgkin disease, and lung cancer, these are still relatively rare events. Therefore, the majority of data regarding the natural history and outcomes for these cancers are based on small case series. Thus, it is difficult to answer specific questions such as whether these neoplastic processes may present at later stages and have a more aggressive course in HIV-infected patients as compared to the general non-HIV-infected population (Sridar et al., 1992; Vyzula, 1996; Powles et al., 2003b). Finally, optimal therapies, including the utilization of HAART during cancer treatment and the initiation of HAART at higher CD4 levels, also continue to evolve. This chapter focuses on the epidemiology and clinical manifestations, including outcomes of non-AIDS-defining malignancies. While the focus of the chapter will be on the more common non-AIDS malignancies such as Hodgkin disease, SCCA, and lung cancer, other non-AIDS-defining malignancies including skin cancer, head and neck cancer, conjunctival cancer, testicular cancer, leiomyosarcoma, hepatocellular carcinoma, breast cancer, multiple myeloma, leukemia, colon cancer, and prostate cancer will also be discussed.

Epidemiology

HAART has significantly changed the epidemiology of HIV disease in the United States, Europe, Australia, and other countries with widespread HAART access. Two studies have shown that the proportion of deaths due to malignancies has increased in the HAART era (Louie et al., 2002; Lewden et al., 2005). Louie et al. (2002) found that non-AIDS-defining malignancies increased as a cause of death in San Francisco County from 6.4% in 1994 to 10.9% in 1998 ($p<0.01$). In addition, in France, Lewden et al. (2005) reported that non-AIDS-associated malignancies were the third leading cause of death and that these malignancies caused 11% of all deaths in the year 2000. Finally, a study conducted by the Data Collection on Adverse Events of Anti-HIV Drugs (D:A:D) evaluated factors associated with

mortality due to non-AIDS-defining malignancies (nADMs) and AIDS-defining malignancies (ADMs) in their cohort of 23,437 patients under active follow-up from December 1999 to April 2001 (Monforte et al., 2008). They found that the overall mortality rate of nADMs was higher than that of ADMs. The death rate from nADMs was 1.8 per 1,000 person-years of follow-up [95% confidence interval (CI) 1.5–2.1] compared to that from ADMs of 1.1 per 1,000 person-years of follow-up (95% CI 0.9–1.2). In addition, they found that in multivariable analysis, both low latest CD4 count and increasing age were associated with an increased risk of death from nADMs and ADMs. Other factors such as combined antiretroviral therapy (cART) utilization increased the risk of death for nADMs only. It is unlikely that cART itself increases the risk for nADMs. Rather it underscores the complex relationship between prolonged survival with HIV disease, immunosuppression, and the diagnosis of and survival from nADMs. Furthermore, several cohort studies performed in the pre-HAART era failed to convincingly show an increased risk of non-AIDS-related malignancy in HIV-infected people (Hajjar et al., 1992; Biggar et al., 1994). These studies add further evidence to support the changing epidemiology of nADMs in the HAART era.

More recent linkage analyses of data from AIDS and cancer registries have shown statistically significant increases in the age-standardized incidence ratio (SIR) or the relative risk (RR) of non-AIDS-defining malignancies for HIV-infected cohorts as compared to standardized populations (Goedert et al., 1998; Franceschi et al., 1998; Grulich et al., 1999; Serraino et al., 2000; Gallagher et al., 2001; Frisch et al., 2001; Allardice et al., 2003; Herida et al., 2003; Dal Maso et al., 2003a). Although there have been some issues with the calculation of age-standardized incidence ratios (SIRs) (Chaturvedi et al., 2008), this technique has been utilized by large AIDS–cancer match registries, the largest of which includes over 150,000 HIV-infected individuals. These studies have shown a relationship between HIV infection and a large number of cancers including Hodgkin disease (Grulich et al., 1999; Serraino et al., 2000; Frisch et al., 2001; Gallagher et al., 2001; Dal Maso et al., 2003a; Patel et al., 2008), anal cancer (Frisch et al., 2001; Gallagher et al., 2001; Grulich et al., 2002; Dal Maso et al., 2003a; Patel et al., 2008), lung cancer (Frisch et al., 2001; Gallagher et al., 2001; Allardice et al., 2003; Dal Maso et al., 2003a), testicular cancer (Frisch et al., 2001; Gallagher et al., 2001), lip cancer (Frisch et al., 2001; Grulich et al., 2002), melanoma (Frisch et al., 2001), liver cancer (Frisch et al., 2001; Allardice et al., 2003), multiple myeloma (Frisch et al., 2001; Gallagher et al., 2001; Grulich et al., 2002), brain cancer (Frisch et al., 2001; Gallagher et al., 2001; Dal Maso et al., 2003a), leukemia (Frisch et al., 2001; Grulich et al., 2002; Dal Maso et al., 2003a), and cancers of the salivary glands, skin (non-KS) (Gallagher et al., 2001; Allardice et al., 2003) and soft/connective tissue (Frisch et al., 2001; Gallagher et al., 2001; Grulich et al., 2002). None of the AIDS–cancer match studies found an increased risk of breast cancer, colon cancer, or prostate cancer. Both Frisch et al. (2001) and Gallagher et al. (2001) found a statistically significant decrease in the risk of prostate and bladder cancer, and Gallagher et al. (2001) found a statistically significantly decreased risk of esophagus and colon cancer.

Because these studies are not analyses of primary data, the relationship between HIV and certain cancers needs to be interpreted with caution due to possible detection bias and/or miscoding. For example, it is unlikely that HIV infection is associated with rare sarcomas of the soft/connective tissue. Rather, it is likely that Kaposi sarcomas were miscoded as soft/connective tissue cancers. These AIDS–cancer registry studies may also be biased because they compare cancer rates of patients with AIDS to age-matched incidence rates in the general population. However, HIV-infected individuals often have specific risk behaviors which distinguish them from the general population, such as higher rates of intravenous drug use and a larger population of men who have sex with men (MSM). Thus, higher liver cancer SIRs may be associated with an increased rate of infection with hepatitis B and C among HIV-infected individuals rather than the immunosuppression associated with HIV infection itself. Thus, prospective studies may have fewer miscoding biases, but the rate ratios or SIRs associated with comparing the rates of cancer among HIV-infected individuals compared to the general population still need to be interpreted with caution.

Studies Comparing the Pre-HAART Era and the HAART Era

Two prospective studies have been performed that included data on the development of non-AIDS-defining malignancies and included data collected after the introduction of HAART (Herida et al., 2003; Patel et al., 2008). Patel et al. (2008) evaluated 54,780 HIV-infected individuals enrolled in the Adult and Adolescent Spectrum of HIV Disease Project (ASD) and the HIV Outpatient Study (HOPS). They evaluated cancer diagnoses from 1992 to 2003 and found 708 non-AIDS-defining cancers. Among the non-AIDS-defining cancers, they found increased SIRs for anal, vaginal, liver, lung, oropharyngeal, colorectal and renal cancers, Hodgkin lymphoma, melanoma, and leukemia. They also found a significantly decreased SIR for prostate cancer. In addition, they evaluated SIRs by year of diagnosis, comparing the pre-HAART era (1992–1995), the early HAART era (1996–1999), and the late HAART era (2000–2003). They found that the incidences of anal, Hodgkin lymphoma, melanoma, colorectal, and prostate cancers all showed an increase in rates when a p-value was calculated for linear trend.

In addition, the authors conducted a multivariable analysis to describe factors associated with each type of cancer among HIV-infected persons. They found that antiretroviral therapy was associated with a decreased risk for AIDS-defining malignancies (Kaposi sarcoma, non-Hodgkin lymphoma, and cervical cancer). However, they also found that the use of antiretroviral therapy was associated with a decreased risk of breast cancer, colorectal cancer, and lung cancer. A low nadir CD4 count was associated with an increased risk of the AIDS-defining malignancies, as well as anal cancer, colorectal cancer, and lung cancer.

In France, Herida et al. (2003) performed a similar study that included 77,025 HIV-infected patients followed from 1992 to 1999. The median follow-up was 32 months. They divided the period of study enrollment into two periods: the

pre-HAART era including 1992–1995 (P1) and the post-HAART era including 1996–1999 (P2). They reported an overall increase of non-AIDS-defining malignancies among HIV-infected men but not among women during both periods of the study. Among men, they found that the incidence of Hodgkin disease was elevated in both P1 and P2. However, oral, colon, rectal and anal cancer, stomach, and CNS cancers were all statistically significantly elevated in P1, but none of these cancers showed an increased incidence in P2. Instead, only lung cancer and kidney cancer were elevated in P2. The incidence of Hodgkin disease was higher in women than in the general population during both P1 and P2 and, as in men, the incidence of lung cancer was higher during P2. Of note, the incidence of breast cancer was significantly lower in HIV-infected women than in the general French population during both P1 and P2. They hypothesized that HAART did not have a measurable impact on the incidence of non-AIDS-defining cancers (Herida et al., 2003).

Studies Linking Risk of Cancer to Level of Immunosuppression

Although using time periods to estimate HAART utilization provides some information regarding the effect of immunosuppression, several studies also attempted to use the timing of cancer diagnosis with respect to the development of an "AIDS diagnosis" as an estimate for evaluating the risk of cancer based on the level of immunosuppression (Frisch et al., 2001; Gallagher et al., 2001). Frisch et al. (2001) defined three criteria that suggested that non-AIDS-defining cancers were associated with immunosuppression: (1) the overall RR for the period from 60 months before to 27 months after AIDS was significantly elevated, (2) the RR in the early post-AIDS period was significantly elevated, and (3) there was a statistically significant increasing trend in the RRs from before to after AIDS onset. Using a test for trend, they found that Hodgkin disease, lung cancer, penile cancer, soft tissue malignancies, lip cancer, and testicular seminoma met the three criteria associated with immunosuppression (Frisch et al., 2001). Mbulaiteye et al. (2003) utilized a subset of the linked AIDS–cancer registry to evaluate the effect of decreasing CD4 count on various AIDS- and non-AIDS-related malignancies. They found that the relative risk for oropharyngeal cancer decreased with worsening immunity. Otherwise, none of the non-AIDS-defining malignancies were affected by the level of immunity (Mbulaiteye et al., 2003).

Gallagher et al. (2001) used similar criteria for defining immunosuppression-related cancers for patients diagnosed in New York state. They divided the time intervals for developing cancer into four time periods: 5–2 years prior to AIDS, 2 years–6 months prior, within 6 months before and 3 months after an AIDS diagnosis, and 3 months to 5 years after an AIDS diagnosis. Using similar criteria to those used by Frisch et al. (2001), they found that cancers of the rectum, rectosigmoid and anus, trachea, bronchus and lung, skin, and connective tissues among males were associated with increasing immunosuppression (Gallagher et al., 2001).

Again, the associations between immunosuppression approximated by time periods and cancer diagnoses need to be interpreted with caution. Because cancer often

takes 5–10 years to develop, all associations that are derived from time-based variables will likely be confounded by length of survival with HIV. Therefore, those patients who have cancer diagnosed 3 months to 5 years after an AIDS diagnosis will likely be more immunosuppressed than those diagnosed with cancer 2 years to 6 months prior to the AIDS diagnosis, but these patients will also likely have survived longer (because a diagnosis of AIDS is highly associated with mortality) (El-Sadr, Lundgren et al., 2006) and thus are inherently at higher risk for cancer. On the other hand, those studies which used temporal associations to estimate the effect of immunosuppression corroborate other studies, including Patel et al., that have specifically evaluated the effect of nadir CD4 count, a more direct measure of immunosuppression. Thus, taken together, it is likely that immunosuppression does increase the risk of several non-AIDS-defining malignancies, including anal and lung cancers.

The Effect of Interrupted Antiretroviral Therapy on the Risk of Cancers

The SMART study was a landmark study evaluating the use of episodic antiretroviral therapy guided by CD4+ count or drug conservation (DC) as compared with continuous antiretroviral therapy or viral suppression (VS). Although the authors initially hypothesized that the continuous use of combination antiretroviral therapy might be associated with adverse events, the study was prematurely terminated because there was a significant increase in all-cause mortality rates among those in the DC arm. In addition, the study showed that continuous use of antiretroviral therapy was significantly protective for not only AIDS-defining illnesses but also non-AIDS-defining illnesses. In a follow-up analysis, Kuller et al. (2008) compared biomarkers of chronic inflammation and coagulation, including high-sensitivity C-reactive protein (hsCRP), interleukin-6 (IL-6), amyloid A, amyloid P, D-dimer, and prothrombin fragment 1+2 in the DC and VS arms of the SMART study, and also performed a nested case–control study to evaluate the relationship between these biomarkers and death. They showed that uncontrolled HIV replication was associated with increases in many markers of inflammation and coagulation, including hsCRP, IL-6, and D-dimers. Markers of chronic inflammation, such as CRP, have been associated with colon cancer (Chiu et al., 2008; Tsilidis et al., 2008).

A follow-up study evaluating all AIDS-defining and non-AIDS-defining malignancies in the SMART study found a significantly increased risk of AIDS-defining malignancies and an insignificant trend toward increased risk of non-AIDS-defining malignancies. Of a total of 5,472 participants, 70 patients developed cancer; there were 13 ADMs and 58 nADMs. The rate of all ADMs was significantly lower in the VS than in the DC arm. The rate of all nADMs was lower in the VS arm, but this difference was not statistically significant. The most common cancers were skin, lung, and prostate cancers, and the factors that predicted nADM included older age, cigarette use, and undetectable viral load at the start of the study. The authors note that because the number of cancers was small, the apparent association

with undetectable viral load may have occurred by chance; however, it is also possible that those with undetectable viral load may be long-term survivors and may have been infected with HIV for a longer time. The authors also note that because the SMART study was prematurely discontinued, it was not powered adequately to observe rare non-AIDS-defining cancers (Silverberg et al., 2007). The mean follow-up was only 16 months, which also decreased the power to detect cancers.

Meta-analysis Comparing HIV-Infected Individuals to Solid Organ Transplant Recipients

To further evaluate the relationship between immunosuppression and incidence of non-AIDS-associated cancers, Grulich et al. (2007) conducted a meta-analysis of cancer risk that pooled data from several different cohorts of HIV-infected individuals and immunosuppressed solid organ transplant recipients. They included seven HIV/AIDS studies and five transplant studies comprising a total of 444,172 individuals with HIV and 31,977 transplant recipients. In both populations, there was an increased SIR for cancers with a known infectious cause, including all three types of AIDS-defining cancer (Kaposi sarcoma, non-Hodgkin lymphoma, and cervical cancer) and other HPV-related cancers (anal, vulvar/vaginal, and penis), as well as Hodgkin lymphoma (EBV-related), liver cancer (hepatitis B/C related), and stomach cancer (*Helicobacter pylori* related).

In addition, cancers possibly associated with HPV, such as non-melanoma skin, lip, esophageal, laryngeal, and eye cancers, were also increased. Finally, the SIRs for lung, kidney, multiple myeloma, leukemia, and melanoma were also elevated for both HIV-infected individuals and transplant recipients. However, cancers of the breast, prostate, and ovary were not elevated in either population (see Table 1). Of note, meta-analysis SIRs were elevated only among transplant recipients for bladder, thyroid, and colorectal cancers. The authors hypothesize that cancers that show elevated SIRs for both transplant recipient and HIV-infected populations share immune deficiency, a contributing factor for the increased risk observed, as opposed to other lifestyle factors. However, the results need to be interpreted with caution because the heterogeneity score for most of these analyses approached significance or was significant.

Table 1 Meta-analysis SIRs for HIV AIDS-defining and non-AIDS-defining cancers (adapted from Grulich et al.)

Type of cancer	Meta-analysis SIR (95% CI)	Observed number of cancers	Number of studies	Heterogeneity *p*-value
EBV-related cancers				
Hodgkin lymphoma	11.03 (8.43–14.4)	802	7	0.00
Non-Hodgkin lymphoma	76.67 (39.4–149)	5, 295	6	0.00
Nasopharyngeal cancer	2.90 (1.80–4.66)		2	Not reported

Table 1 (continued)

Type of cancer	Meta-analysis SIR (95% CI)	Observed number of cancers	Number of studies	Heterogeneity *p*-value
HHV-8-related cancer				
Kaposi sarcoma	3,640.0 (3,326–3,976)	494	1	Not reported
HBV/HCV				
Liver cancer	5.22 (3.32–8.20)	133	7	0.01
H. pylori-related cancer				
Stomach	1.90 (1.53–2.36)	89	7	0.49
HPV-related cancers				
Cervix uteri	5.82 (2.98–11.3)	104	6	0.00
Vulva and vagina	6.45 (4.07–10.2)	21	2	0.55
Penis	4.42 (2.77–7.07)	21	3	0.52
Anus	28.75 (21.6–38.3)	303	6	0.03
Oral cavity and pharynx	2.32 (1.65–3.25)	238	4	0.07
Possibly HPV-related cancers				
Non-melanoma skin	4.11 (1.08–16.6)	121	4	0.00
Lip	2.80 (1.91–4.11)	30	2	0.45
Esophagus	1.62 (1.20–2.19)	48	4	0.53
Larynx	2.72 (2.29–3.22)	142	4	0.55
Eye (conjunctiva)	1.98 (1.03–3.81)	11	2	0.92
Non-infectious related				
Breast	1.03 (0.89–1.20)	194	6	0.60
Prostate	0.70 (0.55–0.89)	202	6	0.22
Colon and rectum	0.92 (0.78–1.08)	224	5	0.34
Ovary	1.63 (0.95–2.80)	30	5	0.34
Trachea, bronchus, and lung	2.72 (1.91–3.87)	1,016	7	0.00
Brain	2.18 (1.29–3.68)	192	7	0.00
Kidney	1.50 (1.23–1.83)	93	6	0.79
Leukemia	3.20 (2.51–4.09)	235	7	0.19
Melanoma	1.24 (1.04–1.48)	200	6	0.37
Multiple myeloma	2.71 (2.13–3.44)	76	6	0.78
Testis	1.35 (1.01–1.79)	216	7	0.16227

In summary, immune suppression from HIV disease appears to increase the risk for AIDS-defining and non-AIDS-defining malignancies. The link between immunosuppression and AIDS-defining cancers appears to be more direct; however, chronic immunosuppression likely plays a role in the development of many non-AIDS-defining malignancies in HIV-infected individuals. The cancers which are associated with viral etiologies, especially Epstein–Barr virus and human papillomavirus, appear to show the strongest association between HIV-associated immunosuppression and cancer incidence. The rest of this chapter will describe

specifics of some of the most common non-AIDS-defining malignancies, including Hodgkin disease, anal, and lung cancers.

Hodgkin Disease

Hodgkin lymphoma (HL) is one of the most common non-AIDS-defining tumors in HIV-infected patients. The risk for developing HL ranged from 4 to 16 times that of the general population in several different prospective cohort and AIDS–cancer registry match studies (Frisch et al., 2001; Grulich et al., 2002; Allardice et al., 2003; Dal Maso et al., 2003a; Clifford et al., 2005; Patel et al., 2008).

HL among HIV-infected individuals appears to have a different etiology and epidemiology compared to HL among HIV-uninfected individuals. First, nearly all of AIDS-associated Hodgkin lymphomas are EBV positive, while less than 50% of HIV-uninfected cases are EBV positive (Uccini et al., 1989). Second, while the incidence of non-AIDS-associated HL has decreased over the past 30 years, the majority of recent studies show an increase in incidence of HL in HIV-infected individuals (Grulich et al., 2002) and a clear relationship between the incidence of disease and immunodeficiency. Glaser et al. (2003) reported that from 1988 to 1998 in the Greater San Francisco Bay area, incidence rates by race of HIV-associated HL were overall higher for whites (11%), blacks (22%), and Hispanics (14%) compared to those with non-HIV-associated HL. Further, data from AIDS cohort and registry matching studies showed a relative risk of HL in HIV that ranged from 2.5 to 8.5 (Franceschi et al., 1998; Spina et al., 1999a, b). Finally, in the HIV population, the HL histologic subtypes often seen are those associated with worse prognosis, including mixed cellularity and lymphocyte-depleted variant (Tirelli et al., 1995a; Glaser et al., 2003; Hoffmann et al., 2004). In a cooperative study in Spain, Rubio and colleagues reported that individuals with AIDS-associated HL showed the following distribution of histologic subtypes: mixed cellularity (41.3%), lymphoid depletion (21.7%), nodular sclerosis (21.7%), and lymphocytic predominance (4.3%) (Rubio, 1994). Biggar et al. (2006) also reported that the incidence of nodular sclerosing HL was higher among those people with AIDS (PWAs) with low CD4 counts and that HL risk significantly increased among those with intermediate CD4 counts (150–199 cells/μl) compared to those with low CD4 counts (<50 cells/μl). Thus, because the incidence of HL is significantly higher among those with higher CD4 cell counts, mixed cellularity and other histologic subtypes of HL appear to occur more frequently among PWAs.

Clinical Presentation of HL

In addition to its distinct epidemiology, HL occurring in the HIV population exhibits clinical features that are distinct from HL in the general population. HIV-infected individuals are more likely to present with advanced-stage disease, extranodal involvement, and systemic symptoms (B symptoms), including fever, night sweats,

and/or weight loss (Vaccher et al., 2001; Glaser et al., 2003; Spina et al., 2003; Doweiko et al., 2004). In the pre-HAART era, approximately 75% of patients were shown to have advanced-stage disease. Bone marrow involvement occurred in 40–50% of individuals and was the first sign of HL in 20% of cases (Ree et al., 1991; Errante et al., 1994; Rubio, 1994; Tirelli et al., 1995a, b).

Treatment of HL

Optimal regimen for HL in HIV-infected individuals has not yet been defined. Antineoplastic treatment of AIDS-associated HL presents challenges because the underlying immunodeficiency caused by HIV may be further compromised by additional chemotherapy. Further, CD4+ counts in these individuals may decrease significantly during treatment, increasing the risk of opportunistic infections (OI) (Berretta et al., 2003).

Prior to HAART, HIV-infected individuals with HL had a median survival of only 8–26 months (Berretta et al., 2003). Gerard et al. (2003) in a retrospective study over 15 years estimated that the 2-year survival probability was 45% in the pre-HAART period and 62% in the post-HAART period. HIV-infected individuals who received and responded to HAART within 2 years of their HL diagnosis were shown to have overall survival of 89% at 24 months (median survival was not reached), whereas median survival time in patients without a response to HAART was only 18.6 months (Hoffmann et al., 2004).

Chemotherapy and HAART

Although several prospective trials have now been performed using antiretroviral drugs together with different chemotherapy regimens, the standard regimen for HIV-uninfected individuals, doxorubicin (Adriamycin), bleomycin, vinblastine, and dexamethasone (ABVD), remains the most commonly used. Several other regimens have been tested, however. The combination of bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisone (BEACOPP) was tested in 12 patients of whom 5 received concurrent HAART. Complete remission (CR) was achieved in all patients. Nine of the twelve patients remained in complete remission for their individual follow-up period, which lasted a median of 49 months. The most common observed toxicity was bone marrow suppression, with grade 3/4 leukopenia in 75% (Hartmann et al., 2003).

Another prospective, non-randomized trial evaluated epirubicin, bleomycin, vinblastine, and prednisone (EBVP) administered with HAART and G-CSF (granulocyte colony-stimulating factor) in 35 previously untreated patients. The median survival was 16 months, the survival rate was 32%, and the disease-free survival was 53% at 36 months. Toxicity was moderate, with grade 3/4 leukopenia observed in 32% of patients and thrombocytopenia in 10% of patients. Forty-eight percent of patients died of HL and 9% died of OI (Errante et al., 1999).

Spina et al. (2002) tested the Stanford V regimen (doxorubicin, vinblastine, mechlorethamine, etoposide, vincristine, bleomycin, and prednisone) and radiotherapy with HAART and G-CSF in a phase II prospective study in 59 HIV-infected individuals. Sixty-nine percent of patients completed treatment with no dose reduction or chemotherapy delays. Bone marrow toxicity and neurotoxicity were the most notable dose-limiting adverse effects. Complete remission was seen in 81% of patients, and 56% of patients were alive and disease-free at median follow-up of 17 months.

Finally, Berenguer et al. (2008) utilized data from the Spanish Group de Estudio del SIDA (GESIDA) clinical trials group to compare treatment and outcomes between HIV-infected HL patients who received HAART and those who did not. The median survival for the entire cohort of 104 patients was 110 months. The 5-year survival was 44% in the group that did not receive HAART and 72% in the HAART group. They found that the following factors were associated with complete remission: (1) appropriate-for-stage therapy: this group used ABVD or mechlorethamine, vincristine, procarbazine, prednisone (MOPP) followed by Adriamycin, bleomycin, vinblastine (ABV); (2) HAART utilization; and (3) CD4 count $\geq$100.

Anal Cancer

Although squamous cell cancer of the anus (SCCA) is a relatively rare cancer in the United States, the rate in the United States is increasing. A recent US population-based analysis of Surveillance, Epidemiology and End Results (SEER) program data found that the incidence of SCCA in the United States among men increased from 1.06 per 100,000 from 1973 to 1979 to 2.04 per 100,000 from 1996 to 2004 (Johnson et al., 2004). Other recent studies have shown a steady increase in the incidence of SCCA during the past three decades (Daling et al., 1987; Melbye et al., 1994), with significant increases among never married men in the San Francisco Bay Area (Melbye et al., 1994; Cress and Holly, 2003). These data likely reflect the significant increased risk of anal cancer among men who have sex with men (MSM) and HIV-infected MSMs.

Among MSM, the incidence of anal cancer has been estimated to be 35 cases per 100,000, comparable to the incidence of cervical cancer in women prior to the introduction of routine cervical Papanicolaou (Pap) screening (Daling et al., 1987; Melbye et al., 1994; Frisch et al., 2000, 2001, 2003). The incidence of SCCA among HIV-infected populations reported from prospective cohort studies ranged from 3.9 to 92 per 100,000 and the relative risks reported ranged from 33.4 to 222 (Grulich et al., 1999; Bower et al., 2004; Clifford et al., 2005).

Clinical Features of Anal Cancer

Several studies have shown that HIV-infected patients with SCCA were younger than HIV-uninfected patients Chiao et al., (2008; Oehler-Janne et al., 2008). Using

national Veteran's Affairs data, our group found that HIV-infected individuals were more likely to be younger (median age 49 for the HIV infected compared to 63 for the HIV uninfected), male, and of African-American race compared to the HIV-uninfected individuals. We did not find a difference in the stage of the disease at presentation; in fact, the HIV-infected patients were more likely to have been diagnosed with in situ disease (Chiao et al., 2008).

Anal Cancer Treatment and Outcomes

Combined chemoradiotherapy is the primary treatment of choice, with surgical treatment reserved for relapsed disease. The 5-year survival in the general population is 70–80% (Northover, 1991; Clark et al., 2004). Only eight small case series (ranging from 4 to 26 patients) describe outcomes of HIV-associated SCCA, with 5-year survivals ranging from 47 to 60% (Doci et al., 1992; Pitcher et al., 1994; Arnott et al., 1996; Flam et al., 1996; John et al., 1996; Gerard et al., 1998; Myerson et al., 2001; Jephcott et al., 2004). The majority of cases were diagnosed at either stage 1 or stage 2 (localized tumor without lymph node invasion) and received combined chemoradiotherapy. Most series reported some toxicity associated with therapy, with one reporting up to 50% of patients unable to complete planned therapy (Bottomley et al., 1996; Hocht et al., 1997; Peddada et al., 1997; Hoffman et al., 1999; Cleator et al., 2000; Stadler et al., 2004; Blazy et al., 2005). In three studies that specifically compared survival among patients with SCCA in the pre-HAART vs. HAART eras, there was a non-significant trend toward improved survival, better tolerability of chemoradiotherapy, and improved local tumor control in the HAART era (Cleator et al., 2000; Bower et al., 2004; Stadler et al., 2004; Blazy et al., 2005). Several studies have shown that individuals with HIV infection and SCCA have similar overall survival and outcomes when compared to HIV-uninfected individuals (Chiao et al., 2008; Oehler-Janne et al., 2008). Oehler–Janne et al. (2008) found that although overall survivals of HIV-infected and HIV-uninfected patients were similar, the 5-year disease-free survival was significantly shorter (38% vs. 87%, respectively). They also found that HIV-infected patients had more acute toxicity associated with chemoradiotherapy compared to HIV-uninfected individuals, thus suggesting that although overall survival was similar in the two groups, a more tolerable chemoradiotherapy regimen will need to be explored for HIV-infected individuals (Oehler-Janne et al., 2008).

Screening for Anal Cancer

SCCA shares many biologic similarities with cervical cancer, including detectable dysplastic precursor lesions and high-risk HPV infection. Thus, the institution of annual anal Pap screening for HIV-infected patients has been recommended (Beckmann et al., 1989; Bosch et al., 1995). Anal Pap smears are obtained by randomly obtaining squamous cells from the anal canal using a Dacron swab. They are then fixed in liquid fixative medium. Similar to cervical cytology protocols,

abnormal anal cytologic findings are confirmed by high-resolution anoscopy (HRA)-directed biopsy of visualized lesions. The 2001 revised Bethesda system of cytologic classification includes a basic primer on anal cytology and uses the system of cervical cytologic classification for classifying anal cytology (Darragh et al., 2004). Anal Pap smears have a similar sensitivity and specificity to cervical Pap smears (Cocchi et al., 1997; Palefsky et al., 1997; Woodhouse et al., 1999; Stoler and Schiffman, 2001; New York State AIDS Malignancy Consortium, 2004). Although there are no definitive clinical studies showing that anal Pap smears decrease SCCA-related morbidity and mortality among HIV-infected individuals, a recent cost-effectiveness analysis found that the incremental cost-effectiveness ratio per quality-adjusted life year saved was $16,000, which is similar to other widely accepted screening procedures (Goldie et al., 1999). Although anal Pap smears are not currently standard of care at this time, individual practitioners are offering anal Pap smear screening and follow-up HRA, but currently there is no consensus regarding the clinical utility of anal Pap smear screening (Chiao et al., 2006).

Effect of HAART on Anal Dysplasia

Like studies evaluating the effect of HAART on cervical dysplasia, studies evaluating the effect of HAART on anal dysplasia report conflicting results, likely related to the significant design and methodological differences between these studies. Palefsky et al. (2001) compared the rates of progression and regression of anal dysplasia after 6 months of HAART. They found that the likelihood of lesion progression or regression was not affected by HAART initiation, but they noted that among patients starting HAART at higher CD4 counts, the use of HAART was associated with a non-significant benefit on anal dysplasia lesions. In a subsequent study, Palefsky et al. (2002) performed a cross-sectional analysis on the prevalence of AIN 3 among a cohort of 433 HIV-positive men. They found that men on HAART had an increased risk of 12.6 (95% CI, 2.4–64) for AIN 2 or 3 after adjustment for CD4 count. In contrast, Wilkin et al. (2004) conducted a cross-sectional study evaluating anal HPV infection and anal dysplasia in 98 HIV-positive men. In a multivariate analysis they found that HAART and higher nadir CD4 count were significantly protective for anal dysplasia by histology but were not protective for anal HPV infection. Therefore, it remains unclear if HAART initiation influences the natural history of AIN in HIV-infected individuals.

Treatment outcomes for anal dysplasia have been reported only for small case series. Current treatment options are similar for HIV-positive and HIV-negative individuals. These treatments include surgical ablation, infrared coagulation, imiquimod, and topical 5-FU (Chang et al., 2002; Kreuter et al., 2004; Goldstone et al., 2005; Graham et al., 2005).

Lung Cancer

The incidence of lung cancer in the United States in the year 2000 was greater than 70 per 100,000, and approximately 160,000 new cases were diagnosed in 2000.

Lung cancer is the leading cause of cancer deaths in the United States among both men and women (American Cancer Society, 2000). Because of the increased risk associated with smoking and because individuals with HIV disease have higher smoking rates (Parker and Leveno, 1998), the incidence of lung cancer appears to be higher among HIV-infected individuals. Although Serraino et al. (2000) and Grulich et al. (1999) did not find an increased risk of lung cancer in two cohorts of HIV-infected patients from Italy and Australia, and Cooksley et al. (1999) did not find an increased risk of lung cancer in a linked AIDS–cancer registry linked study from Harris County, Texas (Cooksley et al., 1999), Frisch et al. (2001), and Gallgher et al. (2001) found an increased SIR/RR when analyzing AIDS–cancer linked registry data. Engels et al. (2001) also conducted an age-stratified analysis of the AIDS–cancer registry match and found that patients under age 50, particularly patients in the 30–49-year age group, had a significantly increased incidence of lung cancer. Also, Parker and Leveno (1998), in a retrospective analysis of the Texas Department of Health cancer and HIV registries, showed a 6.5-fold increase in the incidence of lung cancer in HIV-infected people compared to the general US population. Finally, Goedert et al. (1998) utilized the AIDS–Cancer Match Registry data to evaluate the risk of cancer incidence based on the level of immunosuppression as estimated by the time of cancer diagnosis before (or after) the onset of AIDS. They found that although lung cancer incidence did not increase with increasing immunosuppression, the risk of lung adenocarcinoma was 2.5 (1.0–5.1) in the time period 4–27 months after the diagnosis of AIDS.

Diminished tumor immune surveillance as a result of chronic HIV-related immunosuppression has been postulated as another variable accounting for the increased risk of lung cancer in this population (Vaccher et al., 2001; Engels, 2001). Wistuba et al. (1998) found microsatellite instability in a significantly higher proportion of lung tumor specimens from HIV-infected patients as compared to non-HIV-infected controls and hypothesized that immunosuppressive states may result in genomic instability, which, in turn, may play a role in tumor development. Other factors that have been linked to increased lung cancer risk in HIV-infected patients include opportunistic lung infections (Tirelli et al., 2000), intravenous drug use, and the increasing age of HIV patients in the HAART era (Engels, 2001; Herida et al., 2003).

The Effect of HAART on the Incidence of Lung Cancer

The majority of studies in the post-HAART era show an increased risk of lung cancer, and the consensus is that the incidence of the disease is stable or may be increasing since the introduction of HAART (Bower et al., 2003; Dal Maso et al., 2003b; Herida et al., 2003). In their analysis of a prospective HIV hospital cohort, Herida et al. (2003) found an increased SIR for lung cancer in the HAART period (1996–1999), but not in the pre-HAART period (1992–1995). Bower et al. (2003) analyzed a prospectively acquired database of 8,400 HIV-infected patients between

1986 and 2001. They found 11 lung cancer cases. The incidence of lung cancer increased from 0.8 per 100,000 (0.2–3.2) in the pre-HAART era to 6.7 per 100,000 (3.1–13.9) in the post-HAART era. In the post-HAART era, the relative risk of lung cancer in the HIV-infected population was comparable to that of the general population in southeast England, which was 8.93 (4.92–19.98) (Bower et al., 2003). Dal Maso et al. (2003b), using data from the Italian Cancer and AIDS Registries Study, found that HIV-infected individuals were at higher risk for lung cancer, but the risk did not change in the post-HAART era. The overall SIR was 7.4 (4.6–11.3) in the pre-HAART period and 7.9 (2.1–20.4) in the post-HAART period. A total of 21 lung cancers were identified from 1985 to 1998 (Dal Maso et al., 2003b).

Contribution of Smoking Tobacco to Risk of HIV-Related Lung Cancer

The increased risk of lung cancer seen in large epidemiologic studies has frequently been attributed to increased smoking rates in HIV-positive individuals. Serraino et al. (2000) found that the risk of lung cancer was not significantly increased in a mixed population of male HIV-positive intravenous drug users and non-intravenous drug users but was significantly elevated in a cohort of HIV-negative male intravenous drug users. Additionally, Dal Maso et al. (2003b) reported that the risk of lung cancer was significantly higher in intravenous drug users with HIV compared to other HIV exposure categories. They found that the SIR of lung cancer was 23.9 (11.9–43.0) among intravenous drug users with HIV and was 4.2 (2.0–7.7) for other HIV exposure categories (Dal Maso et al., 2003b). Both Serraino and Dal Maso suggested that intravenous drug users, regardless of HIV status, were at higher risk of lung cancer because of increased tobacco use (Serraino et al., 2000; Dal Maso et al., 2003b). Although smoking information was not specifically collected, Gallagher et al. reported that men whose HIV risk factors were either homosexual contact or intravenous drug use were all at higher risk for lung cancer. In addition, they found that women in all HIV exposure groups were at higher risk for lung cancer (Gallagher et al., 2001).

However, HIV infection may increase the risk of lung cancer independent of tobacco use. Patel et al. (2004) found an increased risk of lung cancer in a prospective cohort of HIV-infected individuals despite controlling for smoking behavior. Furthermore, a recent study by Engels et al. (2006) used an urban HIV cohort from Baltimore, Maryland, to evaluate the risk for lung cancer, specifically controlled for smoking behavior. They found 33 cancers in 5,238 patients, for an incidence of 170 per 100,000 person-years and an SIR of 4.7 (95% CI, 3.2–6.5). When they subsequently adjusted the SIR for smoking rates in the HIV-infected population and a similar urban population (Detroit, Michigan), the SIR decreased to 2.5 (95% CI, 1.6–3.5) but remained significantly elevated. This suggests that HIV infection increases the risk for lung cancer above and beyond other behavioral risks.

Clinical Characteristics of HIV-Positive Patients with Lung Cancer

As is the case for many other cancers, lung cancer in HIV-infected individuals presents differently than in HIV-uninfected individuals. Although the mean age of lung cancer patients in the general population is 68 years Kosary et al., (1995), the mean age at diagnosis in the HIV population ranged from 38 to 49 years Tirelli et al., (2000). In the majority of published series, the most common histology was adenocarcinoma. Adenocarcinoma was diagnosed in between 25 and 60% of cases, while 0–20% showed small cell histology. The authors of these series reported between 30 and 60 pack year median tobacco history. Between 13 and 57% of HIV-positive patients had a history of TB or PCP. A large proportion of cases presented with advanced-stage, unresectable disease (67–100%), and 0–26% were treated with surgery.

Patients in the pre-HAART and HAART eras appear to have similar presentations of disease. Bower et al. (2003) found that the only major difference was the median length of time from HIV diagnosis to lung cancer diagnosis, which was 2 months in the pre-HAART era and 10 months in the HAART era. Another recent HAART era study by Powles et al. (2003a) compared the presentation and outcomes of nine HIV-positive non-small cell lung cancer patients with 27 HIV-negative age- and stage-matched controls. Seven of the nine HIV-positive patients were on HAART at the start of treatment. The median age was similar in the HIV-infected patients and the HIV-uninfected controls (45 and 48 years, respectively). In this study, a similar percentage presented with adenocarcinoma, and a similar percentage presented with stage IV disease (66% of HIV-positive patients and 70% of HIV-uninfected controls). The HIV-uninfected patients had a better performance status than did the HIV-positive patients, with 52% of HIV-negative patients and 22% of the HIV-positive patients presenting with ECOG performance status less than 2.

Treatment and Outcomes of Lung Cancer in the HAART Era

Powles et al. (2003a) found that a similar percentage of the HIV-infected and uninfected lung cancer patients received chemotherapy (66% of HIV-negative patients vs. 88% of the HIV-positive patients). Overall survival was the same for HIV-positive and HIV-negative patients at 4 months ($p = 0.55$). Cancer-related death was similar in the HIV-negative patients and the HIV-positive patients (74% vs. 77%) (Powles et al., 2003a). The findings of Powles et al. (2003a) contrast with previous reports that show inferior survival for HIV-positive patients compared to HIV-negative patients (Vyzula, 1996; Tirelli et al., 2000; Sridar et al., 1992). The earlier studies hypothesized that lung cancer was a more aggressive disease in HIV-positive patients or that fewer HIV-positive than HIV-negative patients were candidates for curative surgical resection because of poor lung function as a result of multiple previous opportunistic infection. Poor performance status may have also contributed to decreased surgical resection or chemotherapy treatment.

The study by Powles et al. (2003b) was not able to address the issue of surgical resection because all the patients in the study presented with advanced-stage disease. However, they hypothesized that the poor outcomes in both the HIV-positive cases and HIV-negative controls may reflect advanced and aggressive disease behavior in young people who present with lung cancer as whole and that lung cancer does not appear to be more aggressive in HIV-positive individuals. They report that advanced non-small cell lung cancer in HIV-infected individuals treated with HAART is still associated with a poor outcome, but the outcomes are similar to HIV-negative controls matched for age and stage (Powles et al., 2003b).

It is unclear if HAART utilization improves outcomes in HIV-infected patients with lung cancer. Although there are no data that directly compare the survival between the pre-HAART era and the HAART era, pooled data from the pre-HAART era indicate that median survival was approximately 2 months (Sridar et al., 1992; Vyzula, 1996; Remick, 1996; Alshafie et al., 1997). Powles et al. (2003b) reported that the survival in the HAART era was 4 months. In addition, Hakimian et al. (2007) reported that median survival in a cohort of 30 HIV-infected individuals was approximately 5.2 months, but the majority of these patients (27/30) were diagnosed with advanced (stage 3B, 4) disease. They hypothesized that HAART may decrease HIV-related mortality and increase ability to tolerate treatment but that ultimately poor performance status and late diagnoses of cancer in HIV patients have led to poor outcomes (Hakimian et al., 2007; Powles et al., 2003b).

In the HAART era, HIV infection per se should not be a contraindication for surgical intervention or palliative therapy. The available data suggest that HIV-positive patients with lung cancer should be treated in a similar fashion to HIV-negative patients based on stage and performance status (Powles et al., 2003a). In addition, palliative chemotherapy and radiotherapy should be considered for possible improvement in quality of life. Hematopoietic growth factors may be necessary to support HIV-positive patients during treatment. Prophylaxis against opportunistic infection during chemotherapy and radiation therapy and avoidance of anemia-inducing antiretroviral medications such as zidovudine should be standard adjunctive treatment measures.

Because lung cancer has such a poor prognosis for HIV-infected individuals and the incidence appears to be stable or on the rise in the HAART era, it is important to evaluate prevention, early detection, and treatment initiatives for these patients. Because surgical resection of early stage disease is the only therapy that has been associated with long-term survival, a low threshold for early diagnostic interventions, including CT scans, to increase early diagnosis of lung cancer in patients can be recommended (White et al., 1995). Finally, smoking is also a significant risk factor for other chronic diseases besides lung cancer for which HIV-positive patients are at risk, including coronary artery disease (Friis-Moller et al., 2003), emphysema (Diaz et al., 2000), and head and neck cancers (Powles et al., 2004). A systematic approach to smoking cessation programs for HIV-positive individuals may be of great benefit in this population.

Conclusions

Antiretroviral therapy has transformed the disease trajectory of AIDS over the last 20 years. Since the widespread use of HAART over the past 10 years, AIDS is no longer an almost inevitably fatal disease and in many individuals behaves as a chronic condition (Louie et al., 2002). However, HIV-infected individuals in the HAART era develop more long-term problems, many of them being associated with chronic low-grade immunosuppression. HAART has decreased the incidence of AIDS-defining malignancies, including Kaposi sarcoma, non-Hodgkin lymphoma, and primary CNS lymphomas Eltom et al., (2002), but the influence of HAART on non-AIDS-defining malignancies is less clear. HAART was introduced less than 15 years ago, and therefore studies that attempt to compare cancer risks in the pre-HAART and post-HAART eras do not include long-term post-HAART follow-up (Herida et al., 2003; Seaberg and Kingsley, 2002; McGinnis et al., 2002). Because chronic immunosuppression may contribute to an increased risk for non-AIDS-defining malignancies in HIV-infected individuals, it will be important to closely monitor this population for emerging epidemiologic trends. The meta-analysis conducted by Grulich et al. comparing cancer incidence in HIV-infected individuals and solid organ transplant recipients underscores the similarity between these two groups and suggests that cancer screening is an important issue for both populations.

In addition, other HAART- or HIV-related adverse effects may also impact on cancer risk factors. Metabolic abnormalities associated with HAART, such as fat redistribution from the periphery to the abdomen and breast, elevated body mass indices, undesirable serum lipid levels, and elevated waist–hip ratios have been associated with breast cancer risk and survival (Agurs-Collins et al., 1998; Schreier et al., 1999; Goodwin et al., 1997). In addition, adjunctive therapies such as testosterone replacement may also be associated with increased risk for tumor growth. Finally, HAART itself has been associated with an increased incidence of cancer (Olivero et al., 2005). Thus, Clinicians need to emphasize the role of smoking cessation, patient education, and age-appropriate cancer screening recommendations in the HIV-infected population. Further studies evaluating the epidemiology, natural history, and optimal treatments for malignancies in HIV-infected individuals are needed to guide this evolving field.

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HIV-Related Cancer Management in Resource-Limited Settings: A Case Study of Malawi

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Abstract The convergence of the AIDS epidemic, HIV-related malignancies and infections, and extreme poverty creates a potentially insurmountable challenge to the overburdened health-care systems of resource-limited countries to provide care to their populations. Yet, given this reality, countries such as Malawi must meet these challenges. In this chapter, we review Malawi's current approach to the management of HIV-related malignancies, address strategies for continued improvement, and highlight research progress that has been made in the area of Kaposi's sarcoma management and goals for the future.

Overview of Malawi Health-Care System

Malawi, a small landlocked country in sub-Saharan Africa (SSA), is among the poorest countries in the world (Bank, 2009). With a population just under 14 million people, Malawi is currently experiencing an annual population growth rate of 2.8% (Population and Housing Census Preliminary Report, 2008). The majority of the population remains in rural areas, although the urban proportion has increased in recent years (Population and Housing Census Preliminary Report, 2008). The southern region of the country is most populous, followed by the central region and then the northern region.

The Malawi government currently spends approximately $15 on health per capita. While a small private health sector exists in Malawi, the public health sector in Malawi, directed by the Ministry of Health (MOH), serves the vast majority of the population. The primary structure of health system consists of central hospitals: Mzuzu Central Hospital (MCH) serves the northern region, Kamuzu Central Hospital (KCH) in Lilongwe serves the central region, and Zomba Central Hospital (ZCH) in Zomba and Queen Elizabeth Central Hospital (QECH) in Blantyre serve

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the southern region. Each of Malawi's 29 districts has at least one district hospital. Within each district, numerous health centers are distributed to provide the most proximal clinic settings for the population. In addition, the Christian Health Association of Malawi (CHAM) provides district-level and health center-level services as a complement to government services. Although public health services are free at central-level hospitals, an option for a private-pay service also exists. In general, patients seek services at the primary health centers and are referred to district or central services as appropriate. Staffs from central hospital departments conduct district visits periodically to assess the need for central-level services. Additionally, government physicians at the central level may apply for external referrals to South Africa for patients with conditions not amenable to locally available resources. Available services vary across health facilities, even at the central hospitals. For example, Kamuzu Central Hospital in Lilongwe is the only hospital in Malawi to provide hemodialysis services. Radiology services such as CT or MRI scanning are available only in Blantyre, which may be up to 12 h by car for those in the most northern districts of Malawi. The highest concentration of specialist physicians is in Blantyre at the Queen Elizabeth Central Hospital, largely due to the association with the Malawi College of Medicine.

HIV in Malawi

Malawi is among the countries with the highest prevalence rates of HIV infection. As of the end of 2007, 11.9% of adults, close to 900,000 individuals, were infected with HIV in Malawi (UNAIDS, 2008). However, in response to the HIV epidemic, the Malawi Ministry of Health launched a massive antiretroviral scale-up program in 2004. As of March 2009, this program, supported by the Global Fund, has used the public health approach to provide antiretroviral treatment to over 150,000 individuals (Malawi Ministry of Health, 2009). There are now 159 ART clinics nationally. Malawi has now reached 25–50% coverage of antiretroviral therapy for those in need (UNAIDS, 2008).

As opposed to many programs in SSA, the Malawi ART program is driven by clinical staging and clinical assessment and does not rely on any laboratory monitoring other than an HIV test. CD4 lymphocyte and HIV RNA testing are limited to only a selected number of sites. Likewise, testing for other potential complications of ART is limited to central locations. This has allowed the Malawi ART program to expand despite the relative lack of health-related infrastructure. In addition to antiretroviral therapy, all ART clinics are expected to have treatment for HIV/AIDS opportunistic complications, including KS. However, while no clinics experienced interruptions in supplies of antiretroviral drugs, a recent survey of ART clinics showed fewer than 50% had a full stock of medications for opportunistic infections, including chemotherapy for cancers such as Kaposi's sarcoma (Malawi Ministry of Health, 2009).

Cancer in Malawi

Malawi's approach to cancer consists of those recommended by the World Health Organization: surveillance, primary prevention and early detection, treatment, and palliative care. However, due to limited resources, each of these components remains underdeveloped.

Surveillance

Since 1989, Malawi has maintained a national cancer registry (Banda et al., 2001). While initially limited to diagnostic material received at the pathology laboratory at Queen Elizabeth Central Hospital (QECH), in 1993 the registry expanded to include population-based assessments for the southern region surrounding the commercial center in Blantyre. For population-based estimates, the cancer registry team collected information on cancer-related diagnoses from all clinics at QECH and seven other hospitals within the Blantyre district. Thus, the registry currently employs pathology-based information for the country and population-based information for the Blantyre district to estimate cancer prevalence and incidence.

In the first report from this registry (1994–1998) (Banda et al., 2001), the overall age-standardized incidence rate (ASR) of cancer in the Blantyre district was 92 per 100,000 for males and 88.8 per 100,000 for females. Compared to other countries in the region (Wabinga et al., 2000; Chokunonga et al., 2000), these incidence rates are markedly lower and may suggest underdetection of cancer cases, given limited diagnostic resources.

HIV-Related Malignancies in Malawi

In both men and women, the most common malignancy overall in Malawi is Kaposi's sarcoma (KS) with an ASR of 39.9 per 100,000 for men and 17.1 per 100,000 for women. KS accounted for 54% of all cancer cases in men and 27% in women (Banda et al., 2001). These KS rates are remarkably similar to those in other SSA countries (Wabinga et al., 2000; Chokunonga et al., 2000) and likely reflect that most KS diagnoses are made clinically and do not rely on histopathology. Notably, these rates are markedly higher than those reported in Malawi prior to the AIDS epidemic in 1976–1980 based on histopathological surveillance (Hutt, 1986). Additionally, KS has also become more frequent among children (Sinfield et al., 2007). Since the advent of ART, introduced widely in 2004 (Harries et al., 2006), there have been no published studies on the incidence of KS in Malawi but recent reports from the registry have not yet shown a decrease.

At KCH, KS is currently a common presenting cancer among the HIV-infected population. In 4 months at the Lighthouse ART clinic, we identified 35 ART-naïve,

chemotherapy-naïve patients presenting with KS. Most patients (60%) presented with ACTG tumor stage T1 (advanced) disease.

As the antiretroviral therapy rollout continues, it is unknown if the incidence of KS will decrease as it has in western countries after the introduction of ART (International Collaboration on HIV and Cancer, 2000). Human herpesvirus 8 (HHV-8, Kaposi's sarcoma herpesvirus) is a primary factor in the development of Kaposi's sarcoma (Schulz, 1999; Dittmer and Damania, 2007). The prevalence of HHV-8 antibodies in the general population in SSA is much higher than that in western settings (Ablashi et al., 1999). While seroprevalence data from Malawi are lacking, the population is likely to show similar infection rates to other countries in the region. Endemic KS existed prior to the AIDS epidemic and relative risks for the development of KS among HIV-infected persons are lower compared to western settings (Parkin et al., 2003), possibly suggesting other pathogenic factors in addition to HHV-8 in the evolution of KS in the African setting.

Squamous cell carcinoma (SCC) of the conjunctiva has also emerged as an HIV-associated malignancy in Malawi (Banda et al., 2001; Waddell et al., 1996), similar to other countries in SSA (Newton et al., 2002; Chokunonga et al., 2000). The age-specific incidence rates for squamous cell carcinoma are similar to those for KS (Banda et al., 2001) and KCH estimates that five new cases are identified monthly in the eye department (Joseph Msosa, personal communication). However, as SCC is not considered a WHO stage-defining condition, routine data on its incidence are not being collected within the ART program.

The age-standardized incidence of non-Hodgkin's lymphoma (NHL) based on the Blantyre cancer registry is 4.3 per 100,000 for men and 3.3 per 100,000 for women. NHL is a WHO stage 4-defining condition (World Health Organization, 2006), but its frequency is not well documented within the ART program and to date, there is no systematic evaluation of NHL among HIV-infected clients in Malawi. Regionally, data are also limited on the incidence of NHL in both HIV-infected and HIV-uninfected populations. Given limited diagnostic resources and the far greater incidence of tuberculosis as an etiology for lymphadenopathy within the resource-limited setting, NHL is undoubtedly frequently misdiagnosed as tuberculosis lymphadenopathy leading to underestimates of its incidence.

Cervical cancer is the second most common malignancy among women in Malawi, with an ASR of 26.2 per 100,000 (Banda et al., 2001). Compared with regional countries (Wabinga et al., 2000; Chokunonga et al., 2000), this rate is markedly lower. However, given the overall absence of cervical cancer screening activities within Malawi, many cases likely go undiagnosed. Even so, cervical cancer constitutes 83.4% of all female genital cancers (Malawi Ministry of Health, 2007). Regionally in countries with high HIV prevalence, evidence suggests an increased incidence of cervical cancer (Chokunonga et al., 2004; Wabinga et al., 2000; Wabinga et al., 1993), although some of the increase preceded the peak of the AIDS epidemic. Higher expression of human papillomavirus (HPV) DNA has been found among HIV-positive women with CD4 counts less than 300 cells compared to HIV-negative women with corresponding higher frequencies of squamous intraepithelial lesions (Motti et al., 1996), although rates of invasive cervical cancer

have shown less consistent associations with HIV (Parkin et al., 2003). Since the scale-up of the Malawi ART program, there has been no systematic evaluation of the frequency of cervical dysplasia or cervical cancer among HIV-infected women.

Prevention and Early Detection

Currently, with the exception of hepatitis B vaccination in infancy, there is no program for the primary prevention of malignancies. While discussion of the human papillomavirus vaccine is underway, the implementation of this vaccine program is likely many years away (Malawi Ministry of Health, 2007).

There is no systematic use of screening/early identification strategies at the national level. The most developed screening procedure to date is visual inspection of the cervix under acetic acid (VIA) which is advocated for cervical screening in Malawi due to its low cost, availability, ease of use, and ability to diagnose and intervene simultaneously (Malawi Ministry of Health, 2007). However, this low-cost intervention is available at only 40 facilities nationally. At KCH, one of the largest providers, nearly 1,200 women accessed this service in the last 18 months (Gift Kamanga, personal communication).

Treatment and Management

Challenges in Diagnosis: Case Reports

The confirmed approach: A 37-year-old HIV-positive female nurse on ART with good response for 3 years presents with new onset of supraclavicular and axillary lymphadenopathy. Additionally, she has noticed a 10 kg weight loss, night sweats, and generalized fatigue. Chest X-ray does not show evidence of tuberculosis and a fine needle aspirate of the node is unrevealing. The patient is referred to the surgical department for lymph node biopsy. However, on her scheduled biopsy date, a road traffic accident and its resulting emergency cases require rescheduling of the procedure. Again, for her next date, the procedure is delayed because of another road traffic accident. Three weeks after her first assessment, the biopsy is performed. The sample is sent to Blantyre, where after another 3 weeks, the diagnosis of NHL is confirmed and CHOP chemotherapy is commenced through her employer-paid program.

The empiric approach: A 28-year-old HIV-positive women presents with marked cervical lymphadenopathy. Based on the lymphadenopathy, the patient is started on tuberculosis treatment. However, after 8 weeks on treatment, the lymphadenopathy has markedly increased and the patient has deteriorated markedly. The patient is admitted to the hospital with a diagnosis of presumed lymphoma and a biopsy is obtained. While awaiting the results of the biopsy, chemotherapy with CHOP is commenced. Unfortunately, the patient expires 3 days after the first cycle of chemotherapy. Biopsy subsequently confirms the diagnosis of lymphoma.

Among the greatest challenges to the management of malignancies in settings such as Malawi is the overall lack of resources for each element of diagnosis, from human resources to technological resources. One of the first obstacles to cancer treatment is obtaining a biopsy. There is an overall human resource shortage. In Malawi, there are an estimated 0.61 nurses/doctors per 100,000 population (UNAIDS, 2008), among the lowest in the world, and this ratio is even lower for specialist trained physicians. Excisional biopsies generally fall to the surgical department of most facilities as elective procedures. However, given the limited number of surgical theatres and staffing surgeons and nurses, elective procedures are often delayed, sometimes by weeks. In a setting where the average biopsy report may take up to 6 months to get a final report, clinicians have at times adopted an empiric approach to management of malignancies based on clinical presentation.

In addition to biopsy difficulties, many standard diagnostic procedures are not available, even in the central hospitals with "tertiary" capability. Currently at KCH, there is no reliable capability to do bronchoscopy and endoscopy has only recently become available through the surgical department. Previously, patients requiring endoscopy attended a CHAM facility approximately 60 km from Lilongwe for this procedure or were referred to Blantyre. The lack of capacity is based on both lack of operational equipment and the maintenance of staff trained in these procedures. Likewise, colposcopy, an essential diagnostic evaluation for cervical cancer, is limited to a research facility on the KCH campus. While trained MOH personnel are permitted to use the colposcope, the availability is such that only a handful of special cases can be evaluated. Similar challenges exist in Blantyre as well although both bronchoscopy and endoscopy services are available in the medical department.

Radiology services are similarly inadequate for complete malignancy evaluation and staging disease. Basic radiography is available at central- and district-level hospitals but not health centers. Central-level and some district-level hospitals may have access to ultrasonography but the accuracy of the result is largely related to the expertise of the interpreting technician. Currently, in the whole of Malawi, there are three CT scanners, all located in Blantyre. Two CT scanners are at private facilities and the costs prohibit the procedure for all but the wealthiest Malawians and expatriates. While the other facility is at the government facility, maintenance issues often render it unavailable for use. From the logistics perspective, movement of patients from the far ends of Malawi to access this service is challenging even when available. Only stable clients can travel the long distances and there must be an operational vehicle to transport the patient. Additionally, human resources again come into play as there may be no trained radiologist to interpret the scans. Currently, there is only one trained radiologist in Malawi. As of July 2008, MRI became available in Malawi, again in Blantyre, largely through a collaboration with Michigan State University and its research projects with the College of Medicine in Malawi and the Queen Elizabeth Central Hospital. While it is expected that many of the challenges that are faced with respect to logistics will remain, there is hope that this collaboration will lead to an improvement in the management of malignancies through improved diagnostic capability.

In western settings, the use of laboratory tumor markers can often help establish a diagnosis of a malignancy. Currently, no government clinic conducts any tumor marker laboratory testing. Two private laboratories perform tumor marker testing including prostate specific antigen, CA 125, CEA, and α-fetoprotein. Similarly, other laboratory testing that might be useful for assessing prognosis is not available at most facilities.

Pathology services for the government program in Malawi are centralized to a single laboratory at the College of Medicine in Blantyre and are currently staffed by two pathologists with responsibilities for diagnostic pathology services, post-mortem evaluations, cancer registry activities, and teaching within the college. As the only histopathology laboratory in the public sector in Malawi, there is a high volume of specimens. Approximately 1,200 samples are received per year for evaluation. Even this represents just a small proportion of the need, as only 39% of registered malignancies in the Blantyre district had histological confirmation (Banda et al., 2001). Given the high volume of diagnostic specimens and the unmet need for pathology services, the waiting time for pathologic interpretation may be months after the biopsy is obtained. In this setting, it has been impossible to begin implementation of routine pathology-based screening procedures for malignant diseases such as Pap smears for the early detection of cervical cancer. However, if finances permit, the use of private pathology services in the country can decrease the biopsy result waiting time to less than 1 week. Additionally, in the past, some biopsy samples have been sent out of country for evaluation, but with the development of services in-country, this has occurred less often but remains a potential avenue to fill the unmet need in the country.

Treatment

Optimal cancer management often requires a multi-disciplinary approach including pathologists, surgeons, radiation therapists, and medical oncologists, and often includes tumor board discussions to individualize patient management. This approach fails when one or more of the components are not available, as in resource-limited settings.

For over half of malignancies in low- to middle-resource settings, primary or adjunctive radiation therapy is commonly utilized as a component of management (Barton et al., 2006). Currently there are no radiotherapy services in Malawi. Any patient requiring this service needs referral to neighboring countries such as Zambia, Zimbabwe, Tanzania, or South Africa. Local physicians must submit a request to an external referral committee that reviews each case and determines if government resources can be allocated to sending the patient abroad. Chemoradiotherapy is the most common indication for external referral, yet most requests are not authorized (Dan Namarika, personal communication), leaving less effective methods for cancer management.

The range of chemotherapeutic drugs in Malawi is limited and clinicians are often faced with non-sustainable supplies. Most clinicians and nurses in Malawi are

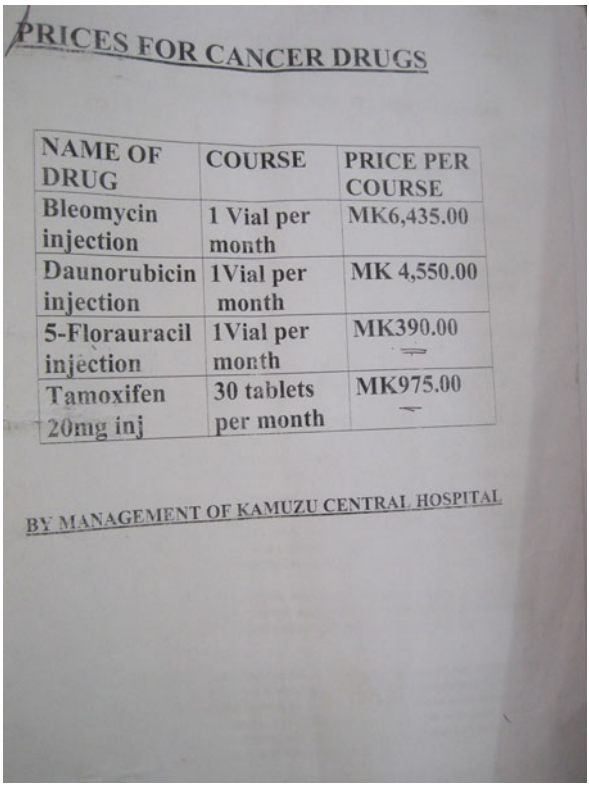
unfamiliar with administration of these medications and their use is largely confined to the central hospital (Photo 1). Currently, there is no medical oncologist at KCH or QECH and chemotherapy is managed by internal medicine specialists or pediatricians. The most widely available chemotherapeutic agents are methotrexate, vincristine, bleomycin, cyclophosphamide, 5-fluorauracil, and doxorubicin, but they are frequently out of stock. In some situations, patients with external sources of income may access additional chemotherapeutic agents from South Africa for administration in Malawi (Photo 2). However, local staff may be unfamiliar with the administration of these agents or lack equipment for continuous infusion drugs.

Photo 1 At Kamuzu Central Hospital, patient records for chemotherapy are recorded in log files and each patient has an individual patient card outlining each cycle. One of the chemotherapy nurses prepares for the day's patient in the current chemotherapy day ward

Chemotherapy is well known to induce cytopenias as a side effect. In 2004, the Malawi Blood Transfusion Service was established with a goal of providing a safe and regular blood supply for Malawians. Previously, blood transfusion was limited to directed donations by family members. Currently, reliable supplies of whole blood are available for management of anemia, although platelet transfusions remain poorly available. There are no colony-stimulating factors available for prevention or management of neutropenia. Rather, doses of chemotherapy are often reduced with subsequent cycles, potentially compromising efficacy.

With cytotoxic chemotherapy, the potential for neutropenic sepsis increases. Malawi maintains a relatively limited number of antibiotics for the treatment of infections, and the emergence of resistant bacteria remains a particular concern for neutropenic cancer patients, particularly in the setting of inadequate infection control practices.

Laboratory monitoring is often necessary in patients receiving chemotherapy. Often, however, even basic tests such as complete blood counts, liver function tests, creatinine, and electrolytes are not available, even in the central hospital setting. Recently, however, the government hospitals have partnered with research


PRICES FOR CANCER DRUGS

NAME OF DRUG	COURSE	PRICE PER COURSE
Bleomycin injection	1 Vial per month	MK6,435.00
Daunorubicin injection	1Vial per month	MK 4,550.00
5-Florauracil injection	1Vial per month	MK390.00
Tamoxifen 20mg inj	30 tablets per month	MK975.00

BY MANAGEMENT OF KAMUZU CENTRAL HOSPITAL

Photo 2 The price of various chemotherapeutic agents is posted in the chemotherapy ward at the Kamuzu Central Hospital. While modestly priced compared with other settings, costs are largely prohibitive for many Malawians

laboratories to ensure more reliable access to safety monitoring for complex patients.

Given the multiple challenges to treatment of cancers in Malawi, the most common external referral requests are for the management of cancer (Dan Namarika, personal communication). However, given the high volume of malignancies, particularly HIV-related KS, most such requests are denied on the basis of cost.

Palliative Care

Palliative care is relatively well developed in Malawi. The Palliative Care Association of Malawi has developed draft guidelines for the appropriate use of analgesics for pain management. Palliative care is a core component of the guidelines for the management of HIV-related illnesses (Malawi Ministry of Health, 2008). Many home-based care programs provide palliative care services, including morphine analgesia. However, morphine usage rates suggest that as a country,

Malawi is likely underutilizing this agent. Unfortunately, due to late detection of many cancers and the previously described barriers to diagnosis and treatment, palliative care is often the primary management for malignancies in Malawi.

Treatment of Common HIV-Related Malignancies in Malawi

The most common malignancy in Malawi, KS, is treated with a combination of ART and vincristine monotherapy. The standard protocol for KS treatment in adults is vincristine 1.4 mg/m^2 (or 2 mg) intravenously weekly for six cycles, followed by the same dose at 2 week intervals for 3 months, and thereafter monthly injections for 6 months (Malawi Ministry of Health, 2008) (Photo 3). More recently, combination therapy with vincristine, doxorubicin, and bleomycin has been used on a small scale at KCH in patients refractory to vincristine or in those with more disseminated KS. Additional sites have been piloting bleomycin and vincristine combination therapy. While not validated by clinical trials in the Malawian setting, vincristine monotherapy is widely available and reports from clinicians and patients suggest fair palliation of symptoms in many cases. To date, comparisons of responses to combination treatment and vincristine monotherapy have not been made.

NHL, while not among the most common malignancies in Malawi, is a WHO stage 4 condition. NHL is commonly treated with standard CHOP chemotherapy (cyclophosphamide 750 mg/m^2, vincristine 1.4 mg/m^2, doxorubicin 50 mg/m^2, and prednisone 100 mg/day). Typically, patients receive 6–8 cycles depending on response. However, given that limited evaluation for the extent of disease is possible, the ability to assess response is challenging. The survival for such patients has remained poor, with many patients succumbing to serious infections during their chemotherapy course.

Photo 3 The Lighthouse pharmacist retrieves a standard dose of vincristine for patient use. Vincristine is the most common chemotherapy available for the treatment of Kaposi's sarcoma. On a daily basis, approximately 15 patients receive vincristine at the Lighthouse clinic

Strategies for Improving HIV-Related Malignancy Care

Surveillance

Currently, the Blantyre cancer registry surveys the Blantyre district and it is critical that this well-established registry continues. However, expanded surveillance to the central region may allow richer evaluation of the incidence of cancer in the country, particularly given the regional variations in HIV prevalence and ART coverage (Malawi Ministry of Health, 2009). Moreover, the current registry has been limited by the relatively small percentage of suspected cases that have been confirmed histologically. Through better estimates of disease burden, projected treatment needs can be estimated and used to formulate national cancer policy.

The Community Health Science Unit (CHSU), a component of the MOH, has traditionally served as a reference laboratory for Malawi, although historically most of its activities have focused on tuberculosis. Currently, the histopathology laboratory is being refurbished and plans are underway to begin pathology services at this location in order to improve diagnostic capability.

Concurrently, the ART program can enhance its routine monitoring and evaluation to include active surveillance for HIV-associated malignancies, including Kaposi's sarcoma, lymphoma and cervical cancer, as well as squamous cell carcinoma of the conjunctiva at a minimum at sentinel sites.

Prevention

Up to 17.8% of malignancies worldwide (26.3% in resource-limited settings) are attributable to infectious causes and could be largely preventable if appropriate steps were implemented (Parkin, 2006). In the resource-limited setting, one of the most obvious preventive interventions is to use vaccines, including the hepatitis B vaccine at birth to prevent hepatitis B infection and the subsequent risk of hepatocellular cancer. More relevant to Malawi would be the ability to prevent cervical cancer-related deaths through HPV vaccination of adolescent women. Until that happens, expansion of the VIA method of screening for cervical cancers and treating pre-invasive lesions may prove highly cost effective in reducing mortality (Goldie et al., 2005). More recent evidence suggests that direct HPV DNA testing may be more effective in reducing cervical cancer mortality and could be implemented without marked expansion of pathology services (Sankaranarayanan et al., 2009). Integration of cervical cancer screening, not only in family planning clinics but also within the ART clinics, in Malawi will reach women at potentially highest risk for the development of cervical cancer and to better characterize the role that HIV plays in the development of cervical cancer, particularly in the setting of ART. The continued rollout of the ART program in Malawi will also likely lead to a reduction in HIV-associated malignancies, and more effective prevention of HIV infection would lead to even greater reductions.

Treatment

Currently, cancer management is largely administered at the central hospitals in Malawi. Over the years, Malawi has discussed the possibility of developing a single cancer centre of excellence equipped with radiotherapy, a broad range of chemotherapeutic agents, diagnostic capability including pathology, and specialists in pathology, surgical, medical, and radiation oncology. In addition to consolidating care in a single location, tracking cancer registrations and treatment outcomes would be simplified. Maintenance of adequate supplies of chemotherapeutic drugs could be centralized with better estimates of need. Training programs for nurses, clinical officers, and medical officers could be developed to increase overall knowledge of cancer prevention and treatment. The centre of excellence model can accommodate visiting professors, while local medical officers pursue post-graduate education. Additionally, research programs could be more easily developed and implemented within such a structured setting, which would lead to evidenced-based treatment. This central model is currently successfully used in the ART program where centers of excellence provide specialized care in addition to performing operations research and providing training and supervision to external sites.

Even now, these ART centers of excellence in Malawi could easily support evaluation of HIV-positive patient cohorts to determine the incidence of malignancies over time and the effect of ART on disease progression. Malignancy treatment protocols for common conditions such as KS can be prioritized as key areas for operations research within the ART program. Such strategies could markedly improve our understanding of HIV-related malignancies and improve clinical management.

The establishment of radiotherapy must be among the first priorities for development of cancer treatment in Malawi. The International Agency for Atomic Energy (IAEA) has been involved in the expansion of radiotherapy services in developing countries and most countries surrounding Malawi have this service available. Malawi seems poised to add radiotherapy in the near future, given that two physicians are currently training in radiation oncology in South Africa. However, the training of technicians needs to closely follow for successful implementation.

Training

Underscoring many of the challenges in the delivery of a comprehensive cancer package is the overall shortage of qualified health-care personnel. One of the immediate interventions to address this physician gap has been expansion of the United Nations Volunteer (UNV) Program, which brings expatriate physicians to Malawi to deliver health care. Similarly, many developed-country government aid agencies partner with the Malawi Ministry of Health to bring specialist physicians to Malawi. However, the long-term strategy to address human resource shortages depends on the expansion of training opportunities for Malawian nationals. In recent years, the Malawi College of Medicine has expanded its medical school class size from 20 to 60 students per year. Medical school departments are developing in-country

post-graduate programs to expand specialist-level care. The medical department has developed a post-graduate program that includes 2 years in Blantyre and 2 years in South Africa to train internal medicine specialists. Through collaboration with the Norwegian government, plans have been approved by the College of Surgeons of East, Central, and Southern Africa to develop a surgical residency program at the KCH. Meanwhile, both governmental and non-governmental programs have realized the need to provide post-graduate training for promising medical officers and increased numbers of Malawian physicians are being supported for training programs in South Africa. To specifically support cancer efforts, Malawian doctors are training in radiology, laboratory hematology, and pathology.

Partnerships

Through strategic partnerships, improvements in cancer management can be achieved. In many instances, the molecular basis for malignancies remains poorly understood. Yet, for example, in the case of Kaposi's sarcoma, the population with this condition in western countries where much basic and translational research is being undertaken is small compared with the SSA setting. Linking basic and translational researchers with the clinical population in need can facilitate research funding to support cancer research programs in developing settings.

For example, the ophthalmology department at KCH has linked with the Princess Alexandra Eye Pavilion in Edinburgh Scotland to study factors associated with the development of squamous cell carcinoma of the conjunctiva and determine the feasibility of using topical mitomycin C and cryotherapy as adjunctive therapy in Malawi. The University of North Carolina Project in Lilongwe Malawi (UNC Project) has partnered with the KCH since the early 1990s to conduct research on sexually transmitted diseases, HIV prevention and treatment, and prevention of mother–to-child transmission. Likewise, the Johns Hopkins Project in Blantyre partners with the QECH and the Malawi College of Medicine. As clinical trial sites for several National Institutes of Health-sponsored networks (AIDS Clinical Trials Group (ACTG), 2009), HIV Prevention Trials Network (HIV Prevention Trials Network, 2009), IMPAACT (International Maternal Pediatric Adolescent AIDS Clinical Trials Group, 2009), Microbicide Trials Network (Microbicides Trials Network, 2009), and Center for HIV Vaccine Immunology (Center for HIV/AIDS Vaccine Immunology, 2009), these projects maintain extensive research infrastructure and are capable of supporting world-class research. Using such infrastructure, Malawi can be poised to take a lead in cancer-related research.

Research

As the most common malignancy in Malawi, KS is logical as a target for development of a research agenda. Next, we highlight research conducted on KS in Malawi to date and outline future research plans.

The characteristics of KS in Malawi have been studied largely due to its strong association with HIV and its designation as an indication for initiating ART within the national program. Early surveys from the antiretroviral fee-paying period (2001–2003) indicated that approximately 11% of all HIV-positive patients presenting to the Lighthouse HIV specialty clinic in Lilongwe had KS as the indication for antiretroviral therapy (Hosseinipour et al., 2006). KS patients were at increased risk for loss to follow-up in this analysis, potentially reflecting increased mortality and/or drop-out from the program due to financial constraints due to payment for vincristine, ART, and increased transport costs due to the increased frequency of visits (Hosseinipour et al., 2006). During this time period, the Lighthouse was one of the few locations in Malawi with vincristine chemotherapy and the high proportion of KS patients may reflect referral bias.

During the fee-paying period of the ART program, a retrospective review of all patients attending the Lighthouse clinic with a diagnosis of KS was conducted (Hosseinipour et al., 2004). The choice of treatment at this time was largely dictated by patient's ability to pay for treatments ($30/month for ART, $10 per vincristine injection) rather than by clinical assessment. The study team evaluated 208 patients with Kaposi's sarcoma, of whom 63% were male. The mean age was 35.7 (SD 9.3) and the median CD4 count was 53 cells/mm^3 (IQR 15–135). Thirty-five percentage of patients received ART only, 32% vincristine only, 27% vincristine and ART, and 6% received neither. Of those patients treated with vincristine, 77% experienced at least short-term partial regression of KS lesions or palliation of symptoms. Overall retention was poor and the median duration of treatment was only 4.2 months. Loss to follow-up was 64% over the 2-year period and documented mortality was 8.6%, although the true mortality was likely much higher. The use of ART with or without vincristine was associated with increased retention in care compared to only using vincristine (Fig. 1).

Again, in the same period, the Lighthouse ART clinic conducted a prospective cohort study of 179 patients initiating ART from January to July 2003 (Hosseinipour

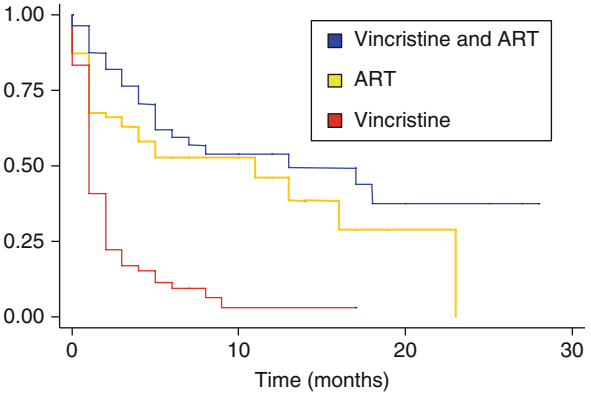


Fig. 1 Kaplan Meier estimates of duration of time retained in care among Kaposi's sarcoma patients receiving care at the Lighthouse clinic from 2001 to 2003 according to the treatment received. Categories of treatment include vincristine only, antiretroviral therapy only, and both vincristine and antiretroviral therapy

Table 1 Characteristics of patients initiating ART at the Lighthouse clinic in Malawi during the pay period and comparison of characteristics according to vital status at 12 months

Baseline characteristics	Overall, $n = 179$	Deaths, $n = 59$ (33%)	Alive, $n = 120$ (67%)	p-value
Age (median)	37	39	36	0.12
Gender (percentage of female)	56.4	47.5	60.8	0.09
Positive history of TB (percentage of yes)	34.6	39.0	32.5	0.39
Kaposi's sarcoma (percentage of yes)	8.9	20.3	3.3	<0.001
History of thrush (percentage of yes)	43.0	54.3	35.0	0.002
History of shingles (percentage of yes)	32.4	23.7	36.7	0.082
WHO stage (%) 1	11.2	1.7	15.8	<0.001
2	11.7	1.7	16.7	
3	62.6	69.5	59.2	
4	14.5	27.1	8.3	
Mean body mass index	21.2 ($n = 158$)	19.9 ($n = 47$)	21.8 ($n = 104$)	<0.005
Median CD4	70	36	108	<0.001
Mean hemoglobin ($n = 176$)	10.4	9.1	11.0	<0.001

et al., 2004). Mortality was high in this cohort (33%) and KS was among the factors strongly associated with death by 1 year (Table 1). Of the 59 deaths, KS was the second most common confirmed cause of death, after tuberculosis. Among KS patients, only 50% survived for 1 year.

More recently, from July to September 2005 during the rollout of free ART, KS was the indication for treatment for 6% of patients in the national ART program in Malawi (Makombe et al., 2008). Among the 7,905 patients initiating ART in Malawi during this time, 488 patients had KS as the indication for ART initiation. Compared to non-KS patients, KS patients had an increased risk of mortality or being lost to follow-up. Similar to the earlier fee-paying period, only 53% of the KS patients were alive and on treatment at 1 year despite access to free ART (Makombe et al., 2008). However, the use of vincristine chemotherapy was unknown for this group due to limitations of the standard ART reporting. Regardless, it is clear that KS disease is among the most important causes of mortality within the Malawi ART program.

In addition, KS progression among survivors poses significant challenges in the evaluation of ART failure. Malawi and WHO ART guidelines (Malawi Ministry of Health, 2006; World Health Organization, 2006) define clinical ART failure as a new or progressive WHO stage 4 condition. Among 203 patients with suspected ART failure at the Lighthouse clinic and the QECH ART clinic between January 2006 and July 2007, 33 (16%) patients had either new ($n = 3$) or progressive KS disease (30 cases) (van Oosterhout et al., 2009; Hosseinipour et al., 2007). All patients

with progressive disease had received regular vincristine chemotherapy during their ART treatment course. The majority of these patients were virologically suppressed (HIVRNA < 400 copies/ml), 77% with progressive KS and 33% with new KS, indicating that KS disease progression may occur despite ART and chemotherapy.

Recent reviews of data from the Lighthouse HIV clinic cohort demonstrate a relatively stable rate of KS presentation as an indicator condition (Hannock Tweya, personal communication). The continued high prevalence of KS among the ART population and its high rates of associated mortality and disease progression underscore the importance of continued research to evaluate risk factors for the development and progression of KS and optimal chemotherapy.

The Lighthouse ART clinic maintains an electronic clinical database of all patients on ART since the rollout of free ART. Data collected include demographics, WHO staging, CD4 count trends, ART initiation dates, and outcome status. As a first step, similar to the evaluation by the HIV unit in the national program, vital outcomes of KS patients should be compared to non-KS patients. However, given the additional information available at the Lighthouse on chemotherapy dosing and immunologic responses, one can also evaluate factors associated with survival including the use of chemotherapy and immunological trends. Given the recent pilot at KCH using combination chemotherapy, it may be possible to compare outcomes of this new regimen to the historical experience at the Lighthouse. However, for optimal evaluation of KS responses, standardized baseline assessment and follow-up procedures need to be better developed.

In 2008, through the National Institutes of Health (NIH) Fogarty International Center ATRIP and the National Cancer Institute-supported AIDS Malignancy Consortium (AMC), KCH and the University of North Carolina partnered to develop a research protocol to determine the prevalence of KS lesions with high-level expression of the Kaposi's sarcoma-associated herpesvirus thymidine kinase (*orf24*) and phosphorylase (*orf36*) genes. With the development of this protocol, the site was able to develop pathology capability in Lilongwe and develop the capacity to measure KSHV viral load. Clinicians working in the HIV clinics received training on appropriate clinical staging of KS and how to perform biopsies.

With these developments, KCH is now poised to conduct KS-related trials with the ACTG and the AMC. Two studies are currently in development and should be ready for enrollment in early 2010. ACTG 5263/AMC 066 will be a randomized controlled trial comparing three different chemotherapeutic regimens (pegylated liposomal doxorubicin monotherapy, etoposide monotherapy, or the bleomycin and vincristine combination) for the treatment of advanced-stage KS in resource-limited settings. ACTG 5264/AMC 067 will compare immediate adjunctive etoposide and ART to ART alone among limited-stage KS patients in resource-limited settings. Both research studies provide access to additional treatment for patients affected by KS and will answer critical management questions for KS patients.

Additionally, the NIH Fogarty International Center has supported personnel from both Malawi and the United States to develop cancer-related projects including expansion of the existing cancer registry.

Summary

The AIDS epidemic in Malawi has resulted in increased rates of HIV-related malignancies and magnified the shortcomings in cancer management in general. While many challenges exist to address both the AIDS and cancer epidemics, expansion of existing activities and partnership with international research programs may allow substantial gains toward the understanding of the association of HIV and malignancy and improve clinical care.

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Immunology of HIV-Associated Kaposi Sarcoma

Ashok Cattamanchi and Corey Casper

Abstract An epidemic of Kaposi's sarcoma (KS) among severely immunocompromised men heralded the beginning of the HIV pandemic in 1981, and since that time considerable efforts have gone into understanding the role of the human immune system in the control of KS tumorigenesis. Infection with human herpesvirus-8 (HHV-8), the etiologic agent of KS, is necessary but not sufficient for the development of the tumor. In this chapter, we review what is known about innate and adaptive cellular immunity to HHV-8 and KS, and consider host, viral and environmental factors which may predispose to the development of KS from chronic HHV-8 infection. Finally, we review the epidemiology of KS in the era of highly active antiretroviral therapy (HAART), where both a dramatic decrease in the incidence of KS but also a newly recognized immune reconstitution inflammatory syndrome (IRIS) has been recognized. In this context, we consider the impact of HAART on the clinical manifestations and natural history of KS.

Introduction

The hypothesis that the immune system prevents the development of cancer through the recognition of tumor cells as foreign was first formally proposed by Burnet and Thomas more than 50 years ago (Burnet, 1970). A large body of evidence in mouse models and humans now overwhelmingly supports the existence of a cancer immunosurveillance system (Dunn et al., 2004). HIV infection subverts this system through a progressive depletion of CD4 T cells, leading to dysfunction in innate, cellular, and humoral immunity. The result is an increased incidence of a broad range of cancers, but particularly with Kaposi sarcoma (KS), which remains the

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most common AIDS-associated cancer in the HAART era (Eltom et al., 2002). In this chapter, we will explore the relationship between immunity and cancer, with a focus on how HIV-related immunodeficiency and immune reconstitution impact KS.

Immunity and Cancer

Murine Models

Advances in immunology and transgenic technology have allowed rigorous testing of the cancer immunosurveillance hypothesis in mouse models. An early seminal study used mice in which the recombinase-activating gene (*RAG*2) was knocked out (Shankaran et al., 2001). This led to a deficiency in T, B, and NKT cells due to an inability to rearrange lymphocyte antigen receptors. RAG2 knockout mice treated with the carcinogen methylcholanthrene (MCA) developed significantly more sarcomas than did wild-type controls, demonstrating that adaptive immunity can control tumor development. Similar experiments with gene knockout mice lacking IFN-γ, perforin (lymphocyte-dependent killing mediator), IL-12, αβ T cells, and γδ T cells have all resulted in increased tumor incidence (Dunn et al., 2004).

Additional studies of murine models have shown that the innate immune system also controls tumor development. Mice with deficiencies in the quantity (Smyth et al., 2001) or quality (Smyth et al., 2000) of NK cells were several-fold more likely to develop MCA-induced tumors than were controls. These and other studies indicate that both innate and adaptive immunities are major determinants of cancer development.

Evidence for the Role of Immunity in Cancer Surveillance in Humans

Considerable evidence in humans also supports the presence of a cancer immunosurveillance system. Perhaps the most convincing is that cancer occurs with increased frequency among immunosuppressed populations. Several studies have reported increased cancer incidence in solid organ transplant (SOT) recipients who are receiving immunosuppression to prevent graft rejection (Birkeland et al., 1995; Pham et al., 1995; Penn, 1996; Grulich et al., 2007; Roithmaier et al., 2007). Among them is a study of 907 heart and/or lung transplant recipients followed from 1989 to 2004. A 7.1-fold increased incidence of cancer compared to the non-transplant population was observed. The most common malignancies documented were lymphoproliferative disorders (26.2-fold increase), head and neck cancers (21.0-fold increase), and lung cancer (9.3-fold increase) (Roithmaier et al., 2007).

Increased cancer incidence also occurs among HIV-infected persons. In fact, the observation of an aggressive form of Kaposi sarcoma (KS) affecting eight young homosexual men in New York in 1981 heralded the beginning of the AIDS

epidemic (Hymes et al., 1981). KS and non-Hodgkin's lymphoma (NHL) were the first cancers to be classified as AIDS defining with cervical cancer added to the list shortly thereafter (1992). All three are caused by viruses: human herpesvirus-8 (HHV-8), Epstein–Barr virus (EBV), and human papillomavirus (HPV), respectively. Now increasing evidence suggests that HIV-related immunodeficiency leads to a much broader range of cancers, including those without any known viral association. Among 54,780 HIV-infected patients followed from 1992 to 2003, several non-AIDS-defining cancers occurred with increased frequency compared to the general population including anal (SRR 42.9, 95% CI 34.1–53.3), vaginal (SRR 21.0, 95% CI 11.2–35.9), Hodgkin lymphoma (SSR 14.7, 95% CI 11.6–18.2), liver (SSR 7.7, 95% CI 5.7–10.1), lung (SSR 3.3, 95% CI 2.8–3.9), melanoma (SSR 2.6, 95% CI 1.9–3.6), oropharyngeal (SSR 2.6, 95% CI 1.9–3.4), leukemia (SSR 2.5, 95% CI 1.6–3.8), colorectal (SSR 2.3, 95% CI 1.8–2.9), and renal (SSR 1.8, 95% CI 1.1–2.7) (Patel et al., 2008).

The increase in immunosuppression-related cancer risk is likely a result of a complex interplay between various arms of the immune system as well as host, viral, and environmental factors. SOT- and HIV-related immunosuppression clearly does not confer the same cancer risks. A meta-analysis comparing cancer incidence among seven studies of persons with HIV/AIDS ($n = 444{,}172$) and five of SOT recipients ($n = 31{,}977$) reported a dramatically higher SIR for KS and NHL among HIV-infected persons (3,640.0 and 76.67, respectively) compared to SOT recipients (208.0 and 8.07, respectively) (Grulich et al., 2007).

Even among the AIDS-defining malignancies, the relationship to immunosuppression is not completely clear. Large differences in SIRs between KS (3,640.0), NHL (76.67), and cervical cancer (5.82) highlight that other factors are involved (Grulich et al., 2007). Both KS and NHL risks increase with decreasing CD4 count, supporting a strong role for immunosuppression in pathogenesis, but cervical cancer risk shows no correlation with CD4 count (Mbulaiteye et al., 2003; Engels et al., 2008). Further complicating the picture is the observation that KS occurs among HIV-infected persons stable on HAART well after immune reconstitution (Maurer et al., 2007). KS also occurs among immunocompetent persons in parts of Africa and the Mediterranean, and more recently has been reported in Western countries among HIV-negative homosexual and bisexual men without any other cellular or humoral immunodeficiency (Kestens et al., 1985; Dukers and Rezza, 2003; Lanternier et al., 2008).

Kaposi Sarcoma

Impact of HIV-Related Immunodeficiency on the Development of KS

Since the beginning of the HIV pandemic in 1981, the incidence of KS worldwide has increased exponentially. In the United States, KS incidence increased nearly 16-fold from 1981 to 1987 (from 0.3 per 100,000 between 1973 and 1981 to 4.7

per 100,000 during its peak in 1987–1991) and today remains twice that of the pre-HIV era (Eltom et al., 2002). In areas where KS was endemic prior to the HIV pandemic, KS incidence increased several hundred fold since 1981 and has yet to decline despite an increasing number of persons starting antiretroviral therapy (Wabinga et al., 2000). In fact, KS has emerged as the most common overall cancer in many sub-Saharan African countries since the start of the HIV epidemic (Sitas et al., 1997; Chokunonga et al., 1999; Sitas et al., 2000; Wabinga et al., 2000).

Several factors contribute to the risk of KS development among HIV-infected persons. Among these, HIV-related immunosuppression is clearly a major factor, with the risk of KS development substantially increased with more severe immunosuppression. A study of 57,350 HIV-infected persons which linked US HIV and cancer registries between 1991 and 2002 showed the impact of immunosuppression on the incidence of KS (Engels et al., 2008). HIV-infected persons who had progressed to AIDS had a markedly elevated SIR of KS (4,800 vs. 800). Impairment of cellular, humoral, and innate immunity against HHV-8 all contributes to this increased risk. Evidence of a role for cellular immunity is suggested by the strong association of KS development with CD4 cell count, with a 28 and 36% increased risk for each decline in CD4 cell count of 50–100 cells/μL, respectively, in two large studies of adults with AIDS (Mbulaiteye et al., 2003; Biggar et al., 2007). Cytotoxic T-lymphocyte (CTL) responses against HHV-8 have also been found to be impaired or absent in persons with AIDS (Bourboulia et al., 2004). HHV-8 likely contributes to CTL evasion through production of lytic-cycle proteins that have been shown in vitro to downregulate MHC class I expression (Ishido et al., 2000).

HIV-infected persons with KS also have qualitatively deficient humoral responses against HHV-8. Despite previous observations showing that persons with KS have high titers of antibodies to HHV-8 latent and lytic antigens (Camera Pierrotti et al., 2000), one study found reduced levels of neutralizing antibodies to HHV-8 in persons with KS when compared to those with asymptomatic HHV-8 infection. Kimball et al. developed an assay for the quantification of HHV-8 neutralizing antibodies using a recombinant HHV-8 which expressed green fluorescent protein (GFP) (Kimball et al., 2004). By determining the minimal serum dilution at which a 50% inhibition of GFP-producing virus in 293 cells was noted, neutralizing antibody titers were measured in persons with HHV-8 infection with and without HIV and KS. KS was independently associated with lower neutralizing antibody titers after adjusting for CD4 count.

The mechanism by which KS is associated with reduced titers of neutralizing antibody is not clear. However, B cells, which are the source of antibody production, may play a key role in KS pathogenesis. In a case series of 21 patients with Castleman disease, a lymphoproliferative disorder caused by HHV-8, the use of the B-cell-ablating monoclonal antibody against CD20 (Rituximab) was associated with a worsening of KS among those with preexisting disease (Bower et al., 2007).

A role for innate immunity in controlling HHV-8 infection is suggested by a study which demonstrated efficient NK-cell-mediated lysis of HHV-8 latently infected

cells in persons without HIV infection or any other immunodeficiency (Sirianni et al., 2002). NK-cell cytotoxicity was shown to be significantly reduced in AIDS patients with progressive KS compared to HIV-negative patients with indolent classic KS or random blood donors. The mechanisms by which HIV and/or HHV-8 may impair NK cells have yet to be elucidated.

Other Factors Affecting KS Risk

Despite the substantial epidemiologic and basic science evidence for the importance of cellular, humoral and innate immunity in the prevention of KS development, it should be noted that plausible evidence supports additional factors which may lead to KS tumorigenesis. In sub-Saharan Africa, endemic KS is well described in children and elderly men without HIV infection or other immune disorders (Kestens et al., 1985). Similarly, classic KS has been well described among HIV-negative men of Mediterranean descent (Dukers and Rezza, 2003). KS has now also been reported in Western countries among HIV-negative homosexual and bisexual men without any other cellular or humoral immunodeficiency (Lanternier et al., 2008). Even in the setting of severe immunosuppression, only 50% of 223 HIV and HHV-8 co-infected men in San Francisco developed KS over 10 years of follow-up, and the risk of developing KS was independent of CD4 count (Martin et al., 1998). In this section, we will explore other factors which may contribute to KS development.

Host Factors

Host genetics is likely a major determinant of KS development. A single nucleotide polymorphism (SNP) in the interleukin-6 (IL-6) gene promoter leading to increased IL-6 production and a pro-inflammatory phenotype has been well described (Fishman et al., 1998). Among a small group of Americans with HIV-associated KS, Greeks who developed KS after renal transplantation, or immunocompetent Israelis with a familial cluster of classic KS, the pro-inflammatory IL-6 phenotype was significantly more common (Foster et al., 2000; Gazouli et al., 2004; Guttman-Yassky et al., 2004).

Other cytokine pathways have also been implicated in KS pathogenesis. Among persons with classic KS compared to controls, diplotypes in IL-8RB and IL-13 conferred decreased (OR 0.49) and increased (OR 1.88) risk, respectively (Brown et al., 2006a). A case report that documented the development of KS in a child with IFN-γ R1 deficiency suggests that the IFN–IL-12 axis may be important (Camcioglu et al., 2004). The influence of immunomodulatory gene SNPs on the immune response against HHV-8 was studied in 172 HHV-8-seropositive adults in Italy without KS (Brown et al., 2006b). High antibody titers to lytic HHV-8 antigens were more common in persons with the functional promoter variant of IL-6 (OR 3.7) and a

haplotype of IL-4 (2.8). High antibody titers to latent HHV-8 antigens were more common in persons with a haplotype in IL-12A.

Human leukocyte antigens (HLAs) have also been analyzed as KS risk factors. Over 100 Mediterranean patients with classic KS were compared to community controls in three studies, all of which found a strong positive association between HLA-DR5 and the presence of classic KS (Pollack et al., 1983; Contu et al., 1984; Kaloterakis et al., 1995). A study of 88 men with HIV-associated KS found that significantly fewer HIV-positive patients with KS were found to have the *HLA-B14* allele when compared with HIV-positive controls, while a meta-analysis of 12 studies comprising 287 HIV-associated KS cases found that persons with HIV-associated KS were more likely to be HLA-DR5 or B35 positive (Ioannidis et al., 1995; Marmor et al., 1995). These older studies are all limited by the failure to account for HHV-8 infection status of controls. A more recent study which assessed HHV-8 serostatus found that polymorphisms at HLA-DB1 were associated with HIV-associated KS (Gaya et al., 2004).

Other host factors associated with KS pathogenesis were explored in a study of persons with classic KS compared to HHV-8-seropositive controls (Brown et al., 2006c). Reduced hematocrit (<37.4%), hemoglobin (<12 g/dL), total lymphocytes (<1,000 cells/μL), CD4-positive T cells (<457 cells/μL), CD8-positive T cells (<213 cells/μL), and increased monocytes (≥638 cells/μL) were associated with an increased risk of classic KS.

Environmental Factors

A role for environmental factors as a cause for the increased KS incidence in sub-Saharan Africa has been proposed to explain the occurrence of classic and endemic KS in isolated geographical regions. A study of 45,000 Jewish Ethiopian immigrants to Israel with a 39% prevalence of HHV-8 infection and without HIV co-infection reported no cases of KS development despite high endemic KS rates in Ethiopia (Grossman et al., 2002). The authors of this study suggested that either protective host factors present in this ethnic group or the requirement of a local environmental cofactor could explain these findings. Other environmental factors which have been suggested to be associated with the risk of developing KS include exposure to soil with elevated silicates (Ziegler, 1993; Montesu et al., 1995; Montella et al., 1996; Simonart et al., 1999; Ziegler et al., 2003), river water (McHardy et al., 1984; Ziegler et al., 1997; Ascoli et al., 2001; Ziegler et al., 2003), animal exposure (Cottoni et al., 1997; Ziegler et al., 2003), malaria (Geddes et al., 1995; Cottoni et al., 1997), nitrites (Haverkos et al., 1985; Archibald et al., 1990; Lifson et al., 1990a, b; Archibald et al., 1992; Beral et al., 1992; Armenian et al., 1993), and tobacco (Goedert et al., 2002; Nawar et al. 2005; Hoover et al. 1993). However, definitive studies showing the role of environmental factors in KS pathogenesis are lacking.

Viral Factors

HHV-8

The distribution of HHV-8 may explain some of the differences in KS epidemiology. For example, among random blood donors, HHV-8 seroprevalence in Kampala, Uganda, is about 45%, whereas in the US general population it is about 1–7%, depending on what antibody assays and cutoffs are used (Hladik et al., 2003; Engels et al., 2007). Uganda also has among the highest incidence of endemic and HIV-associated KS in the world (Dukers and Rezza, 2003). However, the distribution of HHV-8 infection and KS does not always occur in parallel, with high HHV-8 but low KS rates in Alexandria, Egypt, areas of the old and new worlds, and in areas of China (Dukers and Rezza, 2003).

HHV-8 replication appears to be a key risk factor leading to KS development. In prospective clinical trials of intravenous and high-dose oral ganciclovir for the treatment of AIDS-associated cytomegalovirus disease, the rate of new KS development was reduced by 40 and 75%, respectively, among antiviral drug recipients (Mocroft et al., 1996; Martin et al., 1999). As ganciclovir is active against only replicating HHV-8, these studies provide indirect evidence for the role of replicating virus in KS pathogenesis. In addition, the detection of replicating HHV-8 in peripheral blood has been consistently reported as a risk factor for KS development (Whitby et al., 1995; Campbell et al., 2000; Broccolo et al., 2002; Lorenzen et al., 2002; Cannon et al., 2003; Engels et al., 2003b) and higher levels of viremia correlate with disease severity (Laney et al., 2007). HHV-8 produces a number of lytic-phase proteins that appear to be involved in immunoevasion, inflammation, and oncogenesis that may explain in part the importance of replication in KS pathogenesis (Ganem, 2006).

HHV-8 and HIV Synergy

Direct synergy between HIV and HHV-8 independent of immunosuppression has been reported at the molecular level through the HIV *tat* protein. *Tat* has been shown to promote growth of KS tumor cells and cause KS-like lesions in mice (Barillari et al., 1993; Fiorelli et al., 1995). Several studies have shown that *tat* can cause HHV-8 reactivation from latency to lytic replication (Harrington et al., 1997; Huang et al., 2001; Merat et al., 2002; Zheng et al., 2007). *Tat* may activate HHV-8 through modulation of human interleukin-6 (hIL-6) and hIL-6 receptor (Scala et al., 1994; Ambrosino et al., 1997; Zheng et al., 2007). *Tat* has also been shown to enhance HHV-8 entry into endothelial cells, which are the progenitors of KS tumor cells (Aoki and Tosato, 2004). Clinical data also support that failure to suppress HIV replication is a strong risk factor for KS development. A case–control study of 132 HIV-infected patients with 31 KS cases reported that a high HIV RNA level (HR 3.96 per log(10) increase) conferred increased KS risk (Engels et al., 2003a).

These findings support the epidemiologic observations outlined that the risk of developing KS is significantly higher among persons with HIV-associated immunosuppression when compared with organ transplantation and suggest mechanisms independent of immune suppression that may lead to KS development.

Highly Active Antiretroviral Therapy (HAART) and Immune Reconstitution

Quantitative and Qualitative HAART-Associated Immune Reconstitution

HAART has led to a dramatic reduction in mortality among HIV-infected persons (Mocroft et al., 2003; Porter et al., 2003; Bhaskaran et al., 2008). The most recent population-based study of 16,534 HIV-infected persons estimated a decrease in the mortality rate (per 1,000 person-years) from 40.8 prior to the introduction of HAART (pre-1996) to 6.1 for 2004–2006 (Bhaskaran et al., 2008). The hallmark of HIV infection is the steady depletion of CD4 T cells and increased risk of opportunistic infections (OI). HAART initiation leads to both a quantitative and a qualitative immune reconstitution, with increased CD4 T-cell counts and recovery of pathogen-specific antigen recognition.

The kinetics of the CD4 T-cell increase after HAART initiation has been studied using prospective cohorts (Kaufmann et al., 2003; Gras et al., 2007; Moore and Keruly, 2007). These studies report an increase in CD4 T cells by a median of 273–386 cells/μL after a median of 4–7 years of follow-up. The steepest increase occurred during the first 6–12 months and a slower but steady and significant increase over the next 4 years. This general pattern remained the same regardless of nadir CD4 T-cell count. One study reported that the slope of the CD4 T-cell count rise during the first 6 months was higher in patients with a pre-HAART HIV RNA measurement $\geq$4.5 $\log_{10}$ copies/mL compared with <4.5 $\log_{10}$ copies/mL, in women, and in patients with a pre-HAART CD8 count of <1,300 cells/mm^3 compared to >1,300 cells/mm^3 (Gras et al., 2007). In addition, persons with higher nadirs reached higher CD4 T-cell counts at the end of follow-up in all studies. For example, among a prospective cohort of 655 patients, by 6 years the median CD4 T-cell count was 493, 508, and 829 cells/μL among persons with a baseline of $\leq$200, 201–350, and >350 cells/μL, respectively (Moore and Keruly, 2007).

Reconstitution of pathogen-specific immune responses has been demonstrated to numerous agents after HAART initiation including mycobacteria, cytomegalovirus (CMV), hepatitis B, Epstein–Barr virus (EBV), and HHV-8. This reconstitution can occur rapidly, with significant increases in CD4 T-cell proliferation to both *Mycobacterium tuberculosis* and CMV reported within 1–6 months (Autran et al., 1997). One study reported a significant fourfold and an eightfold increase in antibodies to the CMV glycoprotein B 1 and 3 months, respectively, after HAART initiation (Deayton et al., 2002). CMV viremia was also found to decline rapidly, with a median half-life of 5.2 days (Deayton et al., 2002).

The type and kinetics of functional immune reconstitution may vary by pathogen. For example, EBV viremia was found to minimally change in 10 patients followed for 5 years after HAART initiation (Piriou et al., 2005). Another study reported that EBV viremia transiently increased in a subset of 9 out of 17 patients after HAART. All these persons had a rise in CD4 T-cell count and five had incomplete HIV viral load suppression (Righetti et al., 2002). Studies of cellular immune responses against HHV-8 are limited by the number of available epitopes but show delayed cellular immune reconstitution. The most robust study of cellular responses against HHV-8 involved using 309 synthetic overlapping peptides covering three latent and three lytic proteins. T-cell responses were noted to be significantly increased only after 11 months (Bihl et al., 2007). Another study using one latent and three lytic HHV-8 epitopes also found delayed cellular immune reconstitution, with responses absent before HAART and present in some patients after 6 months (Bourboulia et al., 2004).

HAART and KS

The steep decrease in KS incidence since the widespread introduction of HAART is perhaps one of the most dramatic stories of the HIV epidemic. Data from the Surveillance, Epidemiology and End Results (SEER) program showed a 90% decrease in KS incidence during the 1987–1994 period compared to 1998 in major metropolitan areas including San Francisco, Hawaii, Atlanta, and Seattle (Eltom et al., 2002). Another large study which linked HIV/AIDS and cancer registries in Colorado, Florida, and New Jersey reported a decrease in SIR from 2800 (2,300–3,500) in 1991–1995 to 790 (640–980) during the HAART era (1996–2002) (Engels et al., 2008). This decreased risk is not attributable to a change in the burden of HHV-8 infection. The prevalence of HHV-8-seropositive persons among cohorts of homosexual men remained essentially unchanged from 1978 to 1979 (26.5%), 1984 to 1985 (29.6%), and 1995 to 1996 (26.4%) (Osmond et al., 2002). In this section we will explore the various mechanisms by which HAART may impact KS incidence.

HAART-Associated Anti-HHV-8 Immune Reconstitution

Two studies suggest that HAART leads to a delayed and partial reconstitution of anti-HHV-8 cellular and humoral responses. The first study followed 27 HIV-infected patients, with ($n = 4$) and without KS ($n = 23$), for 2 years after HAART initiation (Bourboulia et al., 2004). HHV-8 cellular and plasma viremia significantly decreased after 12 months. Plasma viremia was undetectable only after 24 months. An increase in antilytic, but not antilatent, antibody was seen within 12 months. Cytotoxic T-lymphocyte (CTL) responses were undetectable prior to HAART and detectable after 6 months on HAART.

The second study randomized 33 HIV-infected patients with KS to either HAART alone or HAART plus systemic chemotherapy (Bihl et al., 2007).

Seventy-eight percent showed undetectable HIV viremia and a significant increase in CD4 cell counts over the first 5 months of HAART with or without chemotherapy. HHV-8-specific T-cell responses were significantly increased between baseline and 11 months but not baseline and 5 months. A decreased trend in HHV-8 cellular viremia was noted after 11 months on HAART, but this was not statistically significant.

NK-cell anti-HHV-8 activity also appears to be restored by HAART and may play an important role in controlling KS. Sirianni et al. showed that cells latently infected with HHV-8 were efficiently lysed by NK cells from persons with a normal immune system. NK-cell anti-HHV-8 activity was significantly reduced in AIDS patients with progressing KS compared to HIV-negative patents with indolent classic KS or normal blood donors. NK-cell-mediated lysis of HHV-8 latently infected cells was restored after HAART in HIV-associated KS patients that showed KS regression and clearance of HHV-8 from PBMCs, but not in those with no clinical response and persistent HHV-8 viremia (Sirianni et al., 2002).

HAART Effects on KS Independent of Anti-HHV-8 Immune Reconstitution

Restoration of anti-HHV-8 immunity is likely a major factor in the dramatically reduced incidence of KS in the HAART era. The delayed restoration described by current studies is at odds with the numerous reports of KS regression soon after HAART initiation (Lebbe et al., 1998; Cattelan et al., 1999; Dupin et al., 1999; Pellet et al., 2001; Gill et al., 2002; Paparizos et al., 2002; Bourboulia et al., 2004; Bihl et al., 2007). One explanation for this is that our current understanding of HHV-8 epitopes is incomplete (see Section "HAART-Associated anti-HHV-8 Immune Reconstitution"). Another explanation is that HAART may exert antitumor effects independent of its role in immune restoration. Supporting this is a study including 54,780 persons which linked cancer and HIV registries and found that HAART was independently associated with a decreased risk for Kaposi's sarcoma (RR 0.61, $p < 0.001$), NHL (RR 0.68, $p < 0.001$), cervical cancer (RR 0.48, $p = 0.019$), breast cancer (RR 0.35, $p = 0.013$), colorectal cancer (RR 0.50, $p = 0.027$), and lung cancer (RR 0.52, $p < 0.003$) (Patel et al., 2008).

The exact mechanisms through which HAART may influence KS progression independent of immune restoration are not clear. One possible mechanism is a direct effect of HAART on HHV-8 replication (Casper and Wald, 2007). For example, some of the nucleoside reverse transcriptase inhibitors (zidovudine and stavudine) may be phosphorylated by HHV-8 (Gustafson et al., 2000; Lock et al., 2002). Ongoing natural history studies of HHV-8 replication before and after HAART initiation will help to better answer this question.

Another mechanism may be through direct antitumor effects. Protease inhibitors (PIs) have been shown to have potent antiangiogenic and antitumor effects in a mouse model (Sgadari et al., 2002). Nude mice, genetic mutants which have greatly

reduced T cells, treated by intragastric lavage with the PIs indinavir or saquinavir and injected with KS cells had a 43 and 25% reduction, respectively, in KS lesion development compared to controls. A molecular mechanism for this was demonstrated through PI-mediated inhibition of basic fibroblast growth factor (bFGF)- and vascular endothelial growth factor (VEGF)-induced angiogenesis, endothelial and KS cell invasion, and matrix metalloproteinase-2 (MMP-2), a proteolytic enzyme which can degrade vessel basement membrane, activation (Sgadari et al., 2002). The PI ritonavir also inhibited tumor formation and progression in a KS mouse model. In vitro, ritonavir decreased the production of tumor necrosis factor alpha, IL-6, IL-8, and VEGF, all thought to be important in KS pathogenesis (Pati et al., 2002).

Clinical data regarding the optimal antiretroviral agents to use in persons with HIV and KS show discordant results. A case series of five patients reported relapse of KS after switching from a PI- to a non-nucleoside reverse transcriptase inhibitor (NNRTI)-based highly active antiretroviral therapy (HAART) (Bani-Sadr et al., 2003). The decision to switch was because of virologic failure in three cases and to decrease the pill burden in the other two cases. A prospective observational study of 8,640 HIV-positive patients in the United Kingdom showed no difference in the proportion of PIs and NNRTIs used among 1,204 incident KS cases (Portsmouth et al., 2003). An ongoing clinical trial of persons with epidemic KS randomized to PI- or NNRTI-based HAART in Kampala, Uganda (ClinicalTrials.gov identifier NCT00444379), will help to answer the question of the optimal antiretroviral therapy for persons with KS.

KS Development and Persistence Despite HAART

Progression from HHV-8 infection to KS clearly occurs despite optimal HAART. Maurer et al. described the development of unremitting cutaneous KS in nine patients taking HAART for a median duration of 7 years with sustained CD4 counts of more than 300 cells/mm^3 and suppression of HIV viral load below 300 copies/mL for at least 2 years (Maurer et al., 2007). The overall KS risk in the HAART era among HIV-infected persons is still extremely high, with SIR of 790 (640–980) during 1996–2002 (Engels et al., 2006). The overall risk of the other AIDS-defining illnesses in the HAART era is much lower, with SIR for NHL and cervical cancer of 6.5 and 2.9, respectively (Engels et al., 2006). These data suggest that KS will continue to be a major health problem among HIV-infected persons despite widespread HAART. The impact of HAART on KS incidence in parts of the world with a much higher burden of HHV-8 infection and a predisposition to KS development due to host and environmental factors remains to be seen.

Among persons with KS who are started on HAART with or without chemotherapy, only 44–60% achieve complete resolution of their KS (Blum et al., 1997; Dupin et al., 1999; Dupont et al., 2000; Bihl et al., 2007; Nguyen et al., 2008). The median time to initial improvement and complete response have been reported to be as long as 9 and 33 months, respectively, suggesting that KS response to treatment can be

slow among certain populations (Nguyen et al., 2008). These data suggest that in persons who have KS, current treatment strategies are inadequate. Furthermore, the effect of HAART and chemotherapy in areas with a high burden of KS is unknown.

Immune Reconstitution Inflammatory Syndrome (IRIS)

In 1997, a paradoxical worsening of CMV retinitis was described among patients recently initiating HAART (Jacobson et al., 1997). This syndrome of inflammation both locally and at the site of a previous opportunistic infection has come to be known as "immune reconstitution inflammatory syndrome" (IRIS). IRIS occurs despite improvement in HIV laboratory parameters after HAART initiation, including increased CD4 count and decreased HIV viral load. The pathogenesis of IRIS is still not completely clear but can be thought of as two processes. First, a host inflammatory response causes exacerbation of an ongoing disease process. Falling into this category are multiple case reports of clinical worsening after HAART initiation while on antituberculosis therapy, including worsening pulmonary infiltrates, lymphadenopathy, and intracranial granulomas (DeSimone et al., 2000). The second process involves unmasking of a latent or subclinical infection. Falling into this category are case reports such as disseminated histoplasmosis, including fungemia, 3 weeks after HAART initiation (DeSimone et al., 2000).

Although a standard clinical definition for IRIS does not exist, the incidence of IRIS among persons initiating HAART reported in the medical literature ranges from 10 to 25% (French et al., 2000; Ratnam et al., 2006; Murdoch et al., 2008). In one study looking at persons at potentially higher risk for IRIS due to co-infection with *M. tuberculosis*, *Mycobacterium avium* complex, or *Cryptococcus neoformans*, the incidence was 32% (Shelburne et al., 2005). IRIS events are most commonly described within 2 months of HAART initiation. However, IRIS events much later than this have been well documented. A study of 30 patients with CMV retinitis who responded to HAART reported that 19 developed CMV vitritis after a median of 43 weeks. All had inactive CMV retinitis in the affected eye (Karavellas et al., 1999).

The reported clinical manifestations of IRIS vary widely. For example, a prospective study of 423 persons in South Africa reported tuberculosis (41%), while a retrospective study of 199 persons in London reported genital herpes (50%) as the most common IRIS-related diagnosis (Ratnam et al., 2006; Murdoch et al., 2008). This variation likely reflects in large part the different study populations and underlying epidemiology of potential co-pathogens. Clear risk factors indicating persons at high risk for IRIS have yet to be defined. Severe immunosuppression with a CD4 count less than 50–75 cells/μL has been a consistently reported risk factor (French et al., 2000; Shelburne et al., 2005; Ratnam et al., 2006; Murdoch et al., 2008). One study reported younger age as a risk factor, possibly reflecting the ability to mount a more robust inflammatory response (Ratnam et al., 2006).

There are currently no specific guidelines for management of IRIS. Among 57 of 180 patients co-infected with *M. tuberculosis*, MAC, or *C. neoformans* who

developed IRIS, treatments included non-steroidal anti-inflammatory drugs in 12 (21%), corticosteroids in 16 (28%), invasive measures such as lumbar puncture or lymph node drainage in 44 (77%), intensification of opportunistic infection therapy in 23 (40%), and cessation of HAART in 4 (7%) (Shelburne et al., 2005). Authors reported a low rate of complications with all these measures.

Another option for KS-associated IRIS management is co-administration of HAART and chemotherapy. This has not been explicitly studied in clinical settings. Bihl et al. (2007) randomized 17 patients to HAART alone and 16 to HAART in combination with chemotherapy. They reported a trend toward less frequent progressive disease in persons treated with the combination vs. HAART only (17 and 24%, respectively). More robust clinical trials of combination therapy in areas with a high burden of KS are needed to better answer this question.

IRIS and Cancer

KS is the most commonly reported IRIS-associated cancer (Weir and Wansbrough-Jones, 1997; Rizos et al., 2003; Connick et al., 2004; Crane et al., 2005; Leidner and Aboulafia, 2005; Ratnam et al., 2006; Murdoch et al., 2008). Of the prospective studies of IRIS discussed above, only one was conducted in an HHV-8 endemic region (Murdoch et al., 2008). In this study of 423 persons in South Africa followed for 6 months after starting HAART, the rate of IRIS-associated KS was 5%. Another study of 199 patients in London reported one IRIS-associated KS event (Ratnam et al., 2006).

Most cases have been reported within 2 months of HAART initiation. The clinical course is variable but serious morbidity including death has been reported. One case series of 19 patients with IRIS-associated KS at the University of Washington reported six fatalities, four from pulmonary KS and two from unrelated causes (Leidner and Aboulafia, 2005). Another study including 21 patients with KS prospectively followed after HAART initiation reported that three patients had progressive disease, including one with rapid development of pulmonary KS and death (Gill et al., 2002).

The pathogenesis of IRIS-associated KS is not clear. HHV-8-specific cellular and humoral reconstitution after HAART has been reported to be delayed until at least 5 months after HAART initiation (see Section "HAART-Associated Anti-HHV-8 Immune Reconstitution"). The delay in restoring these responses is at odds with the description of IRIS-associated KS typically within 2 months of HAART initiation. One explanation for this is that studies of HHV-8 immunity are currently limited by an incomplete set of HHV-8 epitopes and thus have to be interpreted cautiously. Another explanation is that IRIS-associated KS is not the result of HHV-8-specific immune responses but instead a product of increased host inflammatory responses to other antigens. For example, a change in the cytokine milieu after immune restoration to another pathogen may lead to KS exacerbation. More studies are needed to better define these mechanisms.

Risk factors for IRIS-associated KS have not been well defined. One prospective study of HAART in 150 therapy-naïve patients with incident KS reported 10 patients (7%) with progressive KS. Risk factors for progression included higher CD4 counts ($p = 0.03$), KS-associated edema ($p = 0.01$), and therapy with both PIs and NNRTIs together ($p = 0.03$). Worsening occurred within 2 months. HAART was successfully continued in all (Bower et al., 2005).

Taken together, the body of evidence to date suggests that KS–IRIS may be a common and morbid condition. However, additional research is needed to define the frequency, clinical manifestations, and pathophysiology of this condition.

Conclusion

Substantial evidence from mouse models and humans shows that the immune system plays a key role in cancer prevention. Immunosuppressed persons are at higher risk for cancer development and the etiology of immunodeficiency may confer different cancer risk profiles. HIV-infected persons are at increased risk for a much broader range of cancers than previously thought, but the largest risk is still for KS. The factors leading to this increased cancer risk, as explored in this review through examination of HIV-associated KS risk, are complex and likely include adaptive and innate immunosuppression, host genetics, viral (HHV-8 and HIV) replication patterns, and environmental factors.

Although HAART-associated immune reconstitution has led to dramatic decreases in morbidity and mortality due to HIV, the problem of cancer among HIV-positive individuals is likely to persist. Unfortunately, the incidence of cancer remains significantly elevated in persons receiving HAART. Furthermore, a potentially devastating consequence of HAART initiation is IRIS. KS is the most common IRIS-associated cancer, and the manifestations of KS-IRIS may be severe. The exact mechanisms which lead to IRIS are not well understood. Now with HAART becoming more widespread in KS-endemic areas, there likely will be increased reports of IRIS-associated KS. Ongoing natural history and treatment studies in HHV-8 endemic areas will provide valuable information about pathogenesis and management of IRIS-associated cancers. Further clinical trials will be needed to determine the pathophysiology and optimal management strategies for KS-IRIS.

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Targeting Signal Transduction Pathways for the Treatment of Kaposi Sarcoma

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Abstract Kaposi sarcoma (KS) is a multi-focal angioproliferative disease driven by infection of cells by Kaposi sarcoma herpesvirus/human herpesvirus-8 (KSHV/HHV8). KSHV/HHV8 infection activates numerous sequential and parallel signaling pathways creating an angiogenic–inflammatory state that leads to the development of Kaposi sarcoma. These pathways can be grouped into KSHV/HHV8- specific pathways and cellular growth/angiogenic pathways that are "pirated" by KSHV/HHV8. Advances in our understanding of the pathogenesis of KS parallel the clinical development of signal transduction inhibitors. This chapter reviews the signaling pathways active in KSHV/HHV8-infected cells and discusses the use of clinical inhibitors of these pathways in the treatment of KS.

Introduction

Kaposi sarcoma (KS) is a multi-focal angioproliferative disease driven by infection of cells by Kaposi sarcoma herpesvirus/human herpesvirus-8 (KSHV/HHV8) (Dupin et al., 1999). The incidence of KS in human immunodeficiency virus (HIV)-infected patients has declined with the introduction of highly active antiretroviral therapy; however, KS remains a leading cause of mortality and morbidity in acquired immune deficiency syndrome (AIDS) (Watson et al., 2004; Aversa et al., 2005).

KSHV/HHV8 infection activates numerous sequential and parallel signaling pathways creating an angiogenic–inflammatory state that leads to the development of Kaposi sarcoma. These pathways can be grouped into KSHV/HHV8-specific pathways and cellular growth/angiogenic pathways that are "pirated" by KSHV/HHV8. Advances in our understanding of the pathogenesis of KS parallel the

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clinical development of signal transduction inhibitors. In this chapter we will review the signaling pathways active in KSHV/HHV8-infected cells and discuss clinical inhibitors of these pathways. This knowledge will allow us to test the importance of specific pathways for KS development in vivo and may translate into effective therapies for KS prevention or treatment.

Biology of Kaposi Sarcoma

The viral and cellular signaling events initiated by KSHV/HHV8 infection of host cells converge on the same second messenger pathways, allowing for potential therapeutic intervention at multiple steps in the pathogenesis of KS.

Interleukin-6/viral interleukin-6/glycoprotein-130 receptor: Interleukin-6 (IL-6) is an inflammatory cytokine that plays an important role in immunity (reviewed in Yoshizaki et al., 1990; Ishihara and Hirano, 2002). IL-6 is induced in HHV8-infected cells by the early lytic genes *Rta*/*ORF50* and the viral G protein-coupled receptor (vGPCR/ORF74) (Pati et al., 2001; Schwarz and Murphy, 2001; Deng et al., 2002). Rta is an HHV8 transcriptional activator that regulates lytic gene expression, and vGPCR is a homologue of the IL-8 receptor (discussed below) (West and Wood, 2003). HHV8 expresses a viral homologue of IL-6, viral IL-6 (vIL-6/ORFK2). vIL-6 is a lytic gene that acts as a growth factor for IL-6-dependent tumor cell lines but is less potent than cellular IL-6, requiring 1,000-fold higher levels to promote growth (Burger et al., 1998).

Both IL-6 and vIL-6 signal through the glycoprotein-130 receptor (gp130) (Neipel et al., 1997; Nicholas et al., 1997; Mullberg et al., 2000). Human gp130 is a shared subunit of the receptor complex for IL-6, leukemia inhibitory factor, ciliary neurotrophic factor, cardiotrophin-1, cardiotrophin-like cytokine, oncostatin M, IL-11, IL-27, and IL-31 (reviewed in Muller-Newen, 2003). The IL-6 receptor complex (IL-6R) is formed by an α-chain subunit (gp80/CD126) that binds IL-6 but does not signal until interaction with the signal-transducing subunit, gp130. Both subunits are required for IL-6 activation of downstream effectors. In contrast, vIL-6 is distinct in that it binds to and requires only gp130 to form a stable, signal-transducing complex (Mullberg et al., 2000). Since gp130 has a broad expression pattern even on cells lacking CD126, vIL-6 has a broader activation potential than does IL-6.

Signaling pathways activated by IL-6 or vIL-6 through gp130 include the Janus kinase/signal transducers and activators of transcription (JAK/STAT), phosphatidylinositol 3-kinase/AKT/ mammalian target of rapamycin (PI3K/AKT/mTOR) (Oh et al., 1998), and mitogen-activated protein kinase (MAPK) signaling pathways (Osborne et al., 1999; Hideshima et al., 2000). Activation of STAT3 induces cell cycle progression via induction of cyclins D2, D3, A, and cdc25 with concurrent downregulation of the cell cycle inhibitors p27 and p21. Activation of the PI3K and MAPK pathways promotes growth and anti-apoptotic pathways (Yao and Cooper, 1995; Fukada et al., 1996; Manning et al., 2002; Tee et al., 2002). PI3K is a lipid kinase that activates AKT, a serine–threonine

kinase that has multiple targets including the mammalian target of rapamycin (mTOR), a kinase that plays a crucial role in cell proliferation and survival. Treatment of vIL-6-expressing cells with an inhibitor of the MAPK pathway blocks vIL-6-mediated proliferation (Hideshima et al., 2000).

IL-6 and vIL-6 induce expression of vascular endothelial growth factor (VEGF), which is the key regulator of angiogenesis (discussed below) (Funamoto et al., 2000; Liu et al., 2001; Naruishi et al., 2003). Human umbilical vein endothelial cells (HUVECs) were shown to proliferate and form tubules in a matrigel assay in response to conditioned media from NIH3T3 cells stably expressing vIL-6, and this proliferation was blocked by anti-VEGF antibody (Aoki et al., 1999). When these stably transfected cells were implanted in athymic mice, the vIL-6-expressing tumors grew more rapidly and were more vascular than control tumors. The vIL-6-expressing tumors also expressed higher levels of VEGF than did the control tumors (Aoki et al., 1999). These data support a critical role for vIL-6 and IL-6 in HHV8-induced angiogenesis, growth, and proliferation.

Viral G protein-coupled receptor (vGPCR): HHV8 encodes a G protein-coupled receptor (GCPR) that is homologous to the IL-8 receptors, CXCR1 and CXCR2 (Arvanitakis et al., 1997; Bais et al., 1998). The *vGPCR* is a lytic gene product harboring a mutation (Asp142Val) that renders it constitutively active (Rosenkilde et al., 2000). The *vGPCR* activates the PI3K/AKT/mTOR pathway as well as members of the MAPK family, which include p38, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK). These kinases are upregulated in response to certain signals such as growth or stress stimuli and can exert both pro- and anti-apoptotic effects. The JNK pathway also has effects on proliferation. Dysregulation in each of these MAPK subfamilies and the PI3K/AKT/mTOR pathways is associated with malignancy (reviewed in Guertin and Sabatini, 2005).

The PI3K/AKT/mTOR pathway also influences angiogenesis through regulation of the transcription factor known as hypoxia-inducible factor-1α (HIF-1α) (Brugarolas et al., 2003, 2004). Tumor cells in environments that are low in oxygen induce angiogenesis via the stabilization of HIFs, which are transcription factors that interact with promoters containing hypoxia response elements (HREs). Two key regulators of angiogenesis, VEGF and VEGF-R1, contain HREs (Takeda et al., 2004). Interestingly, KSHV/HHV8 encodes several genes which upregulate HIFs. For example, vGPCR-dependent activation of both MAPK and p38 kinases leads to the subsequent phosphorylation and activation of HIF-1α, and this is the likely mechanism for vGPCR's induction of VEGF (Sodhi et al., 2000). In addition, KSHV/HHV8 latent gene expression induces the transcriptional upregulation of both HIF-1α and HIF-1β (Carroll et al., 2006).

HREs are also located in the promoter of the heme-degrading enzyme heme oxygenase-1 (*HO-1*) gene. *HO-1* is expressed in KS lesions and induced by KSHV/HHV8 infection of endothelial cells (Cornelissen et al., 2003; McAllister et al., 2004), and its angiogenic and anti-apoptotic activities suggest a potential role in KS development. Given the capacity of KS spindle cells to phagocytose and process erythrocytes (Orenstein 2008), the ability to efficiently metabolize free heme would be advantageous. HO-1 inhibitors have been shown to interfere with

the transforming activity of the vGPCR (Marinissen et al., 2006) and to inhibit the heme-driven growth of KSHV/HHV8-infected endothelial cells (McAllister et al., 2004). These observations suggest that inhibitors of HO-1 should be considered for KS therapy.

In a retroviral vector transduction model, vGPCR was the only HHV8 ORF that was able to induce KS-like lesions in mice (Montaner et al., 2003). In primary effusion lymphoma (PEL) cells, vGPCR induces the angiogenic factors VEGF and IL-6 (Cannon et al., 2003). In endothelial cells, ectopic expression of vGPCR induces expression of IL-6, IL-8, GroA, VEGF-A, and VEGF-R2 (Bais et al., 2003; Montaner et al., 2004). It is unknown if vGPCR mediates the expression of other HIF targets that are expressed in KS such as PDGF-B, TGF-α, and Ang2.

The vGPCR activates the transcription factor nuclear factor-kappa B (NF-κB). NF-κB plays a critical role in the suppression of apoptosis and regulation of the cell cycle (reviewed in Luo et al., 2005; Viatour et al., 2005). NF-κB proteins are tightly regulated and are bound in the cytoplasm by inhibitors of NF-κB (IκB). IκB kinase (IKK)-alpha and IKK-beta phosphorylate IκB, leading to IκB proteolytic degradation. NF-κB then freely moves into the nucleus and regulates transcriptional activity. Constitutive activity of NF-κB, often mediated by abnormal regulation of IKKs, is associated with solid and hematologic malignancies. Inhibitors of both vGPCR and PI3K prevent the activation of NF-κB signaling and block transformation (Dadke et al., 2003). These data suggest that NF-κB activation by the vGPCR leads to cellular transformation and does so through PI3K/AKT/mTOR signaling.

vCCL1, vCCL2, and vCCL3 (vMIP-I, vMIP-II, and vMIP-III): KSHV/HHV8 encodes three viral chemokine homologues, namely vCCL1 (vMIP-I/ORF K6), vCCL2 (vMIP-II/ORF K4), and vCCL3 (vMIP-III/ORF K4.1). These proteins are lytic gene products that are 25–40% homologous to the cellular chemokine macrophage inflammatory protein-1 alpha (MIP-1α) and bind to endogenous chemokine receptors (Moore et al., 1996; Sun et al., 1999; Stine et al., 2000). Chemokines are structurally related glycoproteins that bind to GPCRs and induce leukocyte activation and chemotaxis. When activated, GPCRs signal through downstream effectors such as phospholipase C, MAPKs, and PI3K (reviewed in Sodhi et al., 2004).

The evidence suggests that vCCLs not only modulate host immunity but also play an important role in KSHV/HHV8-induced angiogenesis. vCCL1 activates CCR8, thus promoting migration of monocytes, endothelial cells, and Th2 lymphocytes (Dairaghi et al., 1999). vCCL3 activates CCR4 leading to the recruitment of Th2 T cells. It has been proposed that vCCL2 restricts the immune response to KSHV/HHV8 infection by blocking both CCR1 and CCR5, which are expressed on Th1 lymphocytes (Weber et al., 2001). In addition to their effects on the immune system, all three vCCLs have been shown to be angiogenic in the chick chorioallantoic membrane assay (Boshoff et al., 1997; Stine et al., 2000). vCCL1 induces expression of VEGF-A in PEL cells and both vCCL-1 and vCCL-2 protect PEL cells from chemically induced apoptosis (Liu et al., 2001). The mechanism by which vCCL2 and vCCL3 induce angiogenesis remains to be clarified. Thus, the vCCLs acting in

concert manipulate immune, anti-apoptotic, and angiogenic pathways to facilitate a microenvironment that allows KSHV/HHV8-infected cells to survive.

Interleukin-1: Interleukin-1 (IL-1) is an inflammatory cytokine that acts as an autocrine growth factor for KS spindle cells. IL-1β is upregulated in KS spindle cells compared to normal endothelium, and when IL-1β binding is blocked, the growth of KS spindle cells is inhibited (Ensoli et al., 1989). IL-1β induces fibroblast growth factor 2 (FGF-2 or basic FGF), a potent angiogenic growth factor (discussed below) (Louie et al., 1995). Additionally, in conjunction with platelet-derived growth factor B (PDGF-B), IL-1β increases VEGF expression in KS spindle cells (Cornali et al., 1996). Thus, IL-1β is important in KS spindle cell mitogenesis and KSHV/HHV8-induced angiogenesis.

Vascular endothelial growth factor: A number of effects of the viral cytokines and vGPCR discussed above are mediated through VEGF (or VEGF-A). VEGF is a member of a gene family that includes placental growth factor (PLGF), VEGF-B, VEGF-C, and VEGF-D. VEGF-R1 (Flt1), VEGF-R2 (KDR), and VEGF-R3 (Flt4) are type III receptor tyrosine kinases that are activated differentially by the VEGF family members. KSHV/HHV8 utilizes the VEGF pathway to promote angiogenesis and tumorigenesis. VEGF-R2 is the major mediator of angiogenic and mitotic signaling by VEGF, while VEGF-R1 appears to be important in mediating monocyte chemotaxis. VEGF-R3 is restricted to lymphatic endothelial cells in adults but is upregulated in neo-angiogenesis in tumors as well as KS lesions (reviewed in Ferrara et al., 2003).

Experimental evidence supports a central role for members of the VEGF/VEGF-R family in KS development. VEGF-C and its receptors VEGFR-2 and VEGFR-3 are expressed in KS lesions (Skobe et al., 1999; Folpe et al., 2000; Masood et al., 2002). Recent genomic array studies have confirmed this finding and also demonstrated that PLGF is upregulated in KS lesions (Wang et al., 2004b). HIF-1α activated by HHV8 as discussed above is also a key regulator of VEGF expression. Indeed the angiogenic effects of several viral cytokines and the vGPCR are, either in part or in whole, mediated through HIF-1α-mediated expression of VEGF-A. Additionally, the expression of vGPCR in human endothelial cells leads to activation of VEGF-R2, which in turn activates the PI3K/AKT/mTOR pathway. These series of events immortalize vGPCR-expressing cells and represent a significant transforming mechanism (Bais et al., 2003).

In addition to VEGF-R3, KS spindle cells express other markers of lymphatic endothelium such as LYVE-1 and podoplanin (D2-40) (Kahn et al., 2002; Xu et al., 2004). However, markers of vascular endothelium such as factor VIII and PAL-E are also expressed (Nadimi et al., 1988). The mixed phenotype of KS lesions is further illustrated by a microarray analysis showing a gene expression profile with co-expression of lymphatic and vascular markers (Wang et al., 2004a) and an ultrastructural analysis revealing a spectrum of endothelial cell ultrastructural characteristics with various lymphatic and blood vascular features (Orenstein, 2008). Whether KSHV/HHV8 preferentially infects blood vascular or lymphatic endothelial cells and induces dedifferentiation toward a more convergent phenotype, or infects uncommitted progenitors and influences their lineage commitment, remains

a matter of ongoing debate. Both cell types are susceptible to in vitro infection with KSHV/HHV8 and are subjected to reprogramming to an intermediate phenotype (Carroll et al., 2004; Hong et al., 2004; Wang et al., 2004a). The unusual phenotype of the KS spindle cell suggests additional diversity with respect to signaling pathways, but may also allow unique therapeutic opportunities.

The KSHV/HHV8 *ORF-K1* gene encodes a transmembrane glycoprotein, K1, which is homologous to immunoglobulin receptors and the B-cell receptor. The K1 protein can induce VEGF expression via VEGF promoter activation as well as induce the expression of matrix metalloproteinase-9 (MMP-9) (Wang et al., 2004b). MMPs are essential for digestion of the extracellular matrix that accompanies vascular remodeling and angiogenesis. Recent in vitro studies with KSHV/HHV8-infected endothelial cells suggest that virus-induced secretion and activation of MMP-1, -2, and -9 allows infected cells to invade an extracellular matrix (Qian et al., 2007). Also, the KSHV/HHV8 viral envelope glycoprotein gB can activate VEGFR-3, leading to enhanced endothelial cell migration and proliferation (Zhang et al., 2005). Given the prominent role VEGF expression, secretion, and related signal transduction plays in the development of KS, therapies targeting the VEGF pathway, as well as MMP expression/activation, may play a vital role in tumor therapy.

Angiopoietins: The angiopoietin family of vascular growth factors includes four members: angiopoietins-1, -2, -3, and -4 (Davis et al., 1996; Maisonpierre et al., 1997; Kim et al., 1999; Lee et al., 2004). Tie-2 is a receptor tyrosine kinase that binds all four angiopoietins (Davis et al., 1996; Maisonpierre et al., 1997; Kim et al., 1999; Lee et al., 2004). Angiopoitin-2 (Ang2) is an antagonist to Tie-2, while angiopoietin-1 (Ang-1) is an agonist (Davis et al., 1996; Maisonpierre et al., 1997). The Tie-2/Ang-1 pathway acts in conjunction with VEGF to promote vascular stabilization (Suri et al., 1996). Ang-1 is widely expressed in human endothelial cells, suggesting that it has a role in maintaining vascular endothelial cell stability, while Ang-2 appears to be upregulated at the sites of vascular remodeling. In the presence of high VEGF, Ang-2 antagonism of Tie-2 leads to vessel sprouting; but in the absence of VEGF, Ang-2 expression leads to vascular regression (Oh et al., 1999; Lobov et al., 2002). These data would suggest that Ang-2 disruption of Ang-1-mediated endothelial cell stability might play a role in the neo-angiogenesis seen in tumors. KS has been shown to express Tie-1, Tie-2, Ang-1, Ang-2, and Ang-4 (Brown et al., 2000). However, Ang-2 was expressed at a higher level than Ang-1 in KS lesions (Brown et al., 2000). Recent studies aimed at elucidating the mechanisms of angiopoietin regulation in KS suggest that Ang-2 may be induced by both vIL-6 and vGPCR (Vart et al., 2007) and that this upregulation may be a result of transcriptional activation by AP-1 and Ets-1 (Ye et al., 2007). While the role of angiopoietins in the development of KS remains unclear, the angiopoietin system may be a potential therapeutic target. As an example, adenovirus-delivered anti-Tie-2 intrabody inhibits growth in a KS cell line (Popkov et al., 2005).

Fibroblast growth factors: The fibroblast growth factor (FGF) family is composed of 22 members. FGFs regulate myriad effects in endothelial cells, including mitogenesis, chemotaxis, and angiogenesis (reviewed in Eswarakumar et al., 2005). The FGFs mediate their effects by activating one or more of the FGF receptors.

Binding of FGFs to their receptors is modulated by heparin proteoglycans (Neufeld and Gospodarowicz, 1987). The four FGF receptors (FGF-R1, FGF-R2, FGF-R3, and FGF-R4) are tyrosine kinases that signal through multiple downstream effector pathways including the MAPK and PI3K/AKT/mTOR pathways (Creuzet et al., 1995; Kanda et al., 1997). FGFs implicated in angiogenesis appear to act through multiple mechanisms. FGF-1 (acidic FGF) and FGF-2 (basic FGF) act directly on the endothelial cells to induce migration, proliferation, and tube formation, whereas other family members such as FGF-4 can induce the expression of VEGF.

FGF-2 was one of the first autocrine growth factors described for HHV8 (Sinkovics, 1991). FGF-2 expression is upregulated in KS spindle cells compared to normal vascular endothelial cells, and blocking FGF-2 binding to spindle cells inhibits their proliferation (Sinkovics, 1991). FGF-2 also synergizes with VEGF to induce angiogenesis in KS lesions (Samaniego et al., 1998). Thus, FGF-2 is important in KS spindle cell mitogenesis and KS-induced angiogenesis. FGF-4 was originally isolated from KS cells (hence the name Kaposi sarcoma oncogene) and has been shown to induce expression of VEGF. Also, the HIV-1-transactivating protein Tat induces FGF-1 and FGF-2, which could play an additional role in AIDS–KS (Opalenik et al., 1995; Barillari et al., 1999).

Insulin and insulin-like growth factors: The insulin-like growth factor-I receptor (IGF-IR) is structurally similar to the insulin receptor and its three ligands are insulin, IGF-I, and IGF-II. IGF-IR–ligand interaction is important in the regulation of cell proliferation, anti-apoptosis, differentiation, and cell motility but also plays a crucial role in the anchorage-independent growth of cells (reviewed in Larsson et al., 2005). IGF-IR mediates its effects via interaction with the insulin receptor substrate family of scaffolding proteins, which mediates the activation of PI3K and other downstream signaling pathways (Taniguchi et al., 2006). Activation of the PI3K/AKT/mTOR pathway by IGF-IR then leads to the inhibition of apoptosis.

IGF-IR is expressed in KS lesions. Additionally, IGF-I stimulates cell proliferation and this effect is mediated through IGF-IR. Inhibition of IGF-IR by an anti-IGFR antibody or a specific inhibitor of the IGF-IR tyrosine kinase (picropodophyllin) induces apoptosis of a KS cell line (Catrina et al., 2005). Specific inhibitors of IGF-IR such as genistein and BMS-554417 have also been shown to decrease IGF-IR signaling and may allow the importance of this pathway to be tested in vivo (Kim et al., 2005; Haluska et al., 2006). Endothelial cells infected with KSHV/HHV8 in vitro express elevated levels of the insulin receptor (IR) and treatment with a small molecule inhibitor of IR, HNMPA-(AM_3), inhibits their growth (Rose et al., 2007). Collectively these studies suggest a role for the insulin/IGF axis in KS development.

Platelet-derived growth factor (PDGF): PDGF molecules exist as either homo- or heterodimers of its A, B, C, and/or D isoforms. These dimers bind to the PDGF receptor (PDGF-R), which belongs to the type III receptor tyrosine kinase family. The PDGF-R exists as α and β isoforms, which can form homo- or heterodimers (reviewed in Betsholtz et al., 2001). Activated PDGF-R stimulates multiple signaling pathways through downstream effectors including the PI3K/AKT/mTOR and MAPK pathways (Franke et al., 1995; Brennan et al., 1997; Pearson et al., 1998; Chaudhary and Avioli, 2000). Activation of the PDGF-R by autocrine/paracrine

mechanisms, activating mutations, or by chromosomal translocation has been implicated in the pathogenesis of multiple malignancies.

The PDGF pathway also plays an important role in angiogenesis. Pericytes, which stabilize normal and tumor vasculature, have been shown to require PDGF-B and PDGF-Rβ. Data suggest that pericytes also stabilize tumor vasculature (Lindahl et al., 1997). PDGF-Rβ staining of tumor capillary pericytes has been reported in lung, colon, breast, and prostate carcinomas (Ostman, 2004). Studies of murine models have demonstrated decreased pericyte recruitment to tumor vasculature in mice with a PDGF-Rβ mutant that cannot activate the PI3K/AKT pathway compared to mice with the wild-type receptor (Lindahl et al., 1997). These data would suggest that PDGF is important for both tumor and normal angiogenesis.

Several studies have addressed the role of PDGF-R in KS. When KS spindle cells are cultured, they express both α and β PDGF receptors (Werner et al., 1990). Cultured KS spindle cells undergo growth arrest when placed in PDGF-depleted media, and this arrest can be mitigated by the addition of recombinant PDGF (Roth et al., 1989). It is unclear, however, whether cultured cells reflect KS spindle cell behavior in vivo. Moreover, the source of the PDGF in vivo remains unanswered. Indeed, only PDGF-Rβ appears to be expressed in KS specimens as assayed by in situ hybridization and immunohistochemistry (Sturzl et al., 1992). These data have been confirmed by recent genomic profiling studies, which demonstrated that PDGF-B and PDGF-Rβ are upregulated in HHV8-infected cells and KS lesions (Wang et al., 2004a). Additionally, PDGF-B in conjunction with IL-1β induces the expression of VEGF by cultured KS spindle cells (Cornali et al., 1996). PDGF-R is involved in two critical pathways in KS development: the induction of KS spindle cell growth and tumor-associated angiogenesis.

c-Kit: The c-Kit receptor is another member of the PDGF-R family of type III receptor tyrosine kinases that may be involved in KS. c-Kit is normally expressed on hematopoietic stem cells, mast cells, melanocytes, and germ cells. The receptor is activated by oligomerization, which is triggered by the binding of its ligand, stem cell factor (SCF; also Kit ligand or steel factor). Ligand-dependent autophosphorylation of c-Kit results in a series of downstream signaling events that include the activation of various kinase pathways such as Ras/ERK, PI3K, JAK/STAT, Src family kinase, and phospholipase Cγ (Lennartsson et al., 2005). SCF stimulation of c-Kit and synergy with other cytokines result in cell survival and proliferation. However, oncogenesis can occur when SCF is secreted constitutively or when c-Kit is either overexpressed or mutated to allow ligand-independent activation. c-Kit expression is upregulated in KSHV/HHV8-infected endothelial cells harboring the latent viral genome (Moses et al., 2002). However, we recently found that the phosphorylation of c-Kit in infected cells still requires exogenous SCF (Douglas et al., 2009). In addition, two of the downstream kinase pathways for c-Kit, ERK 1/2, and AKT were significantly activated in KSHV/HHV8-infected cells treated with exogenous SCF. Previous data have shown that KSHV/HHV8 does not significantly increase SCF expression in endothelial cells (Moses et al., 2002). Therefore, these data suggest that KSHV/HHV8's ability to deregulate c-Kit signaling occurs via over-expression of the c-Kit receptor rather than constitutive receptor activation or induction of

SCF secretion. In vitro, both endogenous SCF produced by endothelial cells and exogenous SCF from the culture medium would provide the stimuli to maintain c-Kit activation. In KS lesions, the inflammatory infiltrate comprised of mast cells and macrophages could provide a continual source of elevated SCF to drive lesion development.

Therapeutic Opportunities

The development of targeted signal transduction inhibitors provides the opportunity to test the importance of specific pathways in the development of KS. A number of agents are available that target KSHV/HHV8-specific pathways as well as pathways that are "pirated" by KSHV/HHV8.

Imatinib mesylate (Gleevec; Novartis Pharmaceuticals, East Hanover, NJ) selectively inhibits the tyrosine kinases c-Kit, PDGF-R, and Abl. In an exploratory study, imatinib was administered to patients with AIDS-related KS (Koon et al., 2005). Clinical and histologic regression of cutaneous KS lesions occurred within 4 weeks of therapy. The most common adverse event noted was diarrhea. Tumor biopsies, obtained at baseline and following four weeks of therapy, demonstrated decreased phosphorylation (activation) of the PDGF and MAPK pathways. Only minimal or no activation of AKT was noted in pre-treatment tumor specimens. This study suggests that the MAPK pathway may be more critical than the PI3K/AKT pathway for inducing proliferation of KS lesions. This is in contrast to other cell systems in which signaling from PDGF-R is often transmitted through AKT (Chaudhary and Hruska, 2001). A larger phase II study has recently been conducted by the AIDS Malignancy Consortium to determine the response rate in a multicenter setting and investigate potential markers of response. These results have not yet been published.

The utility of imatinib in the treatment of KS suggests that the use of other targeted agents may be effective in the treatment of KS. Currently a number of inhibitors of the IR/IGF-R axis are in clinical development. Given the effect of inhibition of these pathways in KSHV/HHV8-effected endothelial cells, there is clear evidence to pursue the development of these agents in the clinic (Kim et al., 2005; Haluska et al., 2006).

The remarkable finding that vIL-6, vCCL, and vGPCR induce expression of VEGF provides a missing link to the chain of events by which HHV8 creates an inflammatory–angiogenic environment (Aoki et al., 1999). This observation has led to the testing of a number of agents that target angiogenic pathways in KS.

Both sorafenib (Bay 43-9006) and sunitinib (SU11248) are approved for the treatment of renal cell carcinoma, another highly angiogenic tumor (Motzer et al., 2006; Gollob et al., 2007). These agents inhibit multiple tyrosine kinases including Raf, VEGF-R2, and PDGF-R (Motzer et al., 2006; Gollob et al., 2007). These agents would be expected to demonstrate activity in KS. Sunitinib is being studied in KS patients in east Africa who had not previously received HAART, while a phase I/pharmacokinetic study of sorafenib in patients receiving HAART is currently underway at the NCI.

Another agent that may have therapeutic potential is Veglin (VasGene Therapeutics, Los Angeles, CA), an antisense oligonucleotide to the *VEGF* gene. This agent showed activity in KS in the phase I setting but these observations have not been pursued in a larger study (Levine et al., 2006). A therapeutic trial of bevacizumab (Avastin; Genentech, South San Francisco, CA), a humanized monoclonal antibody to VEGF which is FDA approved for other indications, is currently underway at the National Cancer Institute for classic and AIDS-related KS. We would expect it to have activity in this setting. Inhibitors of the angiopoietin axis are in clinical trials but have yet to be tested in KS.

Since a number of the angiogenic pathways which appear to be activated in KS use common intracellular signaling, these pathways may also represent therapeutic targets. The PI3K/AKT pathway has been shown to regulate mTOR activity through the tuberous sclerosis (TSC 1/2) gene complex (Manning et al., 2002). This complex appears to regulate VEGF expression through mTOR-dependent and mTOR-independent mechanisms (Brugarolas et al., 2003, 2004). Whether this pathway is important for KS-mediated angiogenesis is unknown; however, the recent report of transplant-related KS responding to rapamycin suggests that mTOR may play a critical role in KS development (Stallone et al., 2005). If these results were reproducible in other forms of KS, it would suggest inhibition of other parts of the PI3K/AKT/mTOR pathway are valid therapeutic targets. Alternatively, inhibition of the MAPK and JAK/STAT pathways may be additional targets, given their roles in KS-induced cytokine signaling.

Summary

There are a large number of critical pathways whose importance can finally be tested in vivo using any number of agents in development. Within the next few years, targeted agents such as imatinib, sorafenib, and sunitinib may be alternatives to the use of cytotoxic chemotherapies in the treatment of KS. This “wealth of riches” leaves us with the critical issue of which agents to move forward in future clinical trials and in what sequence. This is a setting where well-designed translational studies may provide insights into the development of subsequent clinical and basic investigations into KS.

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Role of NF-κB Inhibitors in HIV-Related Malignancies

Erin Gourley Reid and Dirk P. Dittmer

Abstract NF-κB is essential for B-cell development and crucial for the survival of B-cell lymphoma. Not surprisingly then NF-κB and other members in this pathway have been developed as anti-cancer drug targets. At the same time, NF-κB is essential for the maintenance and replication of human tumor viruses such as Epstein–Barr virus (EBV) and Kaposi's sarcoma associated herpesvirus (KSHV). The high association of HIV lymphomas with these viruses provides an opportunity to approach these malignancies using strategies that target NF-κB.

The NF-κB Pathway Is Central to Cell Survival

Nuclear factor kappa B (NF-κB) was initially identified as a DNA binding protein that recognized a specific *cis*-element in the kappa light chain enhancer of B cells (Sen and Baltimore, 1986). NF-κB is essential for B-cell development and, not surprisingly, crucial for the survival of B-cell lymphoma. Further studies established its importance as a pro-survival factor in many cancers, not just those of lymphoid origin (reviewed in Ghosh, 2007). NF-κB is a mixed multimer of p50, p65, and other subunits. Subunit composition, posttranscriptional modifications, and other cooperating transcription factors determine target gene specificity beyond recognition of the minimal consensus motif. Different genes are transcriptionally responsive to NF-κB under different cellular conditions and in different tumor types. Hence, mRNA profiling of NF-κB responsive genes can be used to stratify malignancies and to characterize cellular responses to natural (viral infection, growth factor signaling) or artificial (chemotherapy) stimuli. The NF-κB group of transcription factors is involved in inflammatory, immune, and pro- and anti-apoptotic cellular responses (Cahir-McFarland et al., 2004; Hinz et al., 2002; Karin et al., 2002). A very abbreviated overview of NF-κB signaling is provided in Fig. 1.

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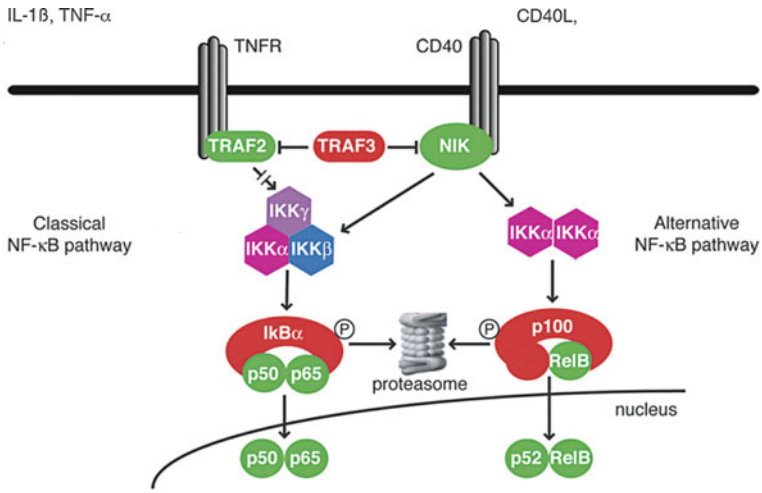

Fig. 1 General overview of NF-κB signaling (adapted from Annunziata et al., 2007)

In the classical or canonical pathway, inhibitor of kappa B (IκB) segregates NF-κB (p50 and p65) in the cytoplasm. In response to a variety of stimuli, including cytokines and viral infection, IκB is phosphorylated by the IκB kinase complex (IKK), typically IKKβ, at specific serine sites and degraded by the ubiquitin-proteasome pathway, allowing for nuclear localization, DNA binding, and transactivation by NF-κB subunits. In the alternative or non-canonical pathway, the p100 precursor is associated with the relB subunit and upon phosphorylation by IKKα cleaved to yield p52. p52 and Rel then translocate into the nucleus and act as a heterodimer. Figure 1 leaves out many additional NF-κB complex partners as well as the novel, third pathway of NF-κB activation. The reader may wish to consult Ghosh (2007) for an exhaustive review of the field. Here, we will confine ourselves to evaluate the evidence which we think justifies and encourages NF-κB-targeted therapies in HIV/AIDS-associated malignancies.

NF-κB in Cancer

NF-κB signaling, IκB, and IKK are targets of ongoing drug development efforts for cancer and other diseases. These have recently been reviewed (Baud and Karin, 2009). A multitude of cancers have alterations in NF-κB signaling. Since there are many different pathways to activate NF-κB, different tumor types rely on these different pathways to varying extents.

Multiple myeloma (MM) exemplifies NF-κB-dependent tumorigenesis. It is the first malignancy for which an agent with NF-κB-inhibitory activity (bortezomib, also known as PS-341/Velcade^{TM}) was approved by the FDA. Analogous to AIDS-associated viral lymphoma, NF-κB activity is elevated in MM, and MM cells in culture are sensitive to NF-κB inhibitors. However, in distinction to AIDS-associated viral lymphomas, NF-κB in MM is activated as a result of genetic

alterations (Annunziata et al., 2007; Keats et al., 2007). In addition to tumor cell autonomous activation, NF-κB is also activated in bone marrow stromal cells on which MM relies for paracrine growth factors.

HIV/AIDS and Malignancies

Approximately 20% of deaths in persons with HIV are due to malignancies in the HAART era. Epidemiological studies have demonstrated an increased incidence of many cancer types in persons with HIV compared to the HIV-seronegative population (Engels et al., 2006). Three categories of malignancies have been classified by the Center for Disease Control as AIDS-defining illnesses: Kaposi's sarcoma (KS), intermediate- or high-grade B-cell lymphomas, and cervical carcinoma. While the risks of each of these "AIDS-associated" cancers are dramatically elevated in the HIV population, many other malignancies have increased incidences in the setting of HIV, some on the order of that seen with the AIDS-associated cancers. A recent WHO survey added cancers of the cervix, anus, and conjunctiva as HIV associated. It further noted that increased risk for cancer of the vulva, vagina, penis, non-melanoma skin cancer, and hepatocellular carcinoma was associated with HIV infection, though evidence in humans was limited (Bouvard et al., 2009). In general, viral-associated cancers show the most dramatic increases in incidence in persons with HIV (see Table 1 and Table 2).

Table 1 Cancer risk in AIDS patients 1996–2002 from Engels et al. (2006)

AIDS-defining cancers	Percent of total cancers in AIDS	Standardized incidence ratio: SIR (95% CI)
Kaposi's sarcoma[a]	30	3,640 (3,330–3,980)
Non-Hodgkin lymphoma[a]	34	22.6 (20.8–24.6)
Diffuse large B-cell NHL[a]	16	29.6 (26.1–33.3)
CNS NHL[a]	7	1,020 (838–1,220)
Cervical cancer[a]	1.8	5.3 (3.6–7.6)

[a]Denotes malignancy commonly associated with a specific virus
Standardized Incidence Ratio (SIR): The ratio of the observed to the expected new cases of cancer; the expected number is based on the age-specific rates for all of US population

NF-κB in AIDS-Associated Viral Oncogenesis

Human viruses are associated with 30% of human cancers (see Table 3) and are central to the pathogenesis of AIDS-defining malignancies (reviewed in Carbone et al., 2008). HIV induces the immune suppression that eventually leads to AIDS; this includes impairment of immune surveillance important for suppression of early malignant clones. HIV may also play more direct roles in promotion of tumors through induction of adhesion molecules, growth factors, and cytokines that support tumors.

Table 2 Cancer risk in AIDS patients 1996–2002 from Engels et al. (2006)

Non-AIDS-defining cancers	Percent of total cancers in AIDS	Standardized incidence ratio: SIR (95% CI)
Anal cancer[a]	2.6	19.6 (14.2–26.4)
Larynx	1.0	2.7 (1.6–4.4)
Lung	6.7	2.6 (2.1–3.1)
Liver[a]	1.2	3.3 (2.0–5.1)
Myeloid and monocytic leukemia	0.7	2.2 (1.1–4.0)
Hodgkin lymphoma[a]	4.4	13.6 (10.6–17.1)
Total non-AIDS-defining cancers	34	1.7 (1.6–1.9)

[a]Denotes malignancy commonly associated with a specific virus

Table 3 Human viruses and their associated cancers

Virus	Associated malignancies
Epstein–Barr virus (EBV)	Lymphoma: Burkitt's, Hodgkin, DLBCL, plasmablastic, immunosuppression related; nasopharyngeal carcinoma
Hepatitis viruses (HBV and HCV)	Hepatocellular carcinoma
Human papillomaviruses (HPVs)	Cervical, anal, penile cancers Squamous cell carcinoma of skin, head and neck
Human T-lymphotrophic virus type 1 (HTLV-1)	Adult T-cell leukemia
Kaposi's sarcoma-associated herpesvirus (KSHV/HHV8)	Kaposi's sarcoma, plasmablastic lymphoma primary effusion lymphoma, MCD
Merkel cell polyomavirus	Merkel cell carcinoma

Human papilloma viruses (HPVs) are associated with anal cancer in HIV-positive men who have sex with men (MSM). Kaposi's sarcoma-associated herpesvirus (KSHV) causes the AIDS-defining malignancies Kaposi's sarcoma (KS) and primary effusion lymphoma (PEL) (an exhaustive review of KSHV is provided in Damania and Pipas, 2009). Epstein–Barr virus (EBV) causes AIDS-defining central nervous system (CNS) lymphoma and is associated with 90% of Hodgkin disease (HD) in HIV-seropositive individuals versus 30% in the HIV-seronegative population (Glaser et al., 2003). HD itself is not technically an AIDS-defining illness, but there has been an increasing incidence of HD in HIV-positive patients since the availability of long-term highly active anti-retroviral therapy (HAART) (Biggar et al., 2005; Engels et al., 2006). EBV is also associated with Burkitt's lymphoma (BL) and diffuse large B-cell lymphoma to a greater degree than is seen in HIV-seronegative populations of North America (Bonnet et al., 2006). Both KSHV and EBV belong to the gamma herpesviridae family.

NF-κB is a central player in the interface of human cells and many viruses – including KSHV, EBV, and HIV as will be detailed below. NF-κB regulates the

latent status of KSHV and EBV, with higher NF-κB levels suppressing reactivation of their viral lytic gene promoters (Brown et al., 2003). In turn, KSHV and EBV both activate NF-κB, further promoting their persistence in a latent state (Izumi et al., 1997; Matta and Chaudhary, 2005). As the latent viral genome expresses few gene products, latently infected cells are less visible to the immune system compared to cells containing viruses in the lytic state, where all viral proteins are expressed.

Gamma Herpesviruses and NF-κB

Upon infection and perhaps initial replication in oral epithelial cells, KSHV, EBV, and their mammalian homologs rhesus rhadinovirus (RRV) and murine gamma herpesvirus 68 (MHV-68) establish long-term latency in B cells (Flano et al., 2000, 2002; Usherwood et al., 1996). In the case of EBV, memory B cells have been identified as the predominant latent reservoir (Babcock et al., 1998; Hochberg et al., 2004). Hence, it is not surprising that the gamma herpesvirus life cycle is linked to B-cell-specific transcription factors such as NF-κB.

The impact of NF-κB inhibition on gamma herpesviruses differs between viruses and between the latent and the lytic life cycle of the virus. Much of our knowledge is influenced by the particulars of the experimental systems that have been developed for each tumor type and virus. The bulk of studies rely on Bay11-7082 as the prototypical experimental NF-κB inhibitor. Because Bay11-7082 is an irreversible inhibitor, any lymphoma that depends on NF-κB activity is exquisitely sensitive to this agent (Cahir-McFarland et al., 2000; Keller et al., 2000; Kim et al., 2005; Mori et al., 2002; Pham et al., 2003). Seemingly conflicting reports exist for the role of NF-κB in gamma herpesvirus pathogenesis and may be attributed to overly generous generalizations from a singular drug and experimental system. A summary of current knowledge on the gamma herpesviruses and their effect on NF-κB activation is shown in Table 4.

Table 4 Human gamma herpesviruses and NF-κB

Virus	Viral protein	Effect on NF-κB	References
KSHV	vFLIP (orf 71, K13)	Homology to caspase-8, interacts with IκB kinase complex to activate NF-κB	Chugh et al. (2005), Guasparri et al. (2004)
	K1	Activate	Lee et al. (2003), Prakash et al. (2002)
	K15	Activate	Brinkmann and Schulz (2006)
	vGPCR (orf 74)	Indirectly activates NF-κB, both autocrine and paracrine	Martin et al. (2008)
EBV	LMP1	TNF receptor homolog; activates both canonical and classical pathways	Shair et al. (2007)

Murine Gamma Herpesvirus 68 (MHV-68)

MHV-68 provides the best in vivo model of gamma herpesvirus infection, because both host and virus can be genetically manipulated. Active NF-κB inhibits lytic MHV-68 replication in cultured fibroblasts which have low endogenous NF-κB activity (Brown et al., 2003).

In the mouse, NF-κB is required for the establishment of MHV-68 latency in lung B cells, since an IκB expressing MHV-68 virus failed to establish latency, whereas acute replication in lung tissue was not affected (Krug et al., 2007). This result is consistent with a B-cell-specific role for NF-κB, since MHV-68 replicates first in lung epithelial cells before seeking a B cell as the latent reservoir. The story is more complicated, however, since administration of MHV-68-IκB virus by i.p. injection was not associated with any phenotype. Administration route (oral, nasal, intraperitoneal)-specific phenotypes are well known for experimental viral infections. These route-specific phenotypes may be a result of the targeting of different cell types, which can differ in their requirement for classical – IκB-dependent – NF-κB pathway activation.

Bortezomib (PS-341/Velcade^{TM}) induces MHV-68 reactivation from latency in MHV-68-infected mice (Hwang et al., 2008), which supports a role for NF-κB in maintaining viral latency and/or the survival of MHV-68 latently infected B cells. Unlike studies in EBV- and KSHV-associated lymphomas, which are driven by confounding genetic alterations in addition to gamma herpesvirus protein expression and which often exhibit abnormally high levels of active NF-κB complexes, these experiments addressed the role of bortezomib in normal murine B cells exhibiting physiological levels of NF-κB.

Kaposi's Sarcoma Herpesvirus (KSHV) and NF-κB

The interaction between KSHV and NF-κB appears to play a direct role in malignant transformation of fibroblasts and endothelial cells. Lytic KSHV replication in fibroblasts induces NF-κB activity, as addition of Bay11-7082 inhibited NF-κB and KSHV gene expression in this context (Sadagopan et al., 2007). KSHV's viral FLICE-inhibitory protein (vFLIP), orf71/K13, has structural homology with the prodomain of caspase-8 and has been shown to protect cells against death receptor-induced apoptosis (Thome et al., 1997). We now know that the viral orf71, also known as vFLIP, is a potent inducer of NF-κB activity and that this, rather than its FLICE-inhibitory activity, may be the reason for its oncogenic phenotype (Chugh et al., 2005; Guasparri et al., 2004). vFLIP has been shown to transform fibroblasts through an NF-κB-dependent mechanism (Sun et al., 2003). It interacts directly with IκB kinase complex to activate NF-κB (Matta et al., 2007). This has been demonstrated consistently in every PEL cell line where vFLIP has been found to be consistently expressed as part of a viral latency loop (Dittmer et al., 1998).

In addition to vFLIP, KSHV encodes at least two transmembrane proteins K15 and K1, which activate NF-κB signaling and support B-cell survival and

proliferation analogous to the LMP proteins of EBV (reviewed in Brinkmann and Schulz, 2006). K1 and K15 are both known to activate NF-κB (Brinkmann and Schulz, 2006; Lee et al., 2003; Prakash et al., 2002). Though both proteins also affect other signaling pathways, e.g., Akt (Wang et al., 2006). It is as yet unclear whether all pathways are needed for their transforming abilities or whether NF-κB activation is an indirect result of cellular transformation.

NF-κB inhibition is promising for the treatment of KSHV-related tumors (see below). PEL cells rapidly apoptose in response to NF-κB inhibition by Bay11-7082 (Keller et al., 2000). The induction of cell death following NF-κB inhibition is likely in part due to KSHV's ability to induce NF-κB activity through the expression of vFLIP/orf71. PEL cells also rapidly apoptose in response to bortezomib (PS-341/Velcade^{TM}) (Abou-Merhi et al., 2007; Matta and Chaudhary, 2005), which inhibits NF-κB and subsequently induces KSHV replication. Case studies have probed the clinical efficacy of bortezomib against PEL, at least in combination with other agents (Boulanger et al., 2008; Siddiqi and Joyce, 2008). However, a definitive answer is missing and a properly controlled clinical trial is urgently needed.

Epstein–Barr Virus (EBV) and NF-κB

EBV-driven tumor cells, such as type III latency lymphoblastoid cell cultures (LCL), depend on constitutively high NF-κB activity, as addition of Bay11-7082 inhibited NF-κB-dependent transcription and cell survival (Cahir-McFarland et al., 2004). Importantly, a dominant negative IκBα mutant had the same effects as Bay11-7082 in this model, lending credence to the NF-κB-specific phenotypes (Cahir-McFarland et al., 2000).

Bortezomib (PS-341/Velcade^{TM}) also is effective against EBV lymphomas in preclinical testing. This was attributed to NF-κB inhibition and proven to induce subsequent EBV replication in culture and in an elegant murine xenograft model (Fu et al., 2008, 2007; Srimatkandada et al., 2008; Zou et al., 2007).

EBV-driven BL cells, which exhibit type I latency during early passage in culture, also depend on constitutively high NF-κB activity. Addition of zidovudine (AZT), which inhibits NF-κB DNA binding activity at least transiently, induced EBV viral reactivation and cell death (Kurokawa et al., 2005; Lee et al., 1999). Importantly, AZT also had clinical anti-tumor activity against EBV-positive CNS lymphoma (Raez et al., 1999).

The EBV-encoded latent membrane protein 1 (LMP1) is a functional homolog of the tumor necrosis factor receptor family. EBV LMP1 constitutively activates NF-κB pathways (Shair et al., 2007). It is believed to mimic CD40L engagement in B-cell activation (Uchida et al., 1999). Both the canonical and non-canonical pathways can be activated by LMP1 depending on cell type, and activation of NF-κB appears essential for transformation by LMP1 in type III latency (Devergne et al., 1998; Kulwichit et al., 1998). NF-κB activation is mediated by either of two essential, non-redundant transforming domains (CTAR-1 or CTAR-2) and involves TRAF and TRADD (Kaye et al., 1995; Shair et al., 2008).

NF-κB, Proteasome Inhibition, and Gamma Herpesviruses

The ubiquitin-proteasome pathway plays an important role in eukaryotic cell cycle regulation and is a crucial regulator of NF-κB signaling (reviewed in Ghosh, 2007). NF-κB is a central player in the interface of human cells and many viruses – including KSHV, EBV, and HIV. NF-κB regulates the latent status of KSHV and EBV, with higher NF-κB levels suppressing reactivation of their viral lytic gene promoters (Brown et al., 2003). In turn, KSHV and EBV both activate NF-κB, further promoting their persistence in a latent state (Izumi et al., 1997; Matta and Chaudhary, 2005). As the latent viral genome expresses few gene products, latently infected cells are less visible to the immune system compared to cells containing viruses in the lytic state, where all viral proteins are expressed. Therefore, NF-κB inhibition may be useful for treating gamma-herpesvirus-related malignancies via induction of the lytic viral state within the tumor cells. In the setting of gamma-herpesvirus-related malignancies, the induction of the viral lytic state would therefore be expected to improve immune recognition of the malignant cells in addition to causing tumor cell lysis.

There is accumulating preclinical data supporting the strategy of NF-κB inhibition for gamma-herpesvirus-related malignancies. Bortezomib (Velcade^{TM}/PS-341) is the only inhibitor of the human 26S proteasome that is FDA approved for clinical use (multiple myeloma and mantle cell lymphoma). Proteasome inhibitors represent a novel class of chemotherapeutic agents with NF-κB-inhibitory activity. Bortezomib has been shown to stabilize cellular proteins involved in suppressing cellular proliferation and promoting apoptosis including p21, p27, p53, and IκBα (Boccadoro et al., 2005). As seen in Fig. 1, IκBα is the principal inhibitor of the NF-κB p50:p65 dimer in the cytoplasm. It is first activated by IκB kinase and then degraded via the 26S proteasome. This frees the NF-κB p50:p65 to translocate into the nucleus and activate pro-growth genes.

Bortezomib inhibited cellular proliferation and induced apoptosis in cell lines derived from KSHV-positive primary effusion lymphoma (PEL), a subtype of non-Hodgkin lymphoma predominately occurring in persons with HIV. In culture, bortezomib demonstrated cytotoxicity against PEL cell lines, often at lower concentrations than for cell lines derived from MM, a disease for which bortezomib is FDA approved (Abou-Merhi et al., 2007; An et al., 2004; Boulanger et al., 2008; Matta and Chaudhary, 2005). Apoptosis induced by bortezomib was associated with inhibition of the classical and alternative NF-κB pathways, activation of the caspase cascade, and tumor suppressors such as p53. Notably p53 is not mutated in PEL and can mediate apoptosis upon activation (Petre et al., 2007). Treatment of these PEL cell lines with bortezomib exerted a synergistic or additive cytotoxic effect in combination with other chemotherapeutic drugs (Matta and Chaudhary, 2005).

Recently an AIDS lymphoma cell line, 2F7-RR1, selected for resistance to rituximab and other standard lymphoma drugs was evaluated for sensitivity to bortezomib and the NF-κB inhibitor dehydroxymethylepoxyquinomicin (DHMEQ). Treatment with either of these two agents resulted in reversal of chemoresistance

to rituximab and other agents (Vega et al., 2008). This is consistent with the idea that NF-κB inhibition triggers cell death by multiple pathways including those that remain functional in otherwise chemoresistant lymphoma cells.

Proteasome inhibition would be expected to have an additional mechanism of activity in gamma-herpesvirus-related malignancies since NF-κB inhibits lytic activation of tumor cells latently infected with the viruses. Indeed, in samples from a patient with an EBV-related polymorphic B-cell lymphoma treated with bortezomib and ganciclovir, EBV viral loads increased after treatment and then fell to nearly 10% of baseline values after four total doses of the combination (Bagni, Whitby, Dittmer, unpublished). Ganciclovir is an inhibitor of viral polymerases and at high intracellular concentrations also has cytotoxic activity. It requires a viral kinase for conversion of the prodrug into its active form. One possible model to explain this clinical observation is that bortezomib, by inducing EBV lytic protein expression in the tumor cells, also induced the EBV kinases, which in turn rendered the infected cell susceptible to killing by ganciclovir.

Bortezomib disrupts KSHV latency and induces apoptosis in KSHV- and EBV-infected lymphoma cell lines (Keller et al., 2000). Bortezomib was identified in a recent screen of 2,700 FDA-approved drugs as the most active in induction of the EBV lytic cycle. In this study, bortezomib led to EBV-TK upregulation and increased EBV viral copy number. Similar viral copy increases occurred with transfection of IκB. Bortezomib also activated lytic EBV and KSHV in vitro and in a murine xenograft model using human lymphoma lines (Fu et al., 2008).

The fact that bortezomib potently induces lytic activation of EBV and HHV-8 and has excellent in vitro activity against PEL cells suggests a possible role for viral lytic activation in tumor cell death. The ability of replicating viral DNA to induce cell death may be due to additional mechanisms rather than lysis due to virion egress. It is well established that lytic viral replication can result in clinical tumor regression in the setting of oncolytic viral therapy. Onyx-015, an adenovirus designed for tumor-selective replication and viral-mediated lysis of tumor cells, has demonstrated lytic activity in a wide variety of tumor cell lines in vitro and robust lytic activity in mouse xenograft models (Heise et al., 1997). Clinical responses have been observed in both metastatic colorectal cancer and head and neck carcinoma, and lytic viral replication was documented in each setting (Nemunaitis et al., 2000; Reid et al., 2005). Notably, inflammatory responses as measured by TNF, gamma interferon, IL1, IL2, and IL6 occurred in treated colorectal cancer subjects (Reid et al., 2001). The colon cancer study of combined Onyx-015 with 5-FU demonstrated persistent viremia and both radiographical and biochemical evidence of inflammation centered at the tumor masses. These parameters demonstrate an immune response to viral-mediated replication and/or tumor cell lysis, which correlated with improved longer term clinical response even in the setting of heavily pre-treated patients who were receiving concurrent cytotoxic chemotherapy.

In summary, there is much preclinical evidence that NF-κB inhibitors are active in different lymphoma cell lines and xenograft lymphoma models associated with gamma herpesviruses. In the setting of EBV- and KSHV-associated malignancies,

NF-κB inhibition has been shown in both preclinical and clinical settings to result in reactivation of these viruses. Viral activation of tumor cells latently infected with viruses would be expected to be beneficial for (i) its direct cytotoxic effects, (ii) increased expression of lytic viral proteins known to elicit a strong adaptive immune response, and (iii) induction of a pro-inflammatory (innate) immune response.

Risks of Viral Lytic Activation Strategies

A potential risk of inducing lytic activation of viruses is raised when considering other syndromes associated with viral replication. KSHV is involved in the pathogenesis of several conditions, some of which, on average, are more life-threatening than KS; these include the plasmablastic variant of multicentric Castleman's disease (MCD) and primary effusion lymphoma (PEL). There are also isolated cases of gamma-herpesvirus-associated viremia and hemophagocytic syndrome (Stebbing et al., 2008; Sullivan et al., 2008).

While KSHV infects endothelial/spindle cells in KS, it infects lymphocytes in both PEL and MCD. KSHV latent gene products, including v-FLIP, appear to dominate the malignant states of both KS and PEL; the virus is considered to be predominantly in a latent state within both of these tumors. By contrast, in the setting of MCD, high levels of KSHV lytic gene products – including ORF5, ORF59, ORF65, and K8 – are typically found, particularly in the mantle zone region of the affected lymph nodes (Abe 2006). Clinically MCD is characterized by systemic inflammation with elevated host IL-6, C-reactive protein, lymphadenopathy, splenomegaly, fevers, hypotension, pancytopenia, hypoalbuminemia, and oligo-/mono-clonal gammopathy. MCD can lead to death via respiratory, renal, or other organ failure related to systemic inflammation. It follows that in driving lytic activation of KSHV, there is a theoretical risk of precipitating MCD or a similar life-threatening systemic inflammatory reaction. It is also possible that the viremia that results may lead to infection of additional lymphocytes and/or endothelial reservoirs which may in turn increase the long-term risk of PEL, other KSHV-associated lymphoproliferative diseases, or KS. Similar risks may apply to lytic activation strategies of other viruses.

Proteasome Inhibition and HIV

Improved control of HIV is associated with improved response rates and survival for many HIV-related malignancies (Bower et al., 1999; Hoffmann et al., 2003). Preclinical data suggest that at least one class of NF-κB inhibitors has activity against the HIV itself: proteasome inhibitors.

Humans have an innate defense mechanism against retroviral infections employing a cytosine deaminase, APOBEC3G (also known as CEM-15). APOBEC3G is incorporated into nascent retroviral virions as they bud from an infected cell

(Mangeat et al., 2003). As the nascent virion infects a cell and begins reverse transcribing its genes into proviral DNA to be integrated into the host genome, APOBEC3G hypermutates the proviral DNA leading to termination of viral replication and further infection (Fig. 2a). HIV has adapted to this antiviral pathway by using one of its accessory genes, *Vif* (virion infectivity factor), to circumvent the human defense mechanism (Mehle et al., 2007). VIF induces ubiquitinization of APOBEC3G, thereby targeting this factor for proteasomal degradation. This effectively shortens the half-life of APOBEC3G within the cell and prevents its incorporation into new virions, antagonizing the host's antiviral response (Fig. 2b). Proteasome inhibition would be expected to reverse the effects of VIF by slowing the degradation of APOBEC3G and allowing it to be incorporated into budding HIV virions. In support of this mechanism, in vitro studies have shown that inhibiting the 26S proteasome impairs HIV viral budding and infectivity (Fig. 2c) (Lassot et al., 2007; Piccinini et al., 2002).

Therefore, proteasome inhibitors may have a role in controlling HIV spread. Additionally, since improved control of HIV infection is associated with better outcomes in AIDS-KS and AIDS lymphoma patients, potential suppression of HIV replication by proteasome inhibitors may provide additional survival benefits to patients with these AIDS-related malignancies beyond that related to any anti-tumor activity.

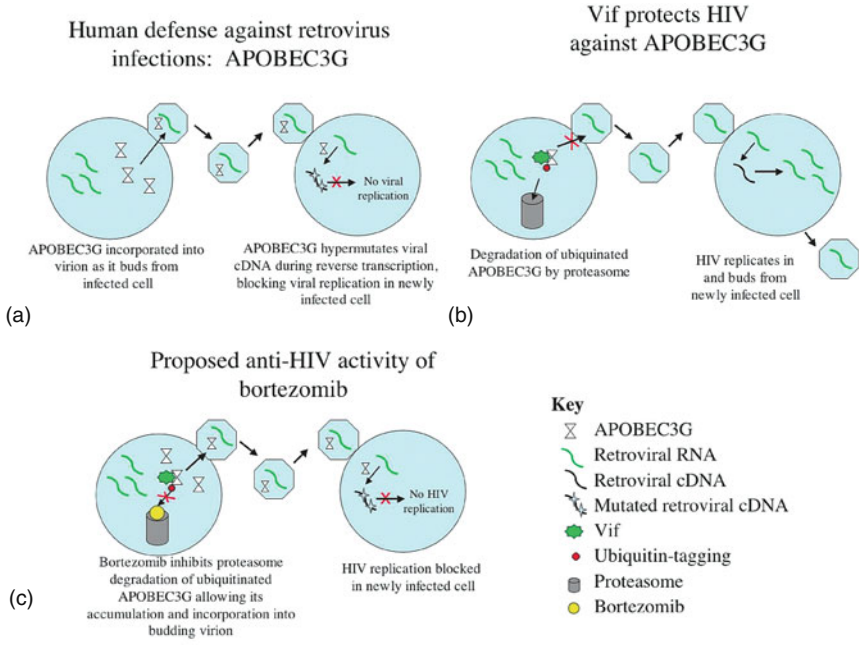

Fig. 2 Mechanism of hypothesized anti-retroviral effects of proteasome inhibitors like bortezomib

Summary and Future Directions

In conclusion, viruses are a common theme in HIV malignancies and NF-κB plays a central role in the interface of human cells and viruses. The high association of HIV malignancies with viruses provides an opportunity to approach these malignancies with strategies that capitalize on their viral associations. The NF-κB pathway and the proteasome-ubiquitin pathway are logical targets for such strategies. The AIDS Malignancy Consortium has developed clinical trials to assess the anti-tumor and viral effects of the proteasome inhibitor, bortezomib, in gamma-herpesvirus-associated malignancies in persons with HIV. While such a strategy is promising based on the preclinical data summarized here, risks related to interruption of viral latency are considerable, underscoring the importance of studying this in the context of formal clinical trials.

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The Molecular Basis of Lytic Induction Therapy in Relation to Gamma herpesvirus (KSHV, EBV)-Associated, AIDS-Related Tumors

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Abstract The frequent presence of the EBV and KSHV genomes in AIDS-related lymphomas has suggested that manipulation of EBV and/or KSHV viral gene expression in these tumors might be used to promote tumor cell death, while sparing uninfected normal cells. Although EBV and KSHV can infect B cells for the life of the host, following recovery from primary infection, very few normal B cells are infected (usually fewer than one in a million B cells in the case of EBV-infected individuals) (Babcock et al., 2001, Methods Mol Biol 174, 103–110; Hochberg and Thorley-Lawson, 2005, Methods Mol Biol 292, 39–56). In contrast, essentially all of the tumor cells of EBV- and/or KSHV-associated malignancies contain the virus. Thus, the development of therapies that can induce killing of all EBV-infected cells in the body would be expected to dramatically reduce tumor cell viability, while potentially having no effect on healthy cells. One of the most promising approaches for inducing specific killing of EBV-infected host cells is commonly referred to as "lytic induction" therapy (Gutiérrez et al., 1996, Cancer Res 56, 969–972; Westphal et al., 1999, Cancer Res 59, 1485–1491; Israel and Kenney, 2003, Oncogene 22, 5122–5130). As the name implies, the goal of this approach is to convert the latent type of EBV infection that normally occurs in tumor cells into the lytic form of viral infection, thereby using the virus itself to kill tumor cells. Although this type of approach is potentially applicable for both EBV-associated and KSHV-associated malignancies, the development of EBV-directed lytic induction therapies is further advanced and thus will be the primary focus of most of the discussion herein.

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Introduction

The two members of the human gamma herpesvirus family EBV and KSHV are the major cause of opportunistic malignancy among patients with AIDS as well as other forms of congenital and acquired T-cell deficiency. In fact, the development of either Kaposi sarcoma (KS) or non-Hodgkin lymphoma (NHL) among young adults with evidence of newly acquired immune compromise became a criterion for the diagnosis of AIDS during the very earliest stages of the HIV epidemic – even before HIV was discovered and before the viral etiology of the respective tumors was confirmed (Cheung et al., 2005). In recent years the widespread introduction of HAART accompanied by T-cell reconstitution has resulted in a marked decrease in new onset of KS and to a lesser extent of NHL. In addition, spontaneous remission of certain cases of seemingly established disease have been noted (Cheung et al., 2005; Wood and Harrington, 2005; Bernstein et al., 2006). The observation that these tumors arise predominantly in the immunocompromised host and that disease progression can often be reversed upon restoration of cellular immunity indicates that many of the defining cellular events leading to tumorigenesis have not fully taken place and that the virus life cycle remains a driving force in cell survival and proliferation.

AIDS-related NHLs are usually of B-cell origin and are much more likely to be associated with EBV than KSHV. The most common AIDS-related lymphoma is diffuse large B-cell lymphoma. This type of lymphoma occurs in patients with very low CD4 cell counts, is often extra-nodal or localized to the central nervous system, and typically contains EBV genomes (Knowles, 1997). Burkitt lymphomas also occur in AIDS patients much more frequently than in the general population (Biggar et al., 2007); however, only half of HIV-related Burkitt lymphomas are reported to contain EBV genomes (Kersten et al., 1998). In contrast, AIDS-related T-cell lymphomas, although rare, do usually contain EBV (Biggar et al., 2001; Arzoo et al., 2004). Children with AIDS have a much increased frequency of the smooth muscle tumor known as leiomyosarcoma, and these tumors (in contrast to leiomyosarcomas occurring in normal hosts) also contain EBV (Jenson et al., 1997). Most Hodgkin lymphomas (HLs) that occur in patients infected with HIV contain EBV genomes (Uccini et al., 1989); however, as with several other malignancies that are detected more often in HIV-infected individuals, HLs are not considered to be AIDS defining as they develop among persons with less profound immunocompromise (Bigger et al., 2006). This may also be the case for some BLs (Clifford et al., 2005). Although EBV genomes are uniformly detected in the tumors of patients with NPC and variably in gastric carcinomas and certain other epithelial cancers, these EBV-associated epithelial tumors do not occur with increased frequency among patients with AIDS (Engels et al., 2008).

In populations where the seroprevalence of KSHV is high, the vascular endothelial cell tumor KS rather than NHL is the most common AIDS-related malignancy. KSHV is uniformly detected in KS (Chang et al., 1994) as well as in two AIDS-associated B-cell proliferative disorders, MCD, a nodal disease characterized by the expression of both lytic and latent KSHV gene products, and a rare AIDS-defining lymphoma known as primary effusion lymphoma (PEL) (Cesarman et al., 1995;

Oksenhendler et al., 2002). These extremely unusual lymphomas occur in serous body cavities and do not typically form a solid mass. Strikingly they are co-infected with EBV in 90% of cases.

Even though the frequency of most types of AIDS-related malignancies (including Kaposi sarcoma, diffuse large B-cell lymphomas, and primary effusion lymphomas) has greatly decreased since the introduction of highly active retroviral therapy (HAART), the risk for these malignancies is still at least 20-fold higher among HAART-treated, HIV-positive patients than for the general population (Clifford et al., 2005). Furthermore, the frequency of Burkitt lymphoma has remained unchanged, and the frequency of Hodgkin lymphoma has actually increased (Clifford et al., 2005; Engels and Goedert, 2005; Biggar et al., 2006). The persistence of AIDS-related Burkitt lymphomas and Hodgkin lymphomas in the era of HAART likely reflects the fact that these tumors occur in largely immunocompetent, as well as immunodeficient, individuals. Indeed, increasing evidence suggests that abnormal immunostimulation (such as occurs during immune reconstitution syndromes following HAART therapy) may contribute to the development of Hodgkin lymphoma (Said, 2007). Thus, AIDS-related malignancies continue to be an important cause of HIV-related morbidity and mortality even in the age of HAART.

Viral proteins involved in the maintenance of the latent state, as well as those that regulate the lytic switch leading either to efficient cell death and/or to expression of viral enzymes that uniquely activate cytotoxic prodrugs (in the event that the lytic cycle is not completed), all provide rational targets for reversing viral tumorigenesis, importantly with far greater specificity than is provided by currently employed treatments. When immune restoration or immune targeting approaches fail or when they are not possible, virus-directed drugs would theoretically limit the need for global approaches such as aggressive chemotherapy (which can produce mortal side effects and predisposes to secondary malignancies) and/or pan-active monoclonal antibodies such as anti-CD20 (which destroys all mature B cells thereby increasing the risk of life-threatening infections). Many laboratories are currently searching for therapeutic agents to directly block the activities of EBNA1 (EBV), LANA (KSHV), and other latent proteins required to maintain viral latency and promote uncontrolled proliferation. In this chapter, we explore how the virus life cycle, its transitions, and the expression of specific virus lytic cycle proteins may also provide a molecular basis for treatment of EBV- and KSHV-associated tumors in patients with AIDS and other forms of profound T-cell compromise.

Latent vs. Lytic EBV Infection

Like all herpesviruses, EBV can infect cells in either a latent or a lytic form (Thorley-Lawson and Gross, 2004). During latent infection, only a small number of viral proteins are expressed, and the viral genome is replicated by the host cell replication machinery once per cell cycle. The viral proteins expressed during latent infection do not kill the host cell but instead may contribute to survival and sometimes tumorigenesis. Drugs that can specifically inhibit the latent form of

EBV infection are not currently available. During lytic infection, EBV expresses approximately 80–100 different lytic viral proteins, and the virus-encoded DNA polymerase replicates the viral genome. Lytic replication is required for the production of infectious viral particles and generally kills the host cell. Certain nucleic acid analogue drugs that can block lytic EBV and KSHV replication are available, as will be further discussed.

Both the latent and lytic forms of EBV infection are required for the long-term persistence of EBV in its human host. Latent EBV infection, which occurs in memory B cells, allows the virus to survive indefinitely in the host, since cells which are latently infected are not efficiently eliminated by the host immune response (Thorley-Lawson, 2001). Thus, the latent form of infection ensures that no host is ever "cured" of EBV infection and provides the reservoir of infected cells from which the virus can periodically reactivate to the lytic form. Since the lytic form of EBV infection is essential for production of infectious viral particles, this form of infection is essential for the ability of the virus to infect new hosts.

In the healthy host, after recovery from primary illness (which can be either asymptomatic or associated with the infectious mononucleosis syndrome), neither the latent nor the lytic forms of EBV infection generally cause clinical illness. However, immunocompromised patients are not able to adequately eliminate cells undergoing either latent or lytic forms of EBV infection. Lytic EBV infection in immunocompromised patients can produce a lesion known as oral hairy leukoplakia, which is due to uncontrolled lytic viral replication within lingual epithelial cells (Greenspan and Greenspan, 1992). This lesion is of low clinical impact and is never life threatening. Although it is usually self-limited, it has been reported to respond to AZT, acyclovir, ganciclovir, foscarnet, and topical podophyllin or isotretinoin. In contrast, the inability of the impaired host immune system to eliminate latently infected B cells, combined with the potent immortalizing properties of certain latent EBV proteins (particularly the combination of viral proteins expressed during the "type III" growth program type of latent infection), can lead to the development of EBV-positive B-cell malignancies (Thorley-Lawson and Gross, 2004).

Control of EBV reactivation. In vivo, the physiologic signal that abrogates latency in the memory B cell and initiates the lytic cycle is believed to be linked to terminal differentiation of the virus-infected B cell into a mature plasma cell. Cellular factors activated as the B cell shifts into its end-stage transcriptional program activate transcription of the viral immediate-early transactivators that can then initiate the lytic cycle cascade. In vitro, EBV infection is converted from the latent to the lytic form in host cells following the expression of the two viral immediate-early proteins, BZLF1 and BRLF1. The BZLF1 and BRLF1 proteins are transcription factors that activate one another's expression, and in combination these two proteins can activate the expression of all the EBV lytic viral proteins (Israel and Kenney, 2003; Amon and Farrell, 2005; Miller et al., 2007). Either the *BZLF1* or the *BRLF1* gene products can convert the virus to the lytic form when transfected into latently EBV-infected cells in vitro under the control of a strong heterologous promoter (Rooney et al., 1988; Zalani et al., 1996; Feng et al., 2002b). However, in latently infected cells, the viral promoters which normally drive expression of the *BZLF1*

and *BRLF1* genes (Zp and Rp, respectively) are not active, and hence the BZLF1 and BRLF1 proteins are not produced (allowing the virus to remain latent). Conversely, when cellular transcription factors, which activate the BZLF1 and/or BRLF1 promoters, are sufficiently expressed in EBV-infected cells, the BZLF1 and BRLF1 proteins are produced, which induces the switch from the latent to the lytic form of EBV infection. Other factors, both cellular and viral, that are currently less well understood determine how efficiently the lytic cycle is completed once it has been robustly initiated by the combined expression of BZLF1 and BRLF1.

Regulation of EBV IE promoters: For lytic induction therapies to be effective, it is apparent that drugs which can efficiently activate either the BZLF1 and/or BRLF1 promoters in tumor cells, ideally without inducing toxicity to normal cells, must be identified. In the normal host, lytic EBV infection can be detected in rare tonsillar B cells that have differentiated into plasma cells. Two reports suggest that the cellular transcription factor XBP-1, which is expressed early in the process of plasma cell differentiation (and is required for such differentiation), activates both the BZLF1 and BRLF1 promoters, helping to explain why EBV often reactivates during plasma cell differentiation (Bhende et al., 2007; Sun and Thorley-Lawson, 2007). In addition to the XBP-1 transcription factor, a number of other cellular transcription factors which positively and negatively regulate the BZLF1 and BRLF1 promoters have been identified. The degree to which these different factors also promote the terminal B-cell differentiation program remains to be defined. Amongst these, cellular factors (including c-jun and ATF-2) which bind to and activate BZLF1 transcription through a CRE motif ("ZII") appear to be critical for reactivation of EBV by a variety of different stimuli (Flemington and Speck, 1990; Speck et al., 1997; Adamson et al., 2000). The transcriptional activity of c-jun and ATF-2 is primarily regulated by phosphorylation, and activation of the kinases which induce phosphorylation of c-jun and ATF-2 (for example, c-jun N-terminal kinase, p38 stress Map kinase, and ERK kinases) is involved in reactivation of EBV by a number of agents, including chemotherapeutic drugs (Feng et al., 2004a, b). A series of MEF2D-binding sites ("ZI" motifs) can function as either positive or negative regulators of the BZLF1 promoter, depending upon whether MEF2D bound to these sites are complexed with type II HDACs (Liu et al., 1997; Gruffat et al., 2002). An important negative regulator of the BZLF1 promoter is the ZEB1 transcription factor, which binds to the "ZV" motif in the promoter (Kraus et al., 2003; Feng et al., 2007). Mutation of the ZV motif in the context of the intact viral genome dramatically enhances the ability of the virus to undergo lytic reactivation in vitro (either spontaneously or in response to TPA/sodium butyrate) (Yu et al., 2007).

Activation of the B-cell receptor (using anti-IgG) also induces the lytic form of EBV infection in many (but not all) EBV-infected B-cell lines in vitro (Takada and Ono, 1989). Furthermore, a large number of other stimuli have been demonstrated to increase the frequency of EBV reactivation in vitro, though with substantial variability and efficacy in different cell types. These include phorbol esters (TPA) (Takada and Zur Hausen, 1984) and other PKC agonists (bryostatin), HDAC inhibitors (sodium butyrate, valproic acid, TSA, and others) (Westphal et al., 2000; Feng and Kenney, 2006; Countryman et al., 2008), calcium ionophores (Daibata et al.,

1990), nucleoside analogues (including iododeoxyuridine and AZT) (Glaser and Rapp, 1972; Kurokawa et al., 2005), certain chemotherapeutic agents (including methotrexate, *cis*-platinum, doxorubicin) (Feng et al., 2002a, 2004a, b), gamma irradiation (Westphal et al., 2000), cytokines such as interferon-α (Tovey et al., 1982), TGF-β (Liang et al., 2002; di Renzo et al., 1994), and other agents. Each of these diverse agents may initiate some part of the cellular transcriptional program that coordinately drives terminal differentiation – and so variably effect lytic induction.

Epigenetic Modifications of the EBV Genome Also Contribute to the Maintenance of Viral Latency

The majority of the viral genome (including the *IE* gene promoters) is methylated in cells with latent infection (Bhende et al., 2004; Chan et al., 2004). Methylation of promoter DNA promotes the formation of an inactive (non-acetylated) chromatin structure and inhibits the ability of cellular transcription factors to activate transcription of nearby genes. Methylation of the BZLF1 and BRLF1 promoters likely inhibits their ability to respond to cellular transcription factors. Consistent with this, 5-azacytidine treatments of certain Burkitt lymphoma lines in vitro (which globally decreases methylation of both cellular and viral DNA) can induce lytic EBV reactivation (Moore et al., 2001). Nevertheless, 5-azacytidine treatments of patients with EBV-positive nasopharyngeal carcinomas were not found to enhance the expression of lytic EBV proteins in these tumors (Chan et al., 2004). This disappointing result likely reflects multiple factors – from differential drug uptake to the distinct effects of azacytidine on chromatin in different cell types. In addition, the observation that the BZLF1 protein (in contrast to cellular transcription factors) preferentially binds to, and transcriptionally activates, the methylated form of the BRLF1 promoter (Bhende et al., 2004) may directly account for why 5-azacytidine treatment decreases the ability of BZLF1 to induce lytic EBV infection in vitro.

Similar to the cellular genome, the EBV genome in latently infected cells is associated with a chromatin structure (Countryman et al., 2008). Thus, viral chromatin modifications such as histone acetylation and histone methylation influence the ability of cellular transcription factors to activate lytic gene expression. Both the BZLF1 and BRLF1 IE promoters are commonly associated with a poorly acetylated (i.e., inactive) chromatin structure in latently infected cells in vitro. Conversely, various histone deacetylase (HDAC) inhibitors have been shown to enhance the degree of lytic reactivation in a number of different latently infected cell lines in vitro, particularly when combined with agents (e.g., TPA or certain chemotherapeutic drugs) which induce the expression of cellular transcription factors known to activate production of BZLF1 and/or BRLF1 RNAs. Nevertheless, in a number of cell lines in vitro, there is a poor correlation between the degree of histone acetylation of the BZLF1 and BRLF1 promoters and their ability to be activated by HDAC inhibitors (Countryman et al., 2008), suggesting that as yet unidentified factors also contribute to the lytic effect of many HDAC inhibitors.

General principles for lytic induction-based therapy of EBV-bearing tumors. The development of lytic induction strategies is based upon the following assumptions, summarized in Fig. 1:

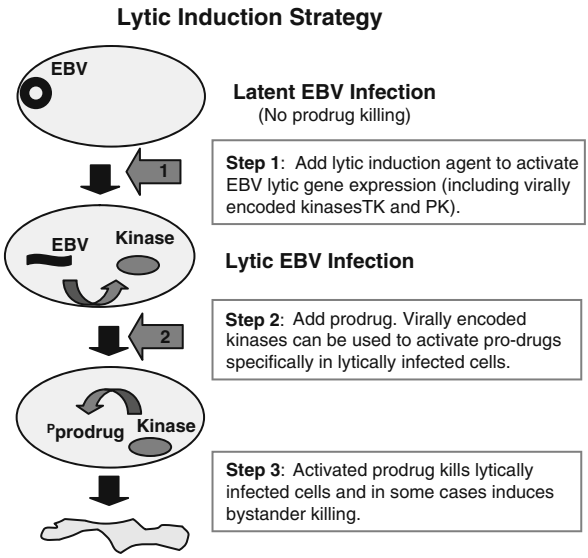

Fig. 1 Lytic induction strategy. The mechanisms by which agents that induce lytic viral gene expression in EBV-positive tumors enhance EBV-dependent cell killing when combined with virally activated prodrugs are summarized

(1) Agents which induce lytic reactivation of EBV-positive tumor cells can be identified, without causing undue toxicity to uninfected cells.

–Lytic EBV reactivation in the tumor cell does not by itself cause serious illness to the host, although it may augment the reservoir of latently infected cells if it is unchecked.

Completion of the EBV lytic cycle will result in the death of the tumor cell and the release of virus. However, it is possible that only a subset of tumor cells will respond or that lytic induction may abort before the virus cycle is completed.

(2) Prodrugs can be coordinately administered that are converted to cytotoxic compounds only by viral protein(s) produced early during the lytic phase in order to augment tumor cell killing.

Additional drugs (this may be the same prodrug or two different drugs) can be administered to eliminate virus production (replication and/or assembly) and prevent dissemination.

Cell death and release of the converted prodrug may produce a bystander effect on nearby tumor cells.

The preclinical and clinical data supporting this paradigm are summarized below.

Identifying drugs which can reactivate EBV in tumor cells. Ample evidence has been provided that the EBV IE promoters are regulated by a variety of different cellular transcription factors (including both positive and negative regulators), as well as by epigenetic modifications such as DNA methylation and histone acetylation. To date, drugs which inhibit HDAC activity have been the most extensively studied agents in regard to their ability to reactivate lytic EBV infection in tumor cells. This initial focus on HDAC inhibitors reflects the fact that a variety of HDAC inhibitors are already FDA approved and known to have an acceptable safety profile (such as the antiseizure drug, valproic acid) or to be in clinical development. Several HDAC inhibitors can increase the number of lytically infected cells in many (but not all) EBV-positive cell lines in vitro. However, in vitro data suggest that the ability of HDAC inhibitors alone to induce the lytic program in most EBV-positive cell lines is relatively weak (with only a minority of cells converting to the lytic form) unless these agents are combined with other lytic-inducing agents (Feng and Kenney, 2006). Nevertheless, somewhat surprisingly the results of a recent small clinical trial suggested that the combination of an HDAC inhibitor (arginine butyrate) plus a nucleoside analogue (ganciclovir) effectively reduced tumor size in some patients with EBV-positive lymphomas that were refractory to conventional therapy (Perrine et al., 2007). This encouraging result, though it must be viewed with considerable caution, suggests that HDAC inhibitors may be more effective in inducing lytic *EBV* gene expression in vivo than would be predicted to be the case from the results in human cell lines, perhaps reflecting the observation that long-term culture selects for cells that cannot be easily converted to the lytic form of infection. Unfortunately, no data were obtained in this study to ascertain whether arginine butyrate actually increased the amount of lytic *EBV* gene expression in patient tumor cells; therefore the proposed mechanism through which this effect was achieved remains to be clinically documented.

Sub-therapeutic doses of several chemotherapeutic agents have also been shown to induce lytic EBV reactivation in vitro, as well as in immunodeficient mice inoculated with EBV-positive tumors (Feng et al., 2002a, 2004a). The killing effect of low-dose chemotherapy is greatly magnified when these drugs are combined with an HDAC inhibitor such as valproic acid (Feng and Kenney, 2006). There is precedence for these agents acting together to elicit tumor cell killing (e.g., preferential uptake by rapidly growing cells followed by cross talk between activated pathways that cause cell cycle arrest, differentiation, and apoptosis). Significantly, however, the synergistic killing effect of low-dose chemotherapy and HDAC inhibitors was shown to be mediated through lytic EBV induction, as it did not occur in cells infected with lytic induction defective EBV mutants. Chemotherapeutic agents activate the BZLF1 and BRLF1 promoters in reporter gene assays performed in EBV-negative cells, and this effect has been shown to be mediated through activation of several different signal transduction mediators, including MAP kinase, PI3 kinase, and p38 kinase (Feng et al., 2002a, 2004a, b). Specific transcription factor-binding sites in the BZLF1 and BRLF1 promoters (including the CRE motif in the BZLF1 promoter) are also required for the chemotherapeutic effect (Feng et al., 2004a, b). Thus, chemotherapeutic drugs induce lytic EBV reactivation by

activating cellular transcription factors that activate the BZLF1 and BRLF1 promoters. Although chemotherapeutic agents have inherent toxicity to normal cells, particularly when given at higher doses, it is important to stress that the discovery that the combination of low-dose chemotherapy and valproic acid induce much more killing of EBV-positive tumor cells in mice transplanted with EBV-positive tumors than does low-dose chemotherapy alone suggests that HDAC inhibitors could likewise be used to allow lower doses of chemotherapy to cure EBV-positive tumors in patients, thereby potentially reducing the toxicity of conventional therapy while simultaneously increasing its efficacy. The specific mechanism(s) through which different HDAC inhibitors potentiate the lytic-inducing effect of chemotherapy has not yet been well studied but is presumed to be mediated through enhanced histone acetylation of the chromatin around the EBV IE promoters.

Similar to the effect of chemotherapy, low-dose gamma irradiation treatment of EBV-positive tumors has been shown to induce the lytic form of EBV infection in EBV-positive tumors cells both in vitro and in mice or rats transplanted with certain EBV-positive tumors (Westphal et al., 2000). The basis for this effect is unknown but may be related to activation of DNA repair pathways and thereby overlap certain effects of chemotherapy. Antibody to the B-cell-specific cell surface antigen CD20, rituximab, which is widely used to treat early stage EBV-positive lymphoproliferative disease, in combination with dexamethasone, also induces the lytic form of EBV infection in at least some B-cell lines (Daibata et al., 2005). The mechanisms responsible for the rituximab/dexamethasone effect have not been defined.

Investigators have also applied small molecule-screening approaches to identify agents with the potential to induce lytic *EBV* gene expression in tumor cells. In a study of 2,700 FDA-approved drugs tested for the ability to activate lytic infection in a Burkitt lymphoma cell line, the antitumor drug bortezomib (a proteasome inhibitor) was identified as the most potent inducer of lytic *EBV* gene expression (Fu et al., 2007). Although bortezomib (which is used to treat mantle cell lymphoma and multiple myeloma) may be a promising agent for reactivating lytic *EBV* expression in patients with Burkitt lymphomas, it does not appear to induce lytic gene expression in EBV-transformed lymphoblastoid cell lines (LCLs) in vitro (a potentially more relevant model for immunoblastic lymphomas in AIDS patients), though it may induce apoptosis in these LCLs by other mechanisms (Zou et al., 2007).

Combining Lytic-Inducing Agents with Lytic Protein-Activated Prodrugs as a Strategy for Maximizing EBV-Dependent Killing of Tumor Cells

Only a subset of EBV-infected cells (generally considerably fewer than 50%) can be converted to the lytic form of EBV infection in vitro using currently available

lytic induction agents (Feng and Kenney, 2006). Therefore, the currently available drugs (such as HDAC inhibitors) may not produce sufficient lytic induction to substantially influence the growth of EBV-positive tumors when used alone. Moreover, accumulating evidence from both in vivo and in vitro studies suggests that the EBV lytic cycle is often abortive – that is to say, although IE proteins are expressed, synthesis of E proteins is not guaranteed and likewise production of late proteins, assembly, and release may not occur (Rea et al., 1994; Laichalk and Thorley-Lawson, 2005). Artificial inducers may also alter the normal stoichiometry of the lytic cascade. At precisely what point a cell that has initiated the lytic switch and more specifically a tumor cell that has initiated the lytic switch is guaranteed to die is not known. Significantly in the course of work by gene therapists who transduced HSV1 thymidine kinase into cells in order to take advantage of the cytotoxic effects of GCV to kill tumors cells, it was successfully demonstrated that even a small number (approximately 10%) of HSV1TK-expressing cells were sufficient to promote efficient killing of an entire tumor mass while sparing normal cells (Culver et al., 1992). These findings imply that released nucleotides (i.e., nucleoside di and triphosphates) can be taken up by nearby cells and are probably preferentially incorporated by tumor cells with augmented metabolic requirements. Despite the long-standing dogma that phosphorylated nucleosides do not cross the plasma membrane, the development of cidofovir, adefovir, tenofovir, and other phosphorylated prodrugs in recent years has shown that this is not uniformly true and that there is a large amount of variation among different cell types (Lee and Martin, 2006). The molecular basis for many of these important differences is just now being uncovered.

Herpesvirus Target Proteins and Candidate Prodrugs: Therapeutic Precedents in Antiviral and Gene Therapy

As knowledge of the functions and enzymatic activities of multiple EBV and KSHV lytic cycle proteins advances, it is likely that many additional drugs that are cytotoxic only to virus-infected cells will be discovered. To date, however, much work has focused on modification of therapeutic strategies that were originally developed to block uncontrolled replication of the human alpha (HSV1/2, VZV) and some beta (primarily CMV) herpesviruses and that were subsequently applied in gene therapy protocols. These antiviral strategies exploit the enhanced ability of the alpha herpesvirus-encoded thymidine kinases and the CMV (which lacks a TK) protein kinase/phosphotransferase (PK/PT) (UL97) to monophosphorylate in a rate-limiting first step the guanine nucleoside prodrugs ACV and GCV compared with relevant cellular enzymes (Dorsky and Crumpacker, 1987; Culver et al., 1992; Crumpacker, 1996). Although the alpha herpesvirus kinases are called thymidine kinases, it is important to note that they are in fact polynucleoside kinases, hence the acceptance of guanine analogues as substrate (Gentry, 1992). The CMV PK/PT (UL97), on the other hand, is a protein rather than a nucleoside kinase and although it does not

phosphorylate dA, dC, dT, or even dG, remarkably it phosphorylates GCV and also ACV (far less efficiently) (Michel et al., 1996; Talarico et al., 1999; Michel and Mertens, 2004). Not surprisingly, UL97 is a less active GCV kinase than are the alpha herpesvirus TKs, which is reflected in clinical practice by greater difficulty in eradicating CMV disease and by the omission of this protein from gene therapy protocols. Following the conversion of ACV and GCV to the triphosphate form which is mediated by cellular nucleoside monophosphate (NMPK) and nucleoside diphosphate (NDPK) kinases, the respective nucleotides are preferentially incorporated into viral DNA by promiscuous viral polymerases. Although ACV and GCV are structurally similar, the presence of an additional hydroxyl group on the acyclic ring of GCV confers greatly enhanced cellular toxicity. ACVTP with its single hydroxyl is a mandatory chain terminator that effectively blocks virus replication on the basis of enhanced incorporation by the viral polymerase. When linked to cellular DNA, ACVTP does prevent chain elongation; however, a new chain free of ACVTP can be preferentially synthesized by cellular polymerases. The mechanism of GCVTP action is distinct. The presence of the additional hydroxyl group facilitates incorporation of GCVTP into viral and cellular DNA. Although DNA replication typically proceeds through one round, at the onset of second-round replication, the aberrant nucleotide (GCVTP) is detected by DNA repair pathways that, once activated, halt further replication and promote apoptosis of the infected cell (Rubsam et al., 1998). After DNA repair systems are recruited, even a single lesion can stimulate apoptosis – hence the enhanced cytotoxicity of GCV. In vivo, major side effects of GCV include suppression of cells that grow or turn over rapidly, particularly white blood cells and platelets. Therefore, similar to many chemotherapeutic agents, but unlike ACV, GCV is a recognized mutagen and teratogen (Thust et al., 2000). Nevertheless, the efficient phosphorylation of and the selective cytotoxicity conferred by GCV following incubation with tumor cells expressing HSV1TK, in addition to the noted bystander effects, have supported ongoing use of this combination in cancer gene therapy protocols. Based on the assumption that the enzymatic activities of the gamma herpesvirus homologues of TK and PK/PT were conserved and would therefore provide endogenous targets for virus-directed therapy of EBV (and KSHV)-associated tumors following lytic induction, therapeutic protocols, some dating back to the early 1990s, and animal studies based on this paradigm were initiated. Though the numbers are limited and data sets incomplete, individual case reports, a small phase 1/2 preclinical trial, and studies in rodents raise the possibility that dual therapy with an inducing agent and GCV may have clinical efficacy (Harrington et al., 1996; Tosi et al., 1997; Mentzer et al., 1998; Raez et al., 1999; Roychowdhury et al., 2003; Perrine et al., 2007; Feng et al., 2004b; Daibata et al., 2005; Wu et al., 2005; Feng and Kenney, 2006; Fu et al., 2007). Surprisingly, however, when the reaction rates and the substrate specificity of the individual gamma herpesvirus enzymes predicted to phosphorylate GCV (TK and PK/PT) were determined by direct experiment, many investigators found that the ability of the homologous EBV and KSHV enzymes to generate GCVMP was poor (Tung and Summers, 1994; Gustafson et al., 1998; Gustafson et al., 2000; Lock et al., 2002). Thus the precise mechanism(s) by which GCV is phosphorylated in cells undergoing

lytic EBV or KSHV infection currently remains a subject of debate and of ongoing investigation. This conundrum may eventually be resolved through understanding of whether additional viral or cellular protein–protein interactions modify TK and PK/PT enzyme function(s). For example, the respective EBV proteins (BXLF1 and BGLF4) were recently reported to interact in a yeast two-hybrid assay (Calderwood et al., 2007) prompting further investigations. Alternatively these enzymes, other lytic viral proteins, or altered expression of cellular proteins resulting from gamma herpesvirus infection or from treatment with inducing agents (or transfection procedures) may independently promote enhanced uptake, phosphorylation, retention, or incorporation of GCVTP into cellular DNA. Definitive knowledge of whether this step is primarily mediated by viral and/or cellular enzymes and of how it is achieved is important for determining how best to direct future research focused on the development of novel antitumor prodrugs that more potently and more selectively destroy virus-infected cells.

Viral Target Proteins Required for Activation of Nucleoside Analogue Prodrugs in Gamma herpesvirus-Infected Tumor Cells

Thymidine kinase (BXLF1 and ORF 21). The EBV genome encodes a homologue of the alphaherpesvirus TKs, localized to the BXLF1 open reading frame (Littler et al., 1986). The C-terminal kinase domain of this enzyme contains five and part of a sixth conserved region that establishes its similarity to alphaherpesvirus TKs (Gentry, 1992). However, a unique 243-amino-acid N-terminus of unknown structure and function is also present. Although the existence of a TK was long suspected, it was not until the late 1980s that the enzyme was cloned and first characterized (Littler et al., 1986; Littler and Arrand, 1988). Preliminary studies using whole lysates from TK-negative bacteria as the source of enzyme suggested that the expressed protein was a polynucleoside kinase like HSV1-TK in that GCV and to a lesser extent ACV could block thymidine phosphorylation (Littler and Arrand, 1988). This distinguished EBVTK from other gamma herpesvirus TKs that had been shown to be strict thymidine kinases (Gentry, 1992). However, when the enzyme was purified from bacterial lysates and subjected to competition assay and later direct kinetic analysis (K_m determination), although EBVTK was confirmed to be a thymidine and even a thymidylate kinase, it demonstrated little additional nucleoside kinase activity (Tung and Summers, 1994; Gustafson et al., 1998). Subsequent investigations of the substrate specificity and kinetics of the related enzyme KSHV TK (ORF 21) yielded similar results, suggesting that the human gamma herpesvirus TKs, like other subfamily members, were predominantly thymidine kinases (Gustafson et al., 2000; Lock et al., 2002). To address the possibility that protein modifications unique to mammalian cells might have altered the properties of these enzymes, both EBVTK and KSHVTK were further investigated. However, when the respective enzymes together with HSV1 TK as a well-characterized control were stably expressed from a human cell line (143B

TK lacking cellular TK1), quantitative analysis confirmed that both the substrate specificity and the K_m of the different proteins were virtually unchanged compared with results from bacteria (multiple clones of the three enzymes were analyzed) (Gustafson et al., 2000). Though semiquantitative in nature, studies from several other laboratories of EBVTK (or KSHVTK) compared with HSV1TK proved consistent – that is, the gamma herpesvirus proteins were far less active TKs than were their alphaherpesvirus orthologues and phosphorylation of cytidine, purines, and their analogues was limited. Ongoing controversy stems not so much from these findings but rather from whether this limited activity can adequately sensitize TK-expressing cells to the guanine nucleoside analogue GCV above the background detected in rapidly growing uninfected cells (given the narrow toxic–therapeutic ratio of GCV). In relation to lytic induction therapy, there is concern that minimally enhanced activity would translate not only into a relatively minor antitumor effect in vivo (a problem for gene therapists using transduced HSV1TK) but also into a relative lack of the desired specificity. On the other hand, these results suggest that GCV may be an active chemotherapeutic agent that could be successfully applied to the treatment of several tumors. EBVTK and KSHVTK do demonstrate enhanced ability to phosphorylate thymidine and many thymidine/uridine analogues, including AZT (Gustafson et al., 2000) and several other analogues that are currently under investigation (Marquez et al., 2006; Prichard et al., 2006). High doses of the widely used HIV reverse transcriptase inhibitor AZT have shown possible combined efficacy together with other agents (e.g. interferon alpha, methotrexate, GCV, hydroxyurea, IL-2) for the treatment of certain AIDS-related virus-associated lymphomas (Harrington et al., 1996; Tosi et al., 1997; Aboulafia et al., 2006), although the major mechanism(s) of action are not entirely known. AZT is poorly phosphorylated in normal uninfected cells and accumulates as AZTMP, leading to feedback inhibition of cellular thymidylate synthetase (TS) (Tornevik et al., 1995). Although TS inhibition blocks cell cycle progression, unlike the lesion produced by GCV, the effect is reversible when drug is removed. However, modest lytic phase stimulation by lytic cycle-inducing agents, including perhaps AZT itself, may significantly increase EBVTK expression causing intracellular accumulation of phosphorylated forms of AZT (Gustafson et al., 2000) and producing marked cytotoxicity in some but not in all virus-infected tumor cell lines (Feng et al., 2004b; Kurokawa et al., 2005). The reasons for the different responses are not currently understood, although they may include differential expression of cellular DNA polymerase beta or of nucleoside and nucleotide transporters, phosphorylases as well as many other cellular enzymes that vary among tumor types (Bouayadi et al., 1997) and may be revealed by future proteomic analyses. Interestingly, recent studies of the virus–virus and virus–cell interactome based on unbiased yeast two-hybrid analyses showed that EBVTK (Calderwood et al., 2007) (and KSHVTK, virion-wide only) (Rozen et al., 2008) interacts with many different viral and cellular proteins. This raises the possibility that some of the cytotoxic activity attributed to direct expression of the TK enzymes may result from protein interactions that occur only in infected cells where these unique interactions can occur.

Protein Kinase/Phosphotransferase (BGLF4 and ORF 36)

All members of the herpesvirus family contain a conserved serine/threonine kinase (Smith and Smith, 1989) that is implicated in a broad range of activities in the course of primary virus infection and virion production (Gershburg and Pagano, 2008). In vitro, these enzymes are reported to phosphorylate a large number of viral and cellular proteins that mediate many different intracellular functions (Bouayadi et al., 1997; Tarakanova et al., 2007; Gershburg and Pagano, 2008; Hume et al., 2008). The extent to which these protein contacts occur in vivo has been variously documented. Interestingly an unbiased proteomic study identified far fewer interactions for BGLF4 than other reports suggest (Calderwood et al., 2007). Among the activities shared by all of the herpesviral PKs/PTs are incorporation into the virion tegument, intracellular localization to the nucleus, and autophosphorylation and phosphorylation of cellular translation factor elongation factor 1 delta (EF-1δ) (Gershburg and Pagano, 2008). Although the HSV1 orthologue UL13 is not required for virus replication (perhaps because the alphaherpesviruses encode a second protein kinase), the CMV orthologue UL97 and EBV BGLF4 do appear to be critical for virus production (Marschall et al., 2001; Gershburg et al., 2004; Gershburg et al., 2007). Significantly, the conserved PKs/PTs do not phosphorylate normal nucleosides and the alpha herpesvirus enzyme is not a GCV kinase; however, in the case of the beta herpesvirus CMV, generation of GCVMP and to a lesser extent ACVMP is mediated by this kinase as documented by examination of the purified enzyme (Talarico et al., 1999) and by detection of viral GCV/ACV resistance mutations mapping to UL97 (and the viral polymerase) (Erice et al., 1997). Although ACV is less efficiently phosphorylated by UL97 than is GCV, in the clinic, ACV has modest ability to prevent CMV infection, although it is ineffective as treatment of established CMV infection (Balfour, 1990). U69 is the orthologue of UL97 in the human beta herpesvirus HHV6. Although U69 does phosphorylate GCV, a comparative analysis of the kinetics of GCV phosphorylation by U69 revealed that U69 is a far less (at least 10-fold less) active enzyme than is UL97 (Ansari and Emery, 1999), and consistent with this finding, the efficacy of GCV for treatment of established HHV6 disease remains arguable (Crawford et al., 2007; Seeley et al., 2007; Ogata et al., 2008).

The EBV orthologue of the conserved protein kinase is BGLF4 and the KSHV orthologue is ORF 36. Multiple protein interactions related in particular to cell cycle regulation, control of cellular and viral DNA replication as well as virus assembly/disassembly have been variably ascribed to these kinases (Tarakanova et al., 2007; Gershburg and Pagano, 2008; Park et al., 2007). Several semiquantitative studies suggested that the respective kinases sensitized cells to GCV-mediated killing (Cannon et al., 1999; Gershburg et al., 2004; Romaker et al., 2006). This has now been clearly established for BGLF4 through development and analysis of genetically modified EBV lacking expression of the protein kinase (Meng et al., 2010). Although 293T cells transiently transfected with either ORF 36 or BGLF4 were sensitized to GCV-mediated killing, when compared with UL97 as a well-characterized control, sensitization to GCV was substantially less and irrelevant

protein controls were absent (Cannon et al., 1999). In related work, BGLF4 was transiently expressed in 293 cells and then compared with positive (UL97) and negative (irrelevant protein) controls (Gershburg et al., 2004; Romaker et al., 2006). Sensitization to GCV-mediated killing was again observed, but only at relatively high drug doses (where comparable protein expression was documented), and at every dose, cells bearing UL97 were killed more efficiently. Whether GCV phosphorylation is mediated by the respective PKs/PTs as in the case of the beta-herpesviruses or is indirect, occurring through secondary protein interactions or through induction of new protein activity, is unknown. However the latter seems unlikely given the overall conservation of the enzyme among herpes family members. Studies that document direct phosphorylation of GCV by the respective protein kinases and demonstration of resistance mutations in the enzyme upon exposure to low dose GCV will close the loop.

Future Directions and Future Targets

Whether the gamma herpesvirus TK and PK/PT enzymes function coordinately and whether a sufficient amount of phosphorylated prodrug can be produced and maintained in virus-infected cells to elicit effective and selective tumor cell killing in vivo remain to be determined. Further investigation of these enzymes and their potential to enhance phosphorylation of different prodrugs to create cytotoxic compounds that can then be evaluated *within the context of virus infection* is an important next step. Although current work has focused on TK and PK/PT as target enzymes, in the future, other viral lytic cycle targets may be identified both in replication-related enzymes (helicase-primase, dUTPase, RNR, and others) and in proteins involved in other key lytic life-cycle steps (RNA export and turnover, assembly, and others) that can be targeted to promote the conversion of unique prodrugs to cytotoxic molecules. Expansion of these additional areas of research is overdue.

Preclinical Trials and Case Reports

Increased herpesvirus replication signals loss of immune control and is associated with many forms of disease in the immunocompromised host. Clinical disease that is based on the development of lytic cycle-derived lesions is associated with alpha and beta herpesvirus replication but also includes oral hairy leukoplakia and early KS in the case of the gamma herpesviruses. Lytic disease can be prevented and treated with antiviral agents that block virus replication, thereby aborting the lytic cycle. Prevention can often be achieved with doses of drug that are inadequate for full treatment (though this may predispose to resistance when disease occurs). The least toxic drugs, which target the virus and spare the cell, are optimal in this setting. In contrast, lytic induction therapy is aimed at destroying tumor cells; thus drugs that result in irreversible damage and cell death are preferred over those that

Table 1 Lytic induction therapy – initial trials

Authors	Major findings
A. Animal studies	
Westphal et al. (2000)	Gamma irradiation and sodium butyrate activated EBV lytic infection in SCID mice grafted with an LCL and an EBV-positive BL. Gamma irradiation, AZT, and GCV alone decreased LCL survival, whereas the combination of radiation and either AZT or GCV further reduced survival of an LCL xenograft
Roychowdhury et al. (2003)	Gamma irradiation induced EBVTK expression in an EBV-LCL implanted into the caudate nucleus of nude rats. Subsequent treatment with AZT and GCV significantly prolonged survival compared with radiation alone or combination antiviral therapy alone
Feng et al. (2004b)	Gemcitabine and doxorubicin, but not 5-azacytidine, initiated expression of early EBV lytic cycle proteins in a SCID mouse tumor derived from an EBV-LCL. Doxorubicin strongly inhibited tumor cell proliferation, whereas gemcitabine, GCV, and AZT modestly inhibited tumor cell proliferation. The combination of doxorubicin or gemcitabine with GCV markedly enhanced inhibition, whereas the combination of gemcitabine and AZT was minimally more inhibitory
Daibata et al. (2005)	Rituximab and dexamethasone in combination induced the lytic cycle and inhibited tumor cell growth in EBV-positive Akata cells (a Burkitt lymphoma) engrafted in nude mice. GCV enhanced the cytotoxic effect
Wu et al. (2005)	NOD/SCID mice engrafted with a KSHV+ EBV-primary effusion lymphoma (PEL) were treated with interferon-α (inducing agent) and AZT. In treated animals the tumor mass was decreased and survival was prolonged; however lytic induction was not assessed
Feng and Kenney (2006)	Both valproic acid and doxorubicin induced early lytic cycle protein expression and together inhibited more tumor growth than did either agent alone (GCV was not utilized) in an LCL xenograft
Fu et al. (2007)	Bortezomib induced expression of EBV lytic cycle proteins in two Burkitt lymphoma cell lines engrafted on SCID mice. Tumor responses were not assessed. In related work, although bortezomib prolonged the survival of SCID mice inoculated with LCLs, no lytic induction was observed (Ping Zouet al., 2007)

Table 1 (continued)

Authors	Major findings
B. Human studies and case reports	
Tosi et al. (1997)	Among 26 HIV-infected patients with high-grade NHL (most had AIDS) treated with methotrexate and AZT, 77% had early complete (46%) or partial responses. Neither EBV infection nor lytic induction was documented
Harrington et al. (1996)	Four patients with AIDS-related small non-cleaved cell lymphoma (SNCC) had a dramatic initial response to interferon-α and AZT. Two HIV-positive patients with large-cell immunoblastic lymphoma did not respond. Neither EBV infection nor lytic induction was documented
Raez et al. (1999)	Five patients with AIDS-related primary CNS lymphoma (EBV positive) were treated with IL-2, AZT, and GCV. Four of the five had excellent initial responses. Lytic induction was not specifically examined
Roychowdhury et al. (2003)	A solid organ transplant recipient with EBV-positive primary CNS lymphoma that was unresponsive to withdrawal of immunosuppression achieved a long-term CR without whole brain irradiation when treated with high-dose AZT and GCV alone. The tumor was documented to express abundant lytic antigens, specifically EBVTK by stereotactic biopsy
Perrine et al. (2007)	In a phase 1/2 trial of arginine butyrate and GCV, eight pretreated patients had documented EBV-positive B lymphoma (four early onset PTLD and two NHLs). Two patients with PTLD had a CR and one had a PR. One patient with diffuse large B-cell lymphoma had a PR. Lytic induction of the tumor cells was not documented, though induction in lines derived from the tumors was sometimes documented
Lechowicz et al. (2009)	Valproic acid treatment of patients with AIDS KS did not induce lytic infection in tumors or affect clinical response

halt proliferation. Interestingly, recent reports suggest that preemptive GCV (often given to prevent CMV disease) may also prevent development of KS (Martin et al., 1999; Casper et al., 2008) and reduce the incidence of lymphoproliferative disease (Darenkov et al., 1997). Whether this is a direct antiviral effect or occurs as a result of its increased uptake by and toxicity to more rapidly growing cells remains to be determined. Irrespective of how different prodrugs are activated in vivo – by the virus and/or by cellular proteins in infected cells, once they accumulate to a level that is adequate in virus-bearing cells, they have the potential to be effective.

Even before "lytic induction therapy" was coined to provide a conceptual framework for this treatment strategy, many physicians were experimenting with therapies that would be considered to fall within the stated paradigm (inducing agent – antiviral prodrug). In fact, the case report literature is extensive. However, major problems in systematic evaluation of this information arise from the fact that as patients are immunocompromised (AIDS patients, immunosuppressed transplant recipients, some patients with chronic infections, autoimmune disease, and cancer), major efforts are typically initiated to decrease immunosuppression and restore T-cell responses at the very same time lytic induction therapy is introduced. This of course confounds the interpretation of results. A second major problem is that actual documentation of lytic induction in primary tumors is uncommon. Preclinical animal studies, particularly studies in immunodeficient rodents, have been informative, although the xenograft model is imperfect and dramatic responses to antivirals alone have sometimes been observed that do not occur in the setting of human disease. In Table 1 we review some of the relevant preclinical and clinical studies, noting that this database is limited and that other potentially pertinent case reports and letters have been omitted.

Summary

Lytic induction therapy though in the early stages of clinical development constitutes a rational strategy for treatment of tumors infected with the human gamma herpesviruses EBV and KSHV. The model/paradigm is particularly relevant to tumors arising in the immunocompromised host, such as AIDS patients, where relatively few cellular mutations have accumulated and the virus may indeed be able to destroy (or destroy with some help) the infected tumor cell upon induced progression through the normal lytic cycle. The stated goal of this therapeutic model is to identify drugs that are non-toxic or are minimally toxic, sparing the effects of standard chemotherapy or pan-B-cell elimination (e.g., anti-CD20 antibody). The variable effects of currently recognized lytic cycle-inducing agents may relate to the stage of differentiation at which the infected tumor cell is frozen by virus infection. Thus, in the future, proteomic analysis of biopsied tumor cells may help to identify the optimal lytic-inducing agent. The production of lytic cycle antigens may additionally serve as an immune stimulus, especially in cases where aggressive reconstitution of the immune response is simultaneously sought. Cytotoxic prodrugs, in particular GCV and AZT, require further study to clarify precisely

how they work in virus-infected cells and to what degree their toxicity is specific, and studies are ongoing in several laboratories. New prodrugs that target the TK, the PK/PT as well as many other enzymes synthesized in the course of gamma herpesvirus replication may provide additional agents that can be combined to produce synergistic killing and decrease the likelihood that resistance will develop. Promising new animal models including variations of the humanized mouse should provide a basis for more physiologic and more systematic preclinical testing that will lead to successful introduction of lytic induction therapy into clinical practice.

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Viral Interleukin-6: Molecular Biology and Pathogenesis

John Nicholas

Abstract Human herpesvirus 8 (HHV-8, also called Kaposi's sarcoma-associated herpesvirus) was discovered in 1994, but interleukin-6 (IL-6) had been implicated in Kaposi's sarcoma and HHV-8-associated multicentric Castleman's disease (MCD) several years prior to this date. The predicted role of IL-6 in KS and MCD pathogenesis was based on its known pro-angiogenic and B-cell mitogenic properties. Thus, when viral IL-6 (vIL-6) was identified in HHV-8, the first reported viral interleukin-6 homologue, this was immediately recognized as a potential contributor to HHV-8-associated pathogenesis. Indeed, the pro-angiogenic, proliferative, and signaling properties of vIL-6 reflected those of its human counterpart. It is now known that there are significant differences in the precise mechanisms of signaling complex formation and in signal transduction induced by the human and viral IL-6 proteins and also that vIL-6 is able to signal intracellularly, which is likely to be highly relevant to its functions in virus biology and viral pathogenesis. The unique properties of vIL-6 suggest that it may be possible to target the viral cytokine specifically with inhibitory agents, but they also highlight special challenges with respect to appropriate targeting of inhibitory agents to the sites of vIL-6 activity. This review discusses the molecular biology of vIL-6, the means by which it may contribute to virus biology and viral pathogenesis, and possible methods of inhibiting the activity of the viral cytokine.

Introduction

IL-6 had been implicated as a contributing factor in Kaposi's sarcoma (KS) and multicentric Castleman's disease (MCD) even prior to the discovery of HHV-8. KS lesions were found to have elevated levels of human IL-6 (hIL-6) levels, KS cells in culture were reported to show increased proliferation rates in response to hIL-6,

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and high serum levels of hIL-6 correlated with increased severity and progression of MCD (Burger et al., 1994; Hsu et al., 1993; Ishiyama et al., 1994; Miles et al., 1990; Yoshizaki et al., 1989). Furthermore, hIL-6 is a known mitogen and survival factor for B cells, and STAT3 (induced by IL-6) is found at elevated levels in many cancers (Benekli et al., 2003; Bowman et al., 2000; Hodge et al., 2005). These properties suggest that IL-6 activity may be important for the development and progression of HHV-8-associated neoplasia, and thus vIL-6, which shares the basic signaling properties of hIL-6, is a likely contributor to viral pathogenesis.

Notwithstanding the likelihood of vIL-6 involvement in virus-associated neoplasia, the precise mechanisms of its contributions are unknown. Direct signaling by the viral cytokine and indirect effects, through the induction of cellular cytokines such as angiogenic and growth factors, could play roles. Of potential importance to the former is activation of STAT3, known to contribute to cell proliferation and cell survival and induced in various cancers (Benekli et al., 2003; Bowman et al., 2000). Another issue of importance when considering the potential role of vIL-6 in viral pathogenesis is when vIL-6 is expressed in the virus life cycle. While it is certain that vIL-6 is expressed at high levels during de novo infection and induced following lytic reactivation (Jenner et al., 2001; Krishnan et al., 2004; Nicholas et al., 1997; Paulose-Murphy et al., 2001), studies of HHV-8^+ malignant tissues and experimental evidence suggest that vIL-6 may be expressed independently of other lytic genes and during latency (Chen et al., 2009a; Chiou et al., 2002; Nicholas et al., 1997; Parravicini et al., 2000). Therefore, vIL-6 may have dual roles, functioning as an autocrine factor to promote proliferation and survival during latency and as a paracrine factor when expressed during lytic cycle, to influence growth and other properties of surrounding latently infected and uninfected cells.

The properties of vIL-6 that bear on its potential relationship to HHV-8-associated pathogenesis and of relevance to future development of strategies to inhibit its function for therapeutic benefit are outlined in this review. While there are many questions remaining, considerable progress has been made on the characterization of this viral cytokine over the past few years, and it is hoped that this and future research in this area will ultimately lead to therapeutic advances.

Activities of vIL-6 and Potential Contributions to Pathogenesis

Not unexpectedly, there are parallels between the activities of vIL-6 and cellular IL-6 proteins, considering their shared use of the gp130 signal transducer. Thus, activities such as support of growth of cytokine-responsive/dependent B cells and other cell types have been reported by several laboratories (e.g., Burger et al., 1998; Hideshima et al., 2000; Jones et al., 1999; Nicholas et al., 1997). The viral cytokine also induces acute-phase gene expression in hepatic cells, another feature of IL-6 activity, with corresponding activation of transcription factor C/EBPβ ("NF-IL6") associated with acute-phase gene induction (Wan et al., 1999). Interestingly, vIL-6 was found to induce expression of hIL-6 in certain cell types, suggesting

the possibility of signal amplification (perhaps particularly important for paracrine effects of the viral cytokine) via this route (Mori et al., 2000).

Notwithstanding similar activities in many assays, when added exogenously to cell culture systems there are detectable differences between vIL-6 and hIL-6 in terms of biological effects. It is worth emphasizing, however, that vIL-6 may not, in vivo, be particularly effective via paracrine signal transduction and activation of gp130 at the cell surface because it is largely sequestered in the ER. Nevertheless, at maximally effective doses, vIL-6 is more active than hIL-6 in support of Baf-130 cell growth (in the presence of soluble gp80, to allow hexameric complex formation) (Hu and Nicholas, 2006), and vIL-6, but not hIL-6, is able to promote the growth and survival of PEL cells in culture (Chatterjee et al., 2002; Jones et al., 1999). Interestingly, anti-sense techniques revealed that the depletion of hIL-6, but not vIL-6, negatively affected clonal growth of PEL cells in soft agar, a phenomenon that could not be mimicked by exogenous addition of hIL-6 neutralizing antibody, and therefore implicating hIL-6 intracellular signaling in this activity (Asou et al., 1998). However, this is difficult to reconcile with the results of biochemical and signal transduction studies that have failed to provide evidence of functional gp130:hIL-6 complexing intracellularly (Chen et al., 2009a).

The role of vIL-6 in virus-associated pathogenesis has generally been presumed to involve effects of paracrine signaling from lytically infected cells to latently infected and uninfected cells. This is because vIL-6 is induced during lytic reactivation, displaying characteristics of an early gene expressed just after the onset of the lytic cascade (Jenner et al., 2001; Lu et al., 2004; Nicholas et al., 1997; Paulose-Murphy et al., 2001; Sun et al., 1999). Thus, vIL-6 may perform functions analogous to those hypothesized for hIL-6 due to its reported promotion of KS cell growth in culture and the correlation of high serum levels of hIL-6 with MCD severity (Burger et al., 1994; Hsu et al., 1993; Miles et al., 1990; Yoshizaki et al., 1989). vIL-6 depletion and supplementation experiments have shown that vIL-6 contributes to the support of PEL cell growth in culture and that it promotes PEL cell growth and dissemination in inoculated mice via pro-angiogenic activities of vIL-6-induced VEGF (Aoki et al., 1999; Aoki and Tosato, 1999; Jones et al., 1999; Zhang et al., 2008). The pro-inflammatory activities of vIL-6 also suggest that it plays a role in KS, as both inflammatory cytokines and angiogenic factors are believed to be involved in KS development (Ensoli and Sturzl, 1998). It should also be appreciated that one of the major consequences of vIL-6 signal transduction is the activation of STAT3, an important contributor to cell survival and a transcription factor induced and implicated in many human cancers (Benekli et al., 2003; Bowman et al., 2000; Hodge et al., 2005).

Notwithstanding the prevailing assumptions about the contribution of vIL-6 to HHV-8 pathogenesis via paracrine (extracellular) signaling, the predominantly intracellular expression of the viral cytokine coupled with its potential expression (at low levels) as a true latent gene (Chen et al., 2009a) suggests another means by which it contributes to HHV-8 neoplasia. This involves intracrine signaling-mediated support of PEL cell proliferation and survival, and vIL-6 could conceivably mediate similar effects in endothelial cells to contribute to KS. Of course, this

mechanism does not preclude the influence of paracrine signaling also, by vIL-6 directly (most likely from cells supporting lytic replication) or by vIL-6-induced cellular cytokines. With regard to the latter, expression of cytokines induced by vIL-6 in latently infected cells would not be restricted by host shutoff functions specified by the ORF37-encoded SOX protein (Glaunsinger and Ganem, 2004a, b).

Role in Virus Biology

Despite extensive studies of vIL-6 activities and signaling mechanisms and investigations using culture and in vivo models of its potential roles in pathogenesis, the function of the viral cytokine in HHV-8 biology is a much under-investigated area of research. Identified positive influences of vIL-6 on the growth of PEL and other cell types indicate that it could contribute to the maintenance of latently infected cell pools, via "lytic-to-latent" paracrine or latent autocrine/intracellular signaling (see below). Clearly, any cellular cytokines induced by vIL-6, produced lytically or during latency, could potentially play positive roles in promotion of cell growth and survival. However, due to the predominant lytic expression of vIL-6, it would be reasonable to speculate that the viral protein also functions to promote virus productive replication, at least in the host, again either directly or via the induction of cellular factors. But, studies using vIL-6-deficient recombinant HHV-8 in culture-based lytic reactivation assays failed to detect any influence of vIL-6 on productive replication efficiency (Chen and Lagunoff, 2007). Unpublished data from this laboratory indicate, in fact, that endogenously produced vIL-6 acts to dampen productive replication in culture. However, paracrine effects in the context of in vivo infection clearly could influence virus replication and spread indirectly, and it seems likely that this is the case. Clearly, further attention needs to be paid to this aspect of vIL-6 research, emphasizing the importance of animal model systems, in particular those utilizing vIL-6-encoding rhesus rhadinovirus (Dittmer et al., 2005; O'Connor and Kedes, 2007; Orzechowska et al., 2008), to gain further insight into in vivo functions of vIL-6.

Signal Transduction and Molecular Biology

HHV-8-encoded vIL-6 shares only 25% amino acid sequence identity with hIL-6 but has a very similar three-dimensional structure (Boulanger et al., 2003; Chow et al., 2001) (Fig. 1). Like hIL-6, vIL-6 possesses two major interfaces (sites II and III) of interaction with its signal transducer, gp130 (β-subunit of the receptor) and another (site I) which interacts with the non-signaling α-subunit, gp80 (Boulanger et al., 2004, 2003) (Fig. 2). However, vIL-6 is unique among IL-6 proteins in its ability to signal directly through the gp130 signal transducer in the absence of gp80 (Molden et al., 1997). Thus, vIL-6 can form functional tetrameric complexes (vIL-6_2:$gp130_2$) in addition to signaling complexes that incorporate the non-signaling α receptor subunit, gp80 (vIL-6_2:$gp130_2$:$gp80_2$) (Boulanger et al., 2004; Hu and Nicholas,

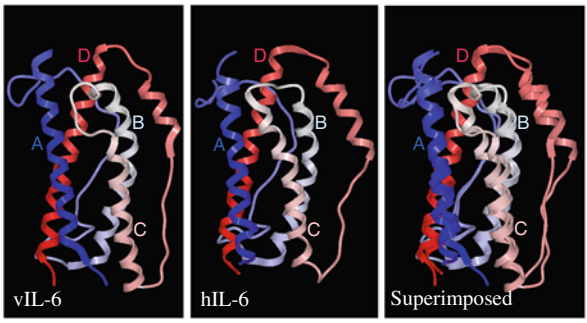

Fig. 1 Structures of vIL-6 and hIL-6. The three-dimensional structures of vIL-6 and hIL-6, as determined by X-ray crystallography (Boulanger et al., 2003; Chow et al., 2001), are shown as ribbon models. The ribbon diagram on the *right* shows the vIL-6 and hIL-6 amino acid backbones superimposed, emphasizing the highly conserved secondary and tertiary structures of the two cytokines. The four major helices (A–D) are labeled, with A and C helices in the front plane

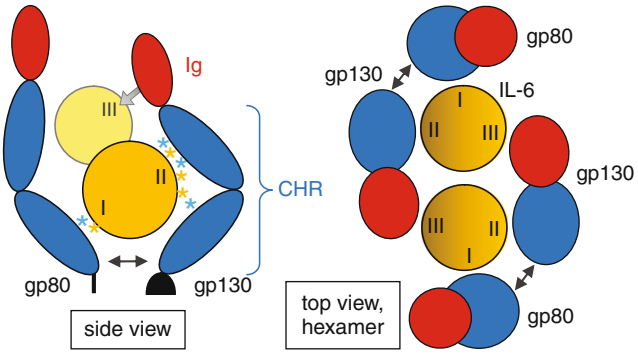

Fig. 2 IL-6:gp130/gp80 interactions. Diagrammatic representation of IL-6 hexameric signaling complex formation (IL-6_2:$gp130_2$:$gp80_2$) via bimolecular ligand bridging by each of the two gp130 receptor subunits (sites II and III interactions with CHR and Ig regions, respectively) and stabilization by gp80 interactions with IL-6 (via site III) and gp130. For clarity, the *left* figure shows only one gp130 and one gp80 receptor subunit; the complete complex and relevant interactions are depicted in the *right* diagram

2006; Wan et al., 1999). While there are notable differences in the specific amino acid residues of vIL-6 and hIL-6 that contact gp130 (Boulanger et al., 2003; Chow et al., 2001), these do not appear to account for the lack of dependence of the viral cytokine on gp80. Evidence from mutational studies suggests that this is due to the particular conformation of vIL-6, rather than the specific composition of the gp130-binding interfaces (Chen and Nicholas, 2006). The three vIL-6-specific tryptophans that form a hydrophobic grove interacting with the cytokine receptor homology region (CHR, domains 2 and 3) of gp130 are not required for gp80 independence [as originally hypothesized from the vIL-6_2:$gp130_2$ crystal structure (Chow et al., 2001)] and vIL-6 helix B that does not interact with gp130 (or gp80) cannot be substituted functionally by hIL-6 helix B (Chen and Nicholas, 2006). Thus, it seems

that vIL-6 naturally adopts a conformation that is conducive to stable gp130 interactions, whereas hIL-6 requires initial binding to gp80 to allow subtle conformational changes in the ligand required for interactions with gp130 and dimerization of the signal transducer. Consistent with such a model is the finding that antibodies directed to a region of the vIL-6 A-B loop which does not bind gp130 abolish its interaction with the signal transducer (Aoki et al., 2001).

The two types of vIL-6-induced signaling complexes have distinguishable characteristics with regard to both signal transduction and biological effects. Incorporation of gp80 leads to accentuated and prolonged signaling and alteration of the ratio of activated STAT1 to STAT3 and greater support of B-cell growth in culture (Hu and Nicholas, 2006). It has also been noted that gp80 is able to stabilize vIL-6-induced gp130 dimerization, reflected in increased amplitudes of signal transduction (Boulanger et al., 2004; Chen and Nicholas, 2006). The molecular basis of the experimentally evidenced ligand- and gp80-dependent differences in IL-6 signaling is unknown, but it is possible that subtle differences in the conformations of the different signaling complexes may result in altered accessibility of signaling tyrosine residues of gp130 to activating JAK-mediated phosphorylation and/or recruitment of STATs, SHP2, or negative regulatory interactions with SOCS proteins (Fig. 3). While the consequences of these different signaling patterns for virus biology, cell biology, and viral pathogenesis remain to be established, it is clear that vIL-6 signaling is qualitatively distinct from signaling induced by hIL-6 and that gp80 can influence vIL-6 signal transduction. Thus, these factors need to be taken into account in analyses of the roles of vIL-6 in virus biology and viral pathogenesis and also in consideration of molecular strategies to interfere with vIL-6 activity.

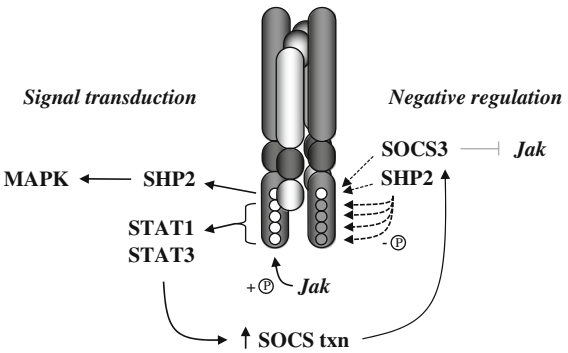

Fig. 3 IL-6 signal transduction. Signaling by vIL-6 and hIL-6 involves gp130 dimerization, hIL-6 requiring prior binding to gp80 to allow hexameric complexing (indicated). vIL-6 can dimerize gp130 either with or without incorporation of gp80 into the signaling complex. Induced dimerization of gp130 leads to *trans*-phosphorylation of gp130 cytoplasmic tyrosine residues by a gp130-associated Janus kinase (Jak). Signaling protein SHP2 and STAT1 and STAT3 transcription factors then bind to specific phosphotyrosines and are themselves phosphorylated and activated. As well as activating MAPK signaling, SHP2 also mediates negative feedback regulation of gp130 signaling via dephosphorylation of tyrosine residues. Negative feedback is also mediated via induction of SOCS (suppressors of cytokine signaling) gene expression

Intracellular Retention and Function of vIL-6

Other than the gp80 independence of vIL-6, the viral and cellular cytokines are also different in their secretion properties. While the half-life of intracellular retention of hIL-6 is <30 min, typical of a secreted cytokine, the equivalent value for vIL-6 is >4 h (Meads and Medveczky, 2004). The result is that vIL-6 is largely retained intracellularly, most of it in the ER compartment, where it is active (Chen et al., 2009a; Meads and Medveczky, 2004). This has important implications for the potential role of vIL-6 in virus biology and pathogenesis, especially when one considers that it is expressed at low levels during HHV-8 latency, at least in PEL cells, and appears to promote cell growth and survival predominantly via intracrine signaling (Chen et al., 2009a). This property of vIL-6 has implications for potential functions of the viral cytokine in HHV-8 biology in addition to virus-induced disease. Low-level expression of the viral cytokine is adequate to mediate biological effects, being concentrated in the ER and restricted functionally to promotion of growth and survival of latently infected cells. With respect to virus biology, this could be particularly relevant to maintenance of latent viral loads within the host, but clearly it could also contribute significantly to malignant pathogenesis. It is notable, in consideration of the differences noted in vIL-6 signaling via tetrameric and hexameric complexes (Hu and Nicholas, 2006), that in the ER vIL-6 functions exclusively via tetramers. This is clearly sufficient to support proliferation and survival of PEL cells (Chen et al., 2009a), notwithstanding previous demonstrations of pro-proliferative activities on PEL and other cell types of exogenously added vIL-6 (Burger et al., 1998; Hideshima et al., 2000; Jones et al., 1999; Nicholas et al., 1997). Such extracellular and paracrine signaling activities of vIL-6 may be particularly important during lytic replication, when the viral cytokine is produced abundantly and can presumably be secreted in biologically significant amounts to promote replication, and likely also contribute to viral pathogenesis via direct effects and by induction of cellular cytokines of particular relevance to KS, PEL, and MCD. Strictly autocrine activities of vIL-6 via ER signaling would be predicted to be the predominant means of vIL-6 function during HHV-8 latency, but again, influence can be exercised via induced cellular cytokines as well as by direct effects of vIL-6 on latently infected cells. Induced cytokines could contribute also in a paracrine manner to pathogenesis, even during latency.

The molecular basis of vIL-6 intracellular retention and ER localization is not understood. It has been established by the elegant studies of Meads and Medveczky (2004) that vIL-6 is N-glycosylated at two residues, consistent with its processing through the ER, but that a significant proportion of the protein escapes later glycan processing and that immature, high-mannose structures can be detected on vIL-6 secreted from transfected cells. Surprisingly, protein stripped of these glycans was found to migrate on SDS-polyacrylamide gels identically with bacterially expressed vIL-6 ORF (K2)-encoded protein (unglycosylated), indicating that the signal sequence of the eukaryotically produced protein is uncleaved, atypical for a secreted protein. However, while this indicated the theoretical possibility of membrane insertion and its possible contribution to ER retention of vIL-6, studies

addressing this issue found no evidence of membrane association of the HHV-8 cytokine (Chen et al., 2009b). Furthermore, there is no classical ER retention motif present in vIL-6 to account for receptor-mediated ER retention. However, the C-helix region of vIL-6 seems to be important for its intracellular retention; substitution of this region with the C-helix of hIL-6 led to markedly increased secretion, and this was seen also after introduction of several paired point mutations within the C-helix (Chen et al., 2009b). However, the C-helix of vIL-6 was not able to confer ER retention to hIL-6. These findings are inconsistent with the presence of a discrete ER retention motif analogous to those found in other ER-resident luminal proteins; rather, they point to the importance of the overall structure of vIL-6 for ER retention by a yet unknown mechanism. It is relevant, however, that vIL-6 has been shown to interact stably with the ER-resident chaperone protein calnexin, and this interaction is dependent on vIL-6 glycosylation (Chen et al., 2009b). The interaction appears to be important for vIL-6 stability, possibly via promoting appropriate protein folding, but it appears not to be involved directly in intracellular retention of vIL-6 (Chen et al., 2009b). Thus, the mechanisms of inefficient secretion and ER localization of vIL-6 remain elusive, but it is clearly an area worthy of further study; interference with these mechanisms in addition to abrogation of vIL-6:calnexin interaction may provide useful antiviral and therapeutic strategies.

Strategies for Therapeutic Targeting of vIL-6

Despite the common association of HHV-8 with KS, PEL, and MCD, these disease entities have distinct clinical, pathological, and molecular characteristics that indicate significant differences in underlying mechanisms of pathogenesis (Schulz, 2006). KS, which itself presents in very diverse ways, is characterized by endothelial cell proliferation to form highly vascular tumors containing spindle-shaped endothelial cells surrounding irregular slit-like spaces containing infiltrating hematopoietic cells, including erythrocytes and inflammatory cells (Ensoli et al., 2001). The endothelial "tumor" cells are not fully transformed, however, and their growth, at least in early stages of KS, appears to be dependent on pro-proliferative, angiogenesis, and inflammatory cytokines present at high levels in the lesions (Ensoli and Sturzl, 1998; Ensoli et al., 2001). PEL and MCD, on the other hand, are B-cell malignancies, the former being derived from post-germinal center B cells and the latter representing a tumor of cells resembling plasma cells. PEL cells are fully transformed and clonal and are often co-infected with Epstein–Barr virus; the disease typically presents as bloody effusions in the pleural or peritoneal cavities (Gaidano and Carbone, 2001; Schulz, 2006). The HHV-8-associated "plasma cell variant" of MCD is a systemic illness characterized by polyclonal expansions of B cells, suggestive of a cytokine-driven process; as mentioned previously, serum IL-6 levels have been correlated with the disease and its progression (Burger et al., 1994; Ishiyama et al., 1994; Yoshizaki et al., 1989). Thus, cytokines may play central roles in KS and MCD by promoting polyclonal hyperproliferation of endothelial

and B cells, respectively, whereas PEL represents a more classical form of neoplasia, most likely driven by transforming genetic alterations. Notwithstanding, PEL cell growth and dissemination is enhanced, in murine models at least, by vIL-6-induced vascular endothelial growth factor (Aoki et al., 1999; Aoki and Tosato, 1999). Thus, inhibitory targeting of vIL-6 could conceivably be of therapeutic benefit for each of these diseases.

Although vIL-6 is likely to be a contributor to HHV-8 disease pathogenesis, the issue of inhibiting its signaling is a difficult one to address practically. Theoretically, one could inhibit activity via application of vIL-6-specific antibodies or binding inhibitory peptides corresponding to vIL-6:gp130 interaction interfaces, highlighting the importance of structural and functional studies addressing the molecular basis of vIL-6:gp130 interactions (Chen and Nicholas, 2006; Chow et al., 2001; Li and Nicholas, 2002; Li et al., 2001). Antibodies to gp130 (and gp80) have been demonstrated to inhibit vIL-6 activity in culture, and assembled vIL-6:gp130 binding site mimetic peptides (corresponding to gp130 sequences) have been shown to be effective under these conditions (Burger et al., 1998; Nicholas et al., 1997; Sudarman et al., 2008). In consideration of clinical applicability of such inhibitory strategies, it is significant that monoclonal antibodies directed to hIL-6 have been used in clinical trials and found to be well tolerated, effective at inhibiting hIL-6 activity, and of some therapeutic benefit (Trikha et al., 2003). Similarly, antibody-based targeting of the IL-6 receptor α-subunit, gp80, has been studied in numerous clinical trials and demonstrated to be effective and of value in the treatment of rheumatoid arthritis (Mircic and Kavanaugh, 2009). However, as outlined above, vIL-6, unlike hIL-6 and other cellular cytokines, is active intracellularly and is likely to contribute during both latency and productive replication to pathogenically relevant signaling via this route. Thus, methods to target vIL-6 in this compartment must be sought. Again, antibody- and peptide-based methods can be employed. Indeed, it has been demonstrated in culture that ER-directed (KDEL-tagged) single-chain antibody to vIL-6 can inhibit vIL-6 signaling in appropriately transduced cells (Kovaleva et al., 2006). Clearly, it would be more realistic to consider application of cell-permeable peptides or structurally related small-molecule analogs to achieve similar effects in vivo. There are potentially alternative and complementary targets for such inhibitors. As vIL-6 stability and protein folding seem to be dependent on the ER chaperone protein calnexin, targeting of this interaction specifically (if possible) may abrogate expression of functional vIL-6. Similarly, if the mechanism of ER retention of vIL-6 can be elucidated, it may be possible to identify small-molecule inhibitors that disrupt the molecular interactions involved. This may be particularly valuable with respect to latency functions of vIL-6, in which the mitogenic and pro-survival effects of intracellular signaling by vIL-6 are likely to be most functionally relevant. Clearly, further understanding of the molecular processes involved in vIL-6 intracellular retention, ER localization, and vIL-6 intracellular signaling is of key importance to the development of such novel vIL-6 inhibitory strategies and treatment options for HHV-8-associated diseases.

Summary and Perspectives

As outlined above, vIL-6-mediated signal transduction via gp130, which activates STAT3 in addition to mitogen-activated protein kinase (MAPK) signaling, is likely to be an important factor in the development of HHV-8-associated KS and B-cell malignancies PEL and MCD. Not only do STAT3 and MAPK promote cell survival and proliferation, via induction of various anti-apoptotic and cell cycle stimulatory proteins (e.g., Mcl-1, Bcl-X_L, c-Fos, and c-Myc), but vIL-6 can trigger the production of cellular cytokines, including mitogenic and angiogenic factors such as hIL-6 and VEGF, that have been implicated in KS and also in the development of PEL and MCD (Aoki et al., 1999; Aoki and Tosato, 1999; Asou et al., 1998; Benekli et al., 2003; Bowman et al., 2000; Hodge et al., 2005; Mori et al., 2000). Thus, these factors may represent appropriate therapeutic targets, as has been recognized in the specific case of vIL-6-induced VEGF and its likely importance in PEL (Aoki and Tosato, 2003).

Unlike its cellular counterpart, vIL-6 can transduce signal via gp130 in the absence of the non-signaling gp80 IL-6 receptor subunit and therefore is able to induce signaling highly promiscuously, in most cell types (Molden et al., 1997). It should be noted, however, that vIL-6 signal transduction appears to be modulated by gp80, which can be incorporated into vIL-6-induced signaling complexes, although the biological significance of this has yet to be determined. Furthermore, it seems apparent that vIL-6 can function both intracellularly, in a strictly autocrine manner, and extracellularly at the cell surface; the former occurs exclusively via gp80-deficient tetrameric complexes (vIL-6_2:$gp130_2$) and may predominate during latency (Chen et al., 2009a). Both autocrine (intracellular) and paracrine (extracellular) mechanisms are known to affect cell survival and proliferation (Burger et al., 1998; Chen et al., 2009a; Hideshima et al., 2000; Jones et al., 1999; Nicholas et al., 1997) and therefore could potentially contribute to HHV-8-associated pathogenesis (Fig. 4). Therefore, in consideration of possible therapeutic strategies targeting vIL-6 for inhibition, it is important to recognize that the viral cytokine may need to be inhibited intracellularly as well as (or instead of) at the cell surface. As discussed above, methods for doing this might include the utilization of small-molecule inhibitors or conceivably cell-permeable peptides representing structures corresponding to vIL-6:gp130 interaction interfaces. These should provide specificity and not affect gp130 signal transduction mediated by other cytokines. It may also be possible to target other interactions that may be required for vIL-6 function, such as those with calnexin (required for vIL-6 stability and probably appropriate folding) and as yet unidentified ER-resident proteins involved in retention of vIL-6 within this signaling compartment (Chen et al., 2009b; Meads and Medveczky, 2004).

In addition to the likely contributions of vIL-6 to HHV-8-associated cancers, it is also important to understand the role of vIL-6 in normal virus biology. At present this is a black box, and one can only speculate about functions of vIL-6 in latency and lytic replication. As discussed above, there is now evidence of functional expression of vIL-6 during latency, and its support of PEL cell growth suggests that it

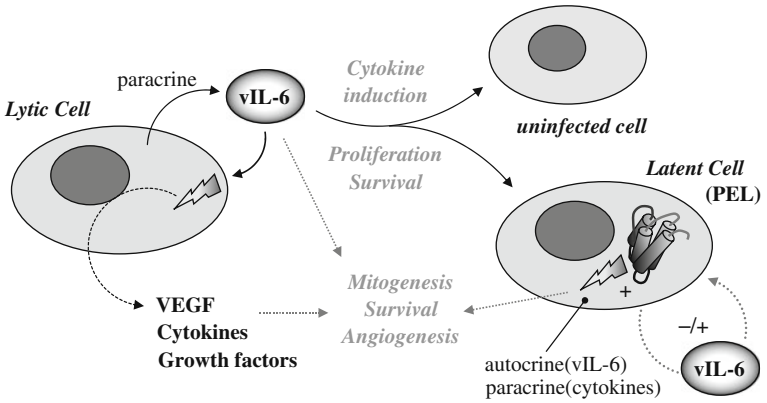

Fig. 4 Envisioned activities of vIL-6. The viral cytokine is expressed most abundantly during productive (lytic) replication, where it is secreted in large amounts and can mediate paracrine effects itself and induce in lytically infected and neighboring cells the production of cellular cytokines that can contribute to pathogenesis via growth promoting and angiogenic activities. In lytically infected cells, vIL-6 signaling can occur via intracellular (intracrine) and extracellular (cell surface) mechanisms (latter indicated). vIL-6 expression during latency has been demonstrated in PEL cells. Expression is very low in comparison to levels of vIL-6 produced during lytic replication, but vIL-6 is functional in latently infected PEL cells and intracrine, strictly autocrine activity is important for cell proliferation and survival (Chen et al., 2009a). Contributions of paracrine signaling in this setting are uncertain, but it is not required for normal growth of PEL cells in culture and relatively little vIL-6 is released extracellularly. Intracellular vIL-6 signaling would be predicted to induce cellular cytokines of relevance to virus-associated pathogenesis, as well as contributing directly to the promotion of cell growth and survival. However, whether vIL-6 is expressed during latency in other cell types, such as endothelial cells, is unknown

may, like other latency proteins, help to sustain the proliferation and survival of latently infected cell pools and virus within the host (Chen et al., 2009a). Thus, it is conceivable that inhibition of vIL-6 activity could provide a way of diminishing latently infected cells and virus in infected individuals. With regard to productive replication, the only available published data from tissue culture-based experiments indicate that vIL-6 does not significantly influence the efficiency of virus production from lytically reactivated cells or establishment of latency following de novo infection (Chen and Lagunoff, 2007). While recognizing that this may not be the case in vivo, at present there is no support for the idea that targeting of vIL-6 or vIL-6 signaling could provide a novel approach to inhibit virus lytic replication and spread, which is known to be important in KS (Campbell et al., 2000; Martin et al., 1999; Quinlivan et al., 2002).

In conclusion, as vIL-6 is a likely contributor to HHV-8-associated endothelial and B-cell cancers KS, PEL, and MCD and may also be important for maintenance of latently infected cells, the viral cytokine and its signaling pathways represent promising therapeutic targets. Further work is required and warranted to elucidate the molecular determinants and mechanisms of intracellular retention, stability, and function of vIL-6 to reveal new avenues to inhibitory targeting of this viral cytokine.

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Targeting the PI3K/Akt/mTOR Pathway in AIDS-Associated Malignancies

Blossom Damania

Abstract The PI3K/Akt pathway is highly activated in a wide variety of human cancers. Dysregulated PI3K signaling plays roles in many aspects of tumorigenesis including apoptotic resistance, uncontrolled proliferation, metastasis, and angiogenesis. AIDS-associated malignancies such as Kaposi's sarcoma (KS) and non-Hodgkin lymphoma (NHL) display activated PI3K, Akt, and mTOR kinases and thus may be susceptible to treatment modalities that inhibit this important signaling pathway. In this chapter, we discuss the use of mTOR inhibitors to treat both KS and NHL.

Introduction

AIDS-Associated Cancers: Kaposi's Sarcoma and Non-Hodgkin Lymphoma

Since the first report of AIDS in the early 1980s, it was noted that there was a close association between HIV infection and the development of a number of cancers (Wood and Harrington, 2005). In fact, one of the reasons AIDS was recognized in 1981 was due to a marked increase in the number of cases of Kaposi's sarcoma (Durack, 1981). AIDS-associated cancers include Kaposi's sarcoma (KS), non-Hodgkin lymphoma (NHL), and invasive cervical carcinomas.

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Kaposi's Sarcoma

Prior to the onset of the AIDS epidemic and the emergence of HIV, Kaposi's sarcoma (KS) had been described in 1872 by Moritz Kaposi, the head of the Dermatology department at the University of Vienna, as *idiopathisches multiples Pigmentsarkom*, a rare angiosarcoma in elderly (non-HIV positive) men of Mediterranean descent (Kaposi, 1872). It is now known that KS can present as four subtypes with distinct, but somewhat overlapping, clinical manifestations. These are classic, endemic, epidemic or AIDS-associated, and iatrogenic KS. Classic KS is most often a disease of elderly Mediterranean and eastern European men, while endemic KS is found in parts of equatorial Africa such as Uganda and Zambia (Martin et al., 1998), where it is responsible for an estimated 1% of all tumors. Hence, in these regions, transmission of KSHV is thought to occur vertically. Certain subtypes of endemic KS are more aggressive than classic KS.

Iatrogenic KS occurs in patients receiving immunosuppressive therapy (Civati et al., 1988), typically after solid organ transplantation. KS comprises an estimated 3% of all tumors associated with transplantation (Mendez et al., 1999). This is seen particularly in regions of high KSHV prevalence, such as southern Italy, Turkey, and Saudi Arabia. Kaposi's sarcoma-associated herpesvirus (KSHV) is the causative agent of Kaposi's sarcoma and KSHV infection in the organ recipient may be acquired prior to receipt of the organ or may be transmitted through the graft itself (Barozzi et al., 2003).

At its peak in the United States, the frequency of KS in AIDS patients was 20,000 times higher than in the general population and the frequency of KS in transplant recipients is estimated to be 500 times higher than in healthy individuals (Stallone et al., 2005). In AIDS-associated KS, incidence rates of KS among men who have sex with men correlated significantly with the lifetime number of sexual partners (Martin et al., 1998). Today, more women are infected with HIV, particularly in areas of high KSHV seroprevalence, such as sub-Saharan Africa, and consequently KS is now seen more commonly in this group.

The KS lesion itself is highly angiogenic and is comprised of spindle-shaped cells, slit-like endothelium-lined vasculature, and infiltrating blood cells. The spindle cells form the majority of the cell population and are thought to arise from lymphatic endothelial cells (Dupin et al., 1995). KS lesions are classified histologically as plaque, patch, or nodular. As the KS tumor progresses clinically, the number of KSHV-infected cells increases and the endothelial cell population within the lesion expands. There is evidence for both polyclonality and monoclonality of the lesions (Kaaya et al., 1998; Rabkin et al., 1997). It is thought that KS probably begins as a polyclonal hyperplasia that may later develop into a clonal neoplasm. Kaposi's sarcoma not only affects the skin but can also involve multiple organs such as the liver, lung, spleen, and gastrointestinal tract. Very aggressive visceral KS can ultimately result in hemorrhage, organ dysfunction, and death. It is important to note that 95% of all KS lesions are positive for KSHV, regardless of clinical type or HIV status.

PEL and MCD

In addition to KS, KSHV is also the causative agent of two lymphoproliferative diseases named primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD). KSHV-associated PEL and MCD incidences are also increased in the HIV-positive population. In fact, the first association of KSHV with MCD was reported in a patient who presented with both KS and MCD (De Rosa et al., 1989). Multicentric Castleman's disease is a B-cell lymphoproliferative disorder that is sometimes referred to as multicentric angiofollicular hyperplasia. Patients usually present with diffuse lymphadenopathy and a constellation of constitutional symptoms. The disease is characterized by vascular proliferation of the germinal centers of the lymph node. There are two forms of MCD: (i) a hyaline vascular form, which presents as a solid mass, and (ii) a plasmablastic variant form that is associated with lymphadenopathy and immune dysregulation. It is the plasmablastic variant of MCD that is associated with HIV infection. Nearly 100% of AIDS-associated MCD is associated with KSHV, whereas only about 50% of non-AIDS-associated MCD contains KSHV DNA. AIDS-associated MCD is often accompanied by the development of KS in the affected individual.

MCD is a polyclonal tumor that is highly dependent on cytokines such as interleukin-6 (IL-6). KSHV itself encodes a viral IL-6 that is also expressed in these lesions (Parravicini et al., 2000). MCD is a polyclonal disorder. Affected patients frequently develop cytopenias, autoimmune manifestations, and other malignancies such as KS and non-Hodgkin lymphoma (Ablashi et al., 2002). The plasmablasts in MCD express monotypic IgM light chains (Du et al., 2001) and viral antigens can be detected in the immunoblastic B cells in the mantle zone of the lymph node.

Primary effusion lymphomas, often referred to as body cavity-based lymphomas (BCBLs), represent a specific subset of NHLs that involve body cavities and form a distinct clinicopathologic group (Nador et al., 1996). Most PELs are KSHV positive and are often co-infected with Epstein–Barr virus (EBV) as well. PELs may involve the peritoneal, pleural, or pericardial cavities. These tumors are typically large-cell immunoblastic or anaplastic large-cell lymphomas that express CD45, clonal immunoglobulin gene rearrangements, and lack *c-myc*, *bcl-2*, *ras*, and *p53* gene alterations (Ablashi et al., 2002; Nador et al., 1996). PELs have the characteristics of a pre-terminal stage of B-cell differentiation. Since PEL cells have mutations in their immunoglobulin genes, they are thought to arise from post-germinal center B cells. Most PELs express CD138/syndecan-1 antigen, which is normally expressed by a subset of plasma cells, but they do not express immunoglobulins.

Primary Central Nervous System Lymphoma (PCNSL) and Systemic Lymphomas

PCNSL is a form of NHL arising within and confined to the CNS. PCNSL accounts for up to 15% of NHLs in HIV-infected patients compared to only 1% of NHLs in the general population. EBV appears to play a pathogenic role in AIDS-associated

PCNSL since the EBV genome is transcribed in more than half of AIDS-associated PCNSLs examined (Cingolani et al., 2005; Ivers et al., 2004). EBV expresses type III latency genes in PCNSL.

Systemic AIDS-associated NHLs are aggressive B-cell lymphomas of high or intermediate grade and are heterogeneous. Approximately one-third can be classified as small noncleaved cell lymphomas, which are Burkitt or Burkitt-like lymphomas. The remaining two-thirds of the lymphomas are diffuse large-cell lymphomas, which are immunoblastic lymphomas or large noncleaved cell lymphomas (Brockmeyer and Barthel, 1998). EBV infection has also been observed in a subset of AIDS-associated systemic NHLs. Type I latency has been detected in AIDS-associated BL and type III latency has been detected in the AIDS-associated DLBCL (Angeletti et al., 2008).

The PI3K/Akt Signaling Pathway

Phosphatidylinositol 3-kinases (PI3Ks) are divided into three classes: Class I PI3Ks exist as heterodimers comprised of one of the four p110 catalytic subunits (α, β, δ, and γ) and one of the three regulatory subunits (p85α, p85β, and p55γ). Class I PI3Ks catalyze the phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP_2) at the 3′ position of the inositol ring to generate phosphatidylinositol-3,4,5-trisphosphate ($PIP3_3$). The p110α, β, and γ catalytic subunits are widely expressed in mammalian tissues, whereas p110δ appears to be restricted to lymphocytes. Similarly, p85α and p85β are widely expressed, whereas p55γ is primarily found in brain cells. Class I PI3Ks can be further divided into class Ia (p110α, β, δ) and class Ib (p110γ) PI3Ks (Engelman et al., 2006; Yuan and Cantley, 2008).

The p110 subunits display homology to serine/threonine kinases and can phosphorylate both phosphoinositides and serine/threonine amino acids on proteins. The p110 subunits have higher activity when not complexed with the p85 regulatory subunits but are also very unstable and the amount of p110 monomeric protein in vivo is low. The class Ia enzymes are generally cytosolic and only localize to the plasma membrane upon cell activation through binding of the SH2 domains of the regulatory subunits to tyrosine-phosphorylated motifs (YXXM) of receptor tails. This binding activates the p110 catalytic subunit. Additional binding of the catalytic subunit to activated Ras enhances its membrane association and activation (Engelman et al., 2006; Yuan and Cantley, 2008).

The generation of PI(3,4,5)P3 by PI3Ks allows proteins that contain a pleckstrin homology (PH) domain to localize to the plasma membrane (Toker, 2000; Toker and Cantley, 1997). The PTEN phosphatase is a negative regulator of this pathway (Stambolic et al., 1998; Wu et al., 1998). PTEN catalyzes dephosphorylation at the D3 position of PIP3, serving to counter the effects of PI3K and resulting in inhibition of this activity. PTEN is itself inactivated by phosphorylation (Torres and Pulido, 2001; Vazquez et al., 2001) (Fig. 1). Among the most well characterized of PH domain-containing proteins is Akt, which when recruited to the plasma membrane is phosphorylated on threonine 308 by phosphoinositide-dependent protein kinase

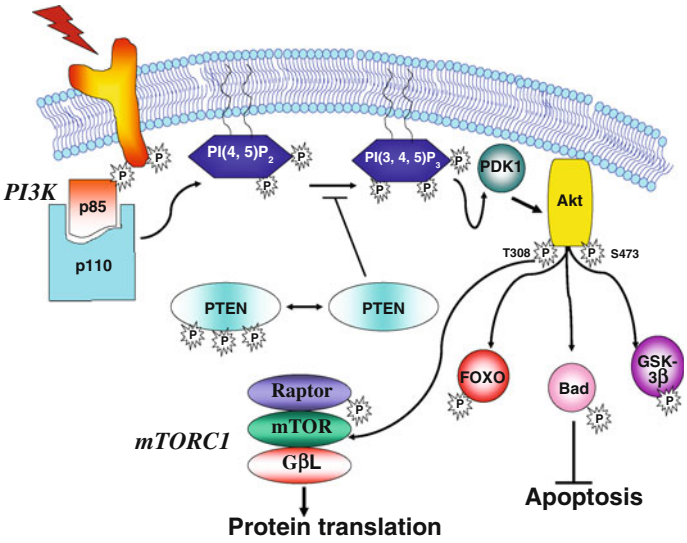

Fig. 1 The PI3K/AKT/mTOR signaling pathway. Activation of cellular (or viral) receptor proteins results in the phosphorylation of tyrosine residues in the receptor cytoplasmic tail. This allows the recruitment of PI3K (p85 and p110) to the receptor. Activated PI3K can generate PIP3 from PIP2. PIP3 subsequently recruits Akt to the cell membrane allowing it to be phosphorylated by PDK1. Activated Akt can phosphorylate and inactivate pro-apoptotic factors like GSK-3β, FOXO, and Bad, leading to cell survival. Activated Akt can also phosphorylate and activate mTOR kinase, leading to increased protein translation and cell proliferation. PTEN is a negative regulator of the PI3K–Akt pathway

1(PDK1), another PH domain-containing protein. Akt is also phosphorylated at serine 473 by a second phosphoinositide-dependent protein kinase 2 (PDK2), which could potentially be one of several different kinases, including the rictor–mTOR complex (mTORC2; see description below).

Active Akt kinase promotes cellular survival mechanisms by directly phosphorylating and inactivating pro-apoptotic factors such as Bad and caspase-9 (Cardone et al., 1998; Datta et al., 1997; del Peso et al., 1997). Additionally, Akt phosphorylates a family of proteins known as the forkhead (FKHR) or FOXO transcription factors (Fig. 1) (Brunet et al., 1999; Kops and Burgering, 1999; Tang et al., 1999). Members of this family that are phosphorylated by Akt include FOXO1, FOXO3a, and FOXO4. The net result of phosphorylation of the downstream targets of Akt is cell survival via inactivation of the FOXO family, GSK-3β, caspase-9, and Bad (Cardone et al., 1998; Cross et al., 1995; Datta et al., 1997; del Peso et al., 1997). Thus, phosphorylation of Bad and FOXO transactivators prevents apoptosis, while phosphorylation of p27, a negative regulator of the cell cycle, prevents cell-cycle arrest.

In addition to its anti-apoptotic role, Akt can also enhance protein synthesis by increasing the phosphorylation of mammalian target of rapamycin (mTOR) (Gingras et al., 1998). A downstream target of Akt is the tuberous sclerosis

complex 2 (TSC2/tuberin). TSC2 interacts with tuberous sclerosis complex 1 (TSC1/hamartin) and is a negative regulator of the small G protein Rheb. Upon phosphorylation of TSC2 by Akt, TSC2 is inhibited from acting as a GTPase-activating protein (GAP) for Rheb, thereby allowing Rheb–GTP levels to accumulate and allowing Rheb–GTP to activate mTOR (Fig. 2) (Manning and Cantley, 2007).

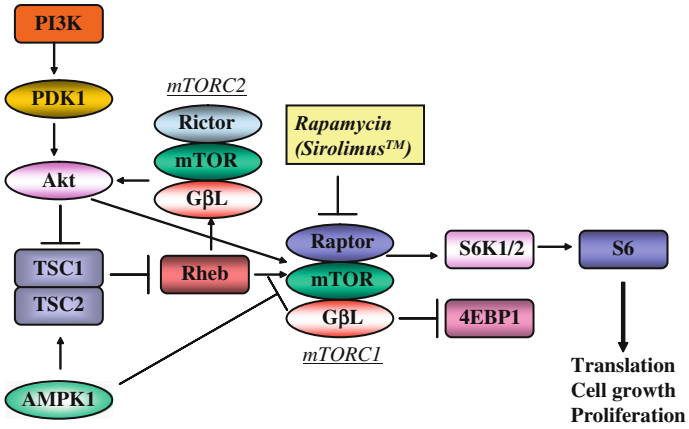

Fig. 2 mTOR signals through two complexes, mTORC1 (raptor complex) and mTORC2 (rictor complex). mTORC1 is rapamycin sensitive, while mTORC2 is rapamycin insensitive. When activated, mTORC2 can phosphorylate Akt in a feedback loop. The activity of the mTORC1 complex is negatively regulated by the TSC1/TSC2 complex. TSC1/TSC2 inactivates Rheb, an enzyme critical for the activation of the mTORC1 complex. TSC2 is a target of Akt kinase and is inactivated upon phosphorylation by Akt. Through this mechanism, the activation of phosphatidylinositol 3-kinase and Akt leads to activation of mTORC1, which promotes cell proliferation through activation of S6 kinase and S6 and inactivation of the eIF4E translational repressor, 4EBP1

Activated mTOR protein can associate with raptor and mLST8/GβL to form the mTORC1 complex, and this complex induces phosphorylation of p70S6 kinase (S6K) leading to activation of the ribosomal protein S6 and inhibition 4E-BP, the repressor of eukaryotic initiation factor eIF4E (Fig. 2). Thus, mTORC1 stimulates translation of TOR-dependent mRNAs and cap-dependent translation (Huang and Manning, 2008). This arm of the mTOR pathway is rapamycin sensitive. However, the mTORC2 complex, which consists of mTOR, mLST8/GβL, Sin, and rictor, is insensitive to the effects of rapamycin. mTORC2 functions in a feedback loop that can phosphorylate and activate Akt at Ser^{473} (Manning and Cantley, 2007) (Fig. 2). The nutrient sensor AMP-activated kinase (AMPK) is a negative regulator of mTOR.

Dysregulated PI3K signaling plays roles in many aspects of tumorigenesis including apoptotic resistance, uncontrolled proliferation, metastasis, and angiogenesis. This occurs through (i) mutations in PI3K itself, (ii) mutations in activated growth factor receptors, (iii) mutations in Ras, or (iv) deletion of the tumor

suppressor gene *PTEN*, which is a negative regulator of the PI3K/Akt pathway (Sansal and Sellers, 2004; Yuan and Cantley, 2008).

Inhibitors of PI3K and mTOR

There have been several attempts to develop inhibitors of PI3K. Wortmannin and a quercetin analogue, LY294002, were developed in the 1990s, but after evaluation in the clinic, they were found to cause hepatotoxicity. Currently there are several new PI3K inhibitors in development for human cancer. These range from single class I PI3K inhibitors such as CAL-101 to multiple class I PI3K isoform inhibitors such as PX-866, XL-147, and GDC-0941 (Ihle and Powis, 2009).

Given its important role in protein synthesis and cell growth, recent advances in cancer research have raised interest in the use of mTOR inhibitors to treat multiple cancers. Rapamycin (also known as sirolimus or rapamune) was first identified as a 914-kDa antifungal bacterial macrolide isolated from *Streptomyces hygroscopicus* (Sehgal et al., 1975). It was initially used in kidney transplant recipients because it was shown to be immunosuppressive (Sehgal et al., 1994). Rapamycin inhibits IL-2 translation and secretion, and thus hinders T-cell proliferation. In the context of organ transplantation, the cell autonomous G1 arrest phenotype of rapamycin is augmented by inhibition of IL-2, which is a paracrine and autocrine growth factor for T cells. In contrast to cyclosporin A, rapamycin did not enhance tumor incidence (Sehgal et al., 1994). Additionally, rapamycin has been shown to sensitize cancers to chemotherapy or radiation therapy (Easton and Houghton, 2006).

Rapamycin and several of its structural derivatives, temsirolimus (Wyeth Pharmaceuticals), everolimus/RAD-001 (Novartis Pharmaceuticals), and AP-23573 (Ariad Pharmaceuticals), are currently in phase I and II clinical trials for a variety of cancers. Temsirolimus was granted FDA approval for the treatment of patients with metastatic renal cell carcinoma and is currently in phase III trials. Similarly, several phase II trials evaluating these novel compounds in non-renal genitourinary malignancies are currently underway and will further define the importance of this pathway as a therapeutic target in these solid tumors.

mTOR Inhibition and Kaposi's Sarcoma

The mammalian target of rapamycin executes essential functions of Akt with regard to cancer cell growth and proliferation. Akt can phosphorylate mTOR directly or phosphorylate and inactivate TSC2, preventing it from heterodimerizing with TSC1 and inhibiting mTOR (Fig. 2). mTOR activation is marked by phosphorylation at Ser^{2441} and Ser^{2448} and association with raptor, the regulatory-associated protein of mTOR. The mTOR:raptor:GβL complex has kinase activity and executes the rapamycin-sensitive functions of mTORC1, such as phosphorylation of the p70 subunit of S6 kinase (p70S6K) at Thr^{389} and Ser^{371}, and phosphorylation of

4E-BP1 at Ser^{65} and $Thr^{37,46}$ (Fig. 2). Phospho-p70S6K phosphorylates small ribosomal protein S6 at $Ser^{235,236}$, which facilitates protein translation. Phosphorylation of 4E-BP1 releases the eukaryotic initiation factor eIF4E from the inactive 4E-BP1:eIF4E heterodimer, which facilitates 40S ribosome assembly and protein translation (Fig. 2). Rapamycin binds to FK506-binding protein 12 (FKB12). The rapamycin–FKB12 complex inhibits mTOR kinase activity (Sabers et al., 1995), preventing activation of S6K and S6. Hence, rapamycin inhibits protein translation, which leads to cell-cycle arrest (cytostasis). Rapamycin typically inhibits cell proliferation with IC_{50}s of 0.5–5 μM. Rapamycin can induce apoptosis but in most instances causes G1 cell-cycle arrest by impeding translation of essential proteins.

Rapamycin is FDA approved to prevent graft rejection following solid organ transplantation. As mentioned above, organ transplant recipients are at an increased risk for developing KS because iatrogenic T-cell immunosuppression activates KSHV, the causative agent of KS. Immunosuppression is thought to disturb host surveillance of this virus, leading to reactivation, increased systemic viral load, and infection of endothelial cells. First-line therapy against transplant-associated KS calls for a reduction in immune suppression upon which some of the KS tumors disappear. However, such an approach risks graft rejection. In a groundbreaking report, Stallone et al. (2005) proved that switching from a regimen that included the immunosuppressant drug cyclosporin A (CsA) to the immunosuppressant drug rapamycin resulted in the regression of cutaneous KS in a group of 15 kidney transplant recipients, whereas graft function was maintained. This was the first indication of the high susceptibility of KS to rapamycin.

Currently, a clinical trial testing rapamycin against HIV-associated KS is being conducted by the AIDS Malignancy Consortium, an NCI/NIH-funded clinical trial group. Several published reports have indicated that rapamycin can be effective against transplant or immunosuppressive therapy-induced KS and that in many (but not all) such patients, KS shows partial or complete response to rapamycin therapy (Campistol et al., 2004; Charfi et al., 2007; Di Benedetto et al., 2008; Gutierrez-Dalmau et al., 2005; Kolhe et al., 2006; Lebbe et al., 2006; Merimsky et al., 2008; Mohsin et al., 2005; Saggar et al., 2008; Sathy et al., 2008; Volkow et al., 2007; Wasywich et al., 2006; Yilmaz et al., 2007; Yuksekkaya et al., 2009). There are also reports suggesting that classic KS is responsive to rapamycin therapy (Merimsky et al., 2008).

mTOR Inhibition and PEL

Activated B cells secrete and are dependent on IL-6 for survival. Secreted IL-6 supports B-cell proliferation in an autocrine fashion which can be inhibited by rapamycin (reviewed in Kishimoto, 2005). PEL cells are exquisitely growth factor dependent in vitro. Depletion of VEGF-1 or IL-6 induces rapid apoptosis in the majority of PEL cultures (Aoki et al., 2000). Most PELs also secrete VEGF-1 and the viral homolog of IL-6, vIL-6. This establishes an autocrine feedback loop. There are some exceptions such as BCBL-1, which secretes very low levels of human IL-6

but higher levels of vIL-6. Sin et al. found that rapamycin inhibited proliferation in the IL-6-dependent BC-1 cell line but less so in the IL-6-independent BCBL-1 cell line (Sin et al., 2007). Rapamycin proved to be efficacious against PEL in culture and in a murine xenograft model (Sin et al., 2007). Rapamycin treatment inhibited downstream mTOR effects, such as S6 phosphorylation. Although it did not affect KSHV transcription, rapamycin inhibited levels of both IL-6 and IL-10 production in PEL cells. FK506 was ineffective in inhibiting PEL growth and CsA required a 20-fold higher concentration (1 μM CsA compared to 50 nM rapamycin). This study suggested the use of rapamycin as a novel therapeutic agent for PEL (Sin et al., 2007). Furthermore, these molecular observations were borne out by two clinical experiences. Dr. David Henry (U. Pennsylvania) and Dr. W. Harrington (U. Miami) each treated one HIV-positive PEL patient with rapamycin. The patients had previously failed to respond to standard chemotherapy but both responded to 0.1 mg/kg/day rapamycin and were alive approximately 1 year after treatment. This is in contrast to most patients with advanced PEL who usually die within weeks.

Most KSHV-associated PELs are uniquely dependent on autocrine growth factors that activate the PI3K/Akt/mTOR pathway. They are therefore uniquely sensitive to rapamycin. However, there are a few exceptions. In one report, two HIV-1-negative patients were described who developed post-transplantation PEL while receiving rapamycin therapy (Boulanger et al., 2008). Thus, it is possible that long-term exposure to rapamycin may have led to rapamycin-resistant PEL in these patients. A combination of rapamycin with other cytotoxic therapies may prove beneficial to prevent the development of rapamycin resistance. Along these lines, our group recently reported that NVP-BEZ235, a dual inhibitor of PI3K and mTOR kinase, was very effective in preventing PEL growth in vitro and in mouse xenograft models (Bhatt et al., 2010).

mTOR Inhibition and EBV-Associated NHL

Rapamycin has also been shown to inhibit the proliferation of Epstein–Barr virus-positive B-cell lymphomas in vitro by decreasing cyclin D2 and D3 protein expression and increasing the levels of p27, a CDK inhibitor (Vaysberg et al., 2007). Additionally, mTOR inhibition of several EBV-positive PTLD lymphomas suppressed their growth (Wlodarski et al., 2005). However, to date, there are no clinical reports on the use of rapamycin to treat EBV-positive primary CNS or systemic lymphomas.

Summary

Literature reports suggest that the PI3K/Akt/mTOR pathway is highly activated in KSHV- and EBV-positive malignancies in HIV-positive people (Sin et al., 2007; Tomlinson and Damania, 2004; Uddin et al., 2005; Wang and Damania, 2008; Wang et al., 2006). This may be due to several viral proteins that have been shown to

activate this pathway, such as KSHV K1, vGPCR, and vIL-6 as well as EBV LMP1 and LMP2 (Bais et al., 1998, 2003; Dawson et al., 2003; Fukuda and Longnecker, 2004; Mainou et al., 2005; Montaner, 2007; Montaner et al., 2003; Moody et al., 2005; Morrison et al., 2003; Mutlu et al., 2007; Scholle et al., 2000; Shair et al., 2007; Sodhi et al., 2006, 2000; Tomlinson and Damania, 2004; Wang and Damania, 2008; Wang et al., 2006).

Treatment with rapamycin and its derivatives has elicited partial or complete responses in KSHV-associated KS and PEL in several patients. However, it is possible that rapamycin resistance may develop after prolonged therapy. Hence, a combination of rapamycin with other treatments may prove more beneficial for AIDS-associated malignancies. For example, a combination of rapamycin with sunitinib (receptor tyrosine kinase inhibitor) might be more effective since rapamycin would block mTORC1 and sunitinib would block mTORC2 feedback, resulting in Akt activation. Indeed, a combination of sunitinib and a rapamycin derivative, RAD001, was proven to be more efficacious against several hematologic malignancies than either drug alone (Ikezoe et al., 2006). Furthermore, additional clinical therapeutic agents that target other arms of the PI3K/Akt/mTOR pathway may also be beneficial for treatment of AIDS-associated KS and NHL, either alone or in combination with rapamycin.

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Restoration of p53 Function by MDM2 Inhibition: Potential Strategy to Treat KSHV-Associated Malignancies

Grzegorz Sarek and Päivi M. Ojala

Abstract Kaposi's sarcoma-associated herpesvirus (KSHV) is a human oncogenic virus associated with Kaposi's sarcoma (KS), the most frequent malignancy in AIDS patients, and primary effusion lymphoma (PEL) as well as multicentric Castleman's disease (MCD). Unlike in several other human cancers, p53 gene mutations are rare in KSHV-induced malignancies and the majority of these tumors contain wild-type p53. Several strategies have recently been developed in an attempt to reactivate p53 tumor suppressor function in malignant tumor cells. These include pharmacological restoration of p53 function by small-molecule inhibitors of the p53–MDM2 interaction. Nutlins represent a class of potent and selective small-molecule antagonists that interact with the hydrophobic cleft representing the p53-binding pocket on the surface of MDM2. Here we discuss the cellular responses to Nutlin and the potential of using restoration of the p53 pathway as a novel treatment modality for KSHV-associated malignancies.

Regulation of p53 by MDM2

The p53 pathway responds to a wide variety of cellular stress signals such as different forms of DNA damage, telomere shortening, mitotic spindle damage, hypoxia, unfolded proteins, improper ribosome biogenesis, and activation of certain oncogenes (Levine, 1997). Activation of the p53 pathway initiates a program leading to cell-cycle arrest, cellular senescence, or apoptosis, all targeted to eliminate potentially malignant cells and to prevent tumor formation (Vogelstein et al., 2000). Stabilization of the p53 protein is a key event in the response to cellular stress and essential for its proper tumor suppressor function (Levine et al., 2004). The p53

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protein is a transcription factor that can either activate or repress the transcription of its target genes. Transcriptional activation by p53 occurs primarily through direct, sequence-specific DNA binding to a site that moderately matches the consensus p53 response element (Riley et al., 2008). Under non-stressed conditions, p53 levels and thus its activity are maintained at very low levels through interaction with its negative regulator MDM2, an E3 ubiquitin ligase that promotes p53 degradation via the ubiquitin-dependent proteasomal pathway (Honda et al., 1997). Furthermore, MDM2 binds the transcription activation domain of p53 and impairs its *trans*-activation capacity (Freedman et al., 1997). Interestingly, MDM2 itself is a p53 target gene whose transcription is induced upon p53 activation, thus generating a feedback mechanism for regulating p53 activity. In addition, MDM2 can also autoregulate its own stability. Upon induction of cellular stress, MDM2 targets itself for degradation and this results in an increase in the half-life of p53 from minutes to hours.

In about 50% of human cancers, the p53-encoding *TP53* gene is altered by mutation or deletion (Lane and Benchimol, 1990; Vogelstein et al., 2000). In the remaining 50% of human cancers, p53 is inactivated by other means such as through binding to oncogenic viral proteins or as a result of alterations in genes whose products interact with p53. A subset of human cancers with wild-type (wt) p53 overexpress the MDM2 protein leading to enhanced p53 degradation (Haines, 1997). These tumors are characterized by rapid tumor progression and resistance to conventional anti-cancer therapies. Intriguingly, MDM2 overexpression is the only mechanism that disables p53 function in these tumors, which suggests that overexpression of MDM2 is an alternative way to inactivate p53 function (Leach et al., 1993) in addition to direct effects on the *TP53* gene.

MDM2 Inhibitors

Several different approaches have been tried over the last few years that aim to identify means to activate the p53 pathway as a targeted cancer treatment (Wang and El-Deiry, 2008). These include small molecules capable of changing the conformation of an inactive mutant of p53 to an active one (CP-31398 and PRIMA-1; Foster et al., 1999; Bykov et al., 2002) or molecules that stabilize and activate p53 (Issaeva et al., 2004). The idea that malignant cells could be eradicated by activation of p53 through inhibition of MDM2 binding has gained particular attention as an attractive strategy for treating cancer with wt p53 expression. To this end, researchers at Roche screened a diverse library of synthetic chemicals to identify compounds that would inhibit binding of MDM2 to p53 (Vassilev et al., 2004). This led to discovery of a series of *cis*-imidazoline analogs that were named Nutlins (for Nutley inhibitor). This class of potent and selective small-molecule antagonists interacts with the hydrophobic cleft that forms the p53-binding pocket on the surface of MDM2 by mimicking the critical amino acids of the helical region of p53 (Vassilev, 2004; Vassilev et al., 2004). By binding to MDM2, the most effective

antagonist, Nutlin-3 prevents it from binding to and targeting p53 for degradation. This leads to stabilization of the p53 protein and activation of the p53 pathway both in vitro and in vivo. Importantly, the efficacy of Nutlins requires wild-type status of the *TP53* gene and intact p53 signaling machinery.

Pleiotropic Cellular Responses to the MDM2 Antagonist Nutlin-3

Nutlin-3 was soon shown to induce apoptosis in a variety of different cancer cell lines, in some primary tumor samples *ex vivo*, and to have strong anti-tumor effects in mouse tumor models for osteosarcoma and prostate cancer (Vassilev et al., 2004; Kojima et al., 2005; Stuhmer et al., 2005; Coll-Mulet et al., 2006; Secchiero et al., 2006; Tovar et al., 2006; Drakos et al., 2007; Logan et al., 2007). The cancer cell specificity and effectors of Nutlin-induced apoptosis have been addressed by several investigations. By using an shRNA barcode screen, Brummelkamp et al. identified a DNA damage sensor protein, the p53-binding protein 1 (53BP1), as a critical mediator of Nutlin-3-induced cytotoxicity (Brummelkamp et al., 2006). Although Nutlin-3 itself does not induce DNA damage (Thompson et al., 2004), the finding suggests that a recently discovered intrinsic property of cancer cells, the activated DNA damage signaling (Bartkova et al., 2005; Gorgoulis et al., 2005), could contribute to the anti-tumor activity of Nutlin-3.

In several cancer cell lines, Nutlin-3 treatment leads to cell-cycle arrest without induction of apoptosis. For example, colon cancer and osteosarcoma cells respond to UVC by apoptosis, while Nutlin-3 treatment induces a cell-cycle arrest. It has been suggested that the response to Nutlin-3 depends on extraordinarily strong induction of *p21Cip1* mRNA levels from the p53 target gene, *CDKN1A*. This study further demonstrated that the transcriptional activity of the *CDKN1A* promoter varies greatly in response to distinct p53-activating stimuli (Donner et al., 2007a, b). In a cell model for cancers with constitutive CDK signaling, Nutlin-3 prevented the anchorage-independent growth of these cells by inducing a growth arrest independently of p21Cip1 (Kan et al., 2007). In this case, the anti-proliferative effect was due to direct interaction of p53 with the *CDC2* and *cyclin B1* promoters. A recent study by Enge et al. (2009) put forward another suggestion for the apparent preference of Nutlin-3 for inducing cell-cycle arrest rather than apoptosis in several cancer cell lines. They showed that when p53 was reactivated by a small-molecule compound, RITA, which binds to p53 instead of MDM2, it led to dissociation of MDM2 from p53 and promoted degradation of both p21Cip1 and a transcriptional cofactor, hnRNP K, which is required for p53-mediated induction of *CDKN1A* gene. Consequently, p21Cip1 was unable to execute its growth-suppressive function, which was shown to favor activation of the p53 apoptotic response. However, when cells were treated with Nutlin-3, this induced binding of hnRNP K to the upstream site of the *CDKN1A* promoter, thus leading to potent up-regulation of p21Cip1 and activation of the cell-cycle arrest function of p53. This suggests that regulation of

p21Cip1 expression represents a major switch between growth arrest and apoptosis in response to p53 reactivation.

In addition to apoptosis and cell-cycle arrest, p53 activation can lead to cellular senescence. Cellular senescence also plays a critical role in early stages of tumorigenesis (Bartkova et al., 2005). Interestingly, recent reports demonstrate that treatment of human and murine normal fibroblasts, oncogenically transformed fibroblasts, fibrosarcoma cell lines, and prostate cancer cells with Nutlin-3 leads to induction of cellular senescence without any signs of apoptosis (Efeyan et al., 2007; Lehmann et al., 2007; Kumamoto et al., 2008). These results suggest that senescence could also be a major cellular outcome of treatment by antagonists of the p53–MDM2 interaction. Whether cancer cells would be more susceptible to Nutlin-3-induced senescence than normal cells needs to be validated.

Recent results have suggested that inhibition of both MDM2 and its close homologue MDMX is necessary for reactivation of p53 in certain tumor cells (Hu et al., 2007). Although MDMX does not possess intrinsic E3 ligase activity, it inhibits the ability of MDM2 to destabilize p53 by forming heterodimers with MDM2 (Jackson and Berberich, 2000). On the other hand, MDMX can also directly interfere with p53 function by forming inactive MDMX–p53 complexes (Michael and Oren, 2003). Despite significant homology between MDM2 and MDMX, Nutlin-3 fails to inhibit MDMX (Hu et al., 2006; Wade et al., 2006). This can therefore severely compromise the therapeutic efficacy of p53 antagonists, especially in cancers overexpressing MDMX (Toledo and Wahl, 2006). To address this challenge, a novel strategy for dual targeting of MDM2 and MDMX was recently reported (Hu et al., 2007) with promising therapeutic potential for cancers involving overexpression of MDMX or both MDMX and MDM2. Importantly, a more recent study by Xia et al. (2008) shows that treatment of 10 solid tumor cell lines with Nutlin-3 substantially decreased the levels of MDMX in a ubiquitin-dependent manner with a subsequent increase of MDM2. These data imply that even though MDMX is not a downstream target of p53, Nutlin-3 can change the ratio between p53 and MDMX. Therefore the remaining MDMX molecules may be insufficient to bind to and inhibit excess p53, while the cellular pool of MDMX is simultaneously targeted to degradation by elevated MDM2 E3 ligase.

Very intriguing recent findings suggest that Nutlin-3 could elicit also its antitumorigenic effects via inhibition of angiogenesis. LaRusch et al. (2007) demonstrated that the anti-angiogenic effects of Nutlin-3 are due to disruption of the interaction between hypoxia-inducible factor 1α (HIF1α) and MDM2. Binding to MDM2 can increase HIF1α activity to induce *VEGF* transcription, and this was shown to occur via a p53-independent mechanism. More recently, Nutlin-3 was also shown to elicit anti-angiogenic activity by preventing migration and tube formation of endothelial cells in 3-D models in addition to inducing cell-cycle arrest in endothelial cells (Secchiero et al., 2008). Importantly, these findings may enable broader application of Nutlin-3 or other MDM2 antagonists in anti-angiogenic regimens.

Combination Therapies

While current chemotherapeutic agents can efficiently induce suppression of tumor growth and regression of tumors, the efficacy of this kind of anti-cancer treatment is limited by acquired drug resistance of tumors, collateral DNA damage, and toxicity to normal tissues. Therefore, the use of non-genotoxic drugs such as MDM2 antagonists, which are also more specific for tumor cells than for normal cells (see below), may help to avoid or reduce severe genotoxic side effects and provide an attractive therapeutic strategy in cancers with wt p53 expression.

Cheok et al. (2007) recently explored the anti-tumor potential of cyclin-dependent kinase (CDK) inhibitors in combination with Nutlin-3. They found that low doses of the CDK inhibitors roscovitine and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) synergized with the MDM2 antagonist in the induction of p53 activity and promoted p53-dependent apoptosis in a dose- and time-dependent manner. This treatment was independent of the DNA damage-induced Ser15 phosphorylation of p53 and did not induce genotoxic stress in the cell. This may thus represent a new therapeutic approach for the use of CDK inhibitors and MDM2 antagonists in combinatorial drug therapy.

Nutlin-3 cytotoxicity can also be enhanced by combining it with genotoxic drugs. Kojima et al. (2005) reported synergistic cytotoxic effects of MDM2 inhibition in combination with cytosine arabinoside and doxorubicin in acute myeloid leukemia blasts but not in normal hematopoietic progenitor cells. Other examples of Nutlin-3 synergism with genotoxic drugs have been reported upon treatment of B-cell chronic lymphocytic leukemia. In these studies, Nutlin-3 synergized with the genotoxic drugs doxorubicin, chlorambucil, and fludarabine (Coll-Mulet et al., 2006; Secchiero et al., 2006). A recent work further demonstrated that Nutlin-3 and doxorubicin, or a selective proteasome inhibitor, bortezomib, display synergistic cytotoxic activity in mantle cell lymphoma (MCL) (Tabe et al., 2009). The intriguing finding of this report was that Nutlin-3 alone, or synergistically with bortezomib, inhibited the growth of an MCL cell line carrying mutated p53. These effects were associated with stabilization and activation of the TP53 homologue p73, increases in p21Cip1 and Noxa, and caspase activation.

p53 Reactivation as a Promising Treatment for Primary Effusion Lymphomas

Primary effusion lymphoma (PEL) is a rare form of non-Hodgkin's lymphoma associated with Kaposi's sarcoma-associated herpesvirus (KSHV) infection. It occurs mostly in HIV-positive patients as lymphomatous effusions, proliferating within pleural, peritoneal, or pericardial body cavities (Cesarman et al., 1995; Ansari et al., 1996; Carbone et al., 1996; Nador et al., 1996). PEL typically manifests as diffuse large B-cell lymphoma and exhibits clonal gene rearrangements and hypermutations

of Ig heavy chain genes, indicating that it represents a pre-plasmacytic stage of B-cell differentiation (Nador et al., 1996; Matolcsy et al., 1998; Gaidano et al., 2000). Despite recent promising leads for novel therapeutic strategies such as inhibition of NF-κB or mTOR signaling (Keller et al., 2000; Wu et al., 2005; Keller et al., 2006; Sin et al., 2006), currently the therapy for PEL remains a challenge. This is primarily due to PEL resistance to conventional chemotherapy or non-chemotherapeutic treatments that are found active against other lymphomas. The prognosis for patients is still very poor and the median survival ranges from 2 to 6 months after diagnosis (Simonelli et al., 2003; Boulanger et al., 2004, 2005). Interestingly, p53 mutations are rare events in all KSHV-associated malignancies (Nador et al., 1996; Carbone et al., 1998; Katano et al., 2001; Chadburn et al., 2004), and the p53 signaling cascade of PEL cells is fully functional in response to a common genotoxic agent, doxorubicin (Petre et al., 2007).

The wt status of p53 in PELs has prompted exploration of the effect of Nutlin-3 in different PEL cell lines (Petre et al., 2007; Sarek et al., 2007). All PEL cell lines harboring the wt p53 allele showed induction of p53 target genes such as p21Cip1, Bax, and MDM2 in response to Nutlin-3 as well as a very efficient growth inhibition and apoptosis. These data provide additional evidence for the functional inactivation of the p53 pathway in these lymphomas, which was previously suggested to be mediated by binding of the KSHV latency-associated nuclear antigen (LANA) to p53 (Friborg et al., 1999). This probably also explains why PELs can retain the wt *TP53* gene as the pressure to mutate p53 is eliminated by LANA.

Nutlin-3 treatment led to rapid induction of p21Cip1 in association with a dramatic G1 arrest in PELs after only 12 h of treatment with Nutlin-3. In contrast to colon cancer and osteosarcoma cells, which respond to Nutlin-3 by a p21Cip-dependent cell-cycle arrest without significant apoptosis (Donner et al., 2007b), Nutlin-3 treatment of PEL cells induced massive apoptosis starting 48 h after treatment (Sarek et al., 2007; Sarek and Ojala, 2007). As high level of p21Cip1 induction was suggested to be a critical determinant of the non-apoptotic cellular response to Nutlin-3 in colon cancers, it is possible that the different outcomes of high p21Cip1 induction in PEL cells could be at least partially influenced by the presence of a virally encoded cyclin that evades the cell-cycle arrest function of p21Cip1 (Swanton et al., 1997; Jarviluoma et al., 2006).

Our results indicate that Nutlin-3 treatment selectively induces massive apoptosis in KSHV-infected lymphomas and lymphoblastoid cells, but not in KSHV-negative, EBV-infected lymphoblastoid cells. In addition, de novo KSHV infection of naïve osteosarcoma cells, which are normally rather resistant to Nutlin-3 cytotoxicity, clearly sensitized them to apoptosis over the uninfected parental cells (Sarek et al., 2007). What could confer the remarkable specificity of Nutlin-3 for KSHV-infected cells? We have identified LANA as a component of the p53–MDM2 complex and demonstrated that Nutlin-3 disrupts this ternary complex in KSHV-positive lymphoma cells (Fig. 1 and Sarek et al., 2007). A second possible factor contributing to Nutlin-3 cytotoxicity toward KSHV-infected cells may be activated DNA damage signaling, which has been recently reported to enhance the selectivity of Nutlin-3-mediated cell killing of cancer cells (Brummelkamp et al., 2006). Intriguingly,

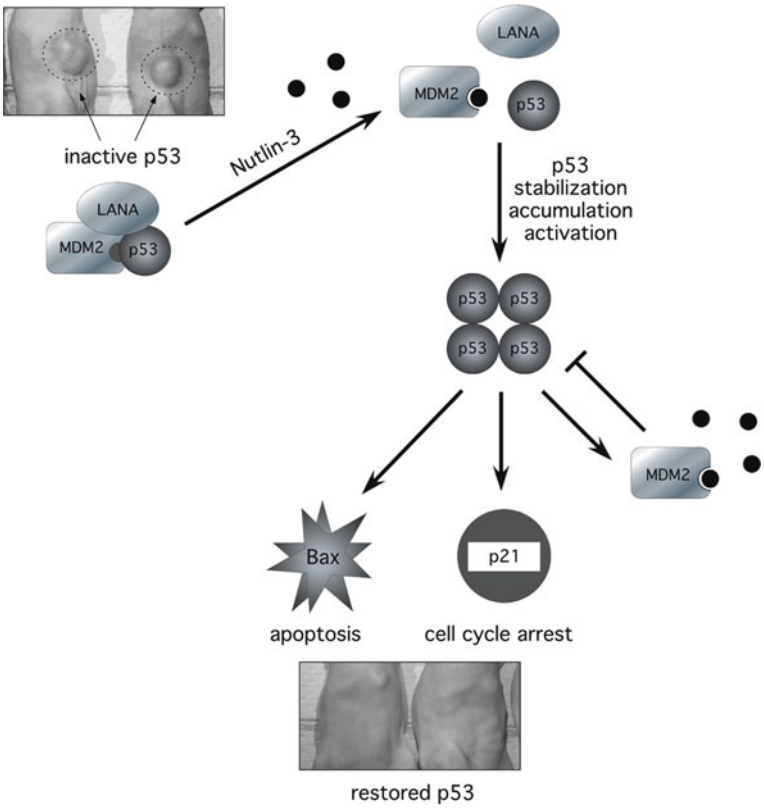

Fig. 1 A model for restoration of p53 function by small-molecule antagonist of MDM-2 in primary effusion lymphomas. In PEL cells LANA interacts with p53 and MDM2, which leads to repression of the p53 function and allows tumor development in mice. Treatment with Nutlin-3 disrupts the p53-MDM2–LANA complex and releases p53 from the repression. Inhibition of MDM2 binding to p53 leads to p53 stabilization, activation, and accumulation as transcriptionally active tetramers. The subsequent activation of the p53 target genes and the p53 pathway results in cell-cycle arrest and massive apoptosis, thus preventing tumor development

an increased number of DNA damage-specific foci of the ATM-Chk2 checkpoint components were detected in PEL cells, but not in EBV-infected lymphoblastoid cells (Sarek and Ojala, 2007). Furthermore, when the function of the ATM-Chk2 pathway was compromised by treatment with caffeine, the extent of apoptosis was suppressed in PEL cells, suggesting that activated DNA damage signaling is contributing to the Nutlin-3 specificity in PELs. High-level MDM2 expression has also been suggested to promote the specific cytotoxic effects of Nutlin-3 (Patton et al., 2006; Tovar et al., 2006). Further support for this concept in relation to KSHV lymphomas was provided by the study of Petre et al. (2007), which examined *MDM2* mRNA levels using data representing the transcriptional profile of over 60 B-cell tumors. Interestingly, PEL cases were clearly distinguished from all other B-cell

tumors by elevated *MDM2* mRNA levels, supporting the role of amplified MDM2 levels in contributing to the Nutlin-3 specificity for PELs.

PELs are often dually infected with both KSHV and EBV, which could influence the response of cells to p53 reactivation. Nutlin-3 was recently shown to activate the p53 pathway in all Burkitt's lymphoma (BL) cell lines harboring wild-type p53, regardless of their EBV status. Interestingly, Nutlin-3 was able to induce apoptosis in EBV(–) or latency I EBV(+) cells, while cells displaying latency III EBV(+) appeared much more resistant to Nutlin-3-induced cytotoxicity (Renouf et al., 2009). The dually infected PEL cell lines have been shown to exhibit a restricted EBV latency gene expression pattern (Horenstein et al., 1997; Szekely et al., 1998), while the EBV lymphoblastoid cell lines (LCLs) display type III latency (Lewin et al., 1995; Szekely et al., 1998). It is thus possible that EBV infection can contribute to cytotoxicity by Nutlin-3 in PEL cells. This is supported by our observation of the rapid cell death response of BC-1 cells to Nutlin-3 (Sarek et al., 2007).

While the results described above are encouraging and demonstrate the potential of MDM2 inhibition to kill PEL cells, an essential feature of an anti-cancer therapeutic agent is not only high efficiency in vitro but more importantly inhibition of tumor proliferation in vivo. We therefore investigated the effect of p53 restoration in a human xenograft mouse model for PEL. This was accomplished by implanting BC-3 PEL cells in matrigel (Staudt et al., 2004) to create xenografts in Balb/c nude mice. Nutlin-3 was administered to the tumor-bearing mice intraperitoneally at 20 mg/kg every second day for a period of 14 days (Sarek et al., 2007). Inhibition of tumor growth was achieved already after three doses, and a subset of PEL tumors completely regressed after two consecutive weeks of treatment. Some tumors with partial response in this group resumed growth 9 days after the treatment was stopped. These tumors, however, were substantially smaller in size and had a slower growth rate when compared to tumors in control animals. Importantly, additional treatment with Nutlin-3 led to tumor regression again without signs of recurrence during the follow-up period (Sarek et al., 2007). This study suggests that p53 reactivation by MDM2 antagonists could be a potentially effective treatment for KSHV-associated lymphomas.

To extend the application of p53 reactivation as a therapeutic strategy in vivo we are currently investigating the effect of Nutlin-3 in an intraperitoneal PEL tumor model in severe combined immunodeficient mice (unpublished data). In this model we are using BC-3 PEL cells carrying an NF-κB-regulated luciferase reporter gene (BC-3/NF-kB-luc) (Keller et al., 2006). This model mimics human disease progression better than subcutaneous xenografts and allows us to follow tumor formation and the therapeutic effect of MDM2 inhibition by non-invasive whole-body in vivo imaging.

The goal of an optimal anti-cancer treatment is to efficiently eradicate malignant cells without causing extensive damage to cells in normal tissues. This has also been a concern in the development of MDM2 inhibitors for cancer treatment. Ringshausen et al. (2006) have recently shown that reactivation of p53 function in the absence of the *MDM2* gene (in MDM2-deficient mice) results in atrophy and massive apoptosis in radiosensitive tissues leading to animal death.

Conversely, reports by Ventura et al. (2007) and Shangary et al. (2008) demonstrate that restoration of p53 function by means other than the deletion of *MDM2* gene induced complete tumor growth inhibition without any damage to either radiosensitive or radio-resistant organs. In accordance with these findings, our preliminary results also suggest that accumulation of p53 following Nutlin-3 treatment does not cause substantial apoptosis in radiosensitive tissues of treated mice (our unpublished data). These findings provide further hope for efforts to treat human cancers by pharmacological reactivation of p53.

Restoration of p53 Function in KS Tumors

The encouraging results from Nutlin-3 treatment of PEL mouse models suggest that the efficiency of pharmacological inhibition of MDM2 should also be evaluated in suitable preclinical models for Kaposi's sarcoma (KS). Similar to PEL and solid KSHV lymphomas, mutations in p53 are very infrequent in KS (Chadburn et al., 2004). Further support that Nutlin-3 might also elicit potent anti-tumor activity in KS is provided by our recent finding that DNA damage signaling is activated in KSHV-infected endothelial cells as well as in early-stage lesions of KS tumors (Koopal et al., 2007). We have also shown that Nutlin-3 treatment specifically increases apoptosis of KSHV-infected endothelial cells but has a minimal effect on the viability of the parental non-infected cells (Koopal et al., 2007). The anti-tumor activity of Nutlin-3 against KS in vivo models could be tested either in mice bearing subcutaneous xenografts of KSHV-infected endothelial cells (An et al., 2006) or in a recently reported mouse model, in which KS-like tumors form upon introduction of a recombinant GFP-expressing KSHV into mouse bone marrow endothelial lineage cells (Mutlu et al., 2007).

Future Prospects for Pharmacological Inhibition of MDM2

As discussed above, targeting the MDM2–p53 interaction could be a promising therapeutic strategy for KSHV-associated malignancies. It would therefore be important to invest in development of MDM2 antagonists with high specificity, optimal pharmacokinetic profile, and bioavailability. Employing a de novo structure-based approach, Shangary et al. (2008) recently reported identification of spiro-oxindoles as a new class of inhibitors for the p53–MDM2 interaction. They demonstrated that one of the ideal antagonists, MI-219, effectively and selectively induced apoptosis in cancer cells expressing wt p53 but had minimal effect on normal cells. Moreover, upon administration of a single oral dose, MI-219 inhibited cell proliferation and induced apoptosis in a mouse xenograft model for human cancer. This further confirms that established tumors with non-mutated p53 remain persistently vulnerable to the pro-apoptotic activity of p53 and suggests that pharmacological inhibition of the p53–MDM2 interaction by ideal antagonists warrants clinical evaluation as a new form of cancer therapy.

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Interferon in Kaposi's Sarcoma Biology and Therapy

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Abstract Interferon alfa (IFN-α) was one of the first drugs shown to have activity against AIDS-associated Kaposi's sarcoma (KS). Multiple clinical studies have defined IFN-α's role as a single agent or in combination with antiretroviral drugs against KS and have indicated that preserved immune function and the absence of activation of the endogenous IFN system are markers for improved outcome. Infection with both human immunodeficiency virus (HIV) and the KS-associated herpesvirus (KSHV) has been associated with perturbations in the IFN system, but it is not known precisely how this influences the development of KS, its clinical course, or response to exogenous IFN therapy. Multiple mechanisms have been proposed for IFN-α's antitumor activity against KS including inhibition of cell proliferation, targeted inhibition of pathways leading to aberrant angiogenesis, and inhibition of both HIV and KSHV, but its precise mechanism of action is not known, nor is it known why some tumors fail to respond. The future development of IFN therapy for KS should include combinations of IFN with other agents and/or the use of IFN preparations that require less frequent administration.

Introduction

The appearance of Kaposi's sarcoma (KS) in previously healthy young men in New York and San Francisco was one of the first heralds of the AIDS epidemic in the early 1980s (Centers for Disease Control, 1981). Long considered a rare neoplasm that usually presented in elderly individuals in developed countries, KS was also known to affect indigenous Africans across a spectrum of ages and to occur at increased rates in individuals who were immunosuppressed (Penn, 1979). The

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link to iatrogenic immunosuppression, together with the observation, first reported in the early 1970s, of herpesvirus-like particles in KS tumor biopsies (Giraldo et al., 1972, 1975, 1978), provided a context in which to consider KS as an opportunistic neoplasm and a rationale for attempts to use interferon therapeutically in this disease. It was reasoned that interferon, by virtue of its antiviral, antineoplastic, and immunomodulatory actions, might have activity against a neoplasm possibly linked to a herpesvirus and arising in the setting of profound but unexplained immunosuppression. This, in fact, proved to be the case, as many of the initial patients treated with recombinant interferon alfa (IFN-α) showed regression of their KS lesions (Krown et al., 1983; Groopman et al., 1984). It was only much later, however, that a more nuanced, but as yet incomplete understanding of the role of the interferon system in AIDS and KS was attained.

This chapter reviews the results of clinical trials of interferons in AIDS-associated KS, what is known about perturbations in the interferon system in patients with AIDS and KS, and our current understanding of how interferon treatment may affect the disease process. The potential future place of interferon in the therapeutic armamentarium for KS will also be discussed.

Kaposi's Sarcoma Background

KS is an angioproliferative neoplasm that is characterized pathologically by the presence of spindle-shaped endothelial cells, inflammatory cells, expression of growth and angiogenic factors and inflammatory cytokines, and angiogenesis. The tumor usually arises in the skin, but lesions can also involve multiple other sites including the oral cavity, the gastrointestinal tract, lymph nodes, the lung parenchyma, endobronchial mucosa and pleural surfaces, the liver and other visceral organs, and bone, but does not involve the brain. Considerable evidence indicates that the spindle cells comprising KS lesions are of endothelial origin and show a gene expression profile most closely resembling that of lymphatic endothelial cells, but blood vascular endothelial cell markers are also expressed (Wang et al., 2004). Development of KS requires infection with a human herpesvirus, human herpesvirus-8, also known as the KS-associated herpesvirus (KSHV) (Chang et al., 1994), but other co-factors are involved in KS lesion development.

Therapeutic Trials of Interferons in Kaposi's Sarcoma

Interferon-α monotherapy: Interferons were among the first therapeutic agents tested against AIDS-associated KS (KS/AIDS). By chance, the availability in 1981 of the first recombinant alfa IFNs for clinical testing, and shortly thereafter of highly purified lymphoblastoid IFN preparations, coincided with the rapidly increasing numbers of people diagnosed with KS/AIDS. The ability of high-dose recombinant IFN-α to induce regression of KS/AIDS was first reported in a small group of

patients in 1983 (Krown et al., 1983) and confirmed over the next several years in larger numbers of patients treated with both recombinant and highly purified lymphoblastoid IFN-α preparations (Groopman et al., 1984; Gelmann et al., 1985; Rios et al., 1985; Real et al., 1986; Kern et al., 1987; Fischl et al., 1987; Volberding et al., 1987; Lane et al., 1988; deWit et al., 1988; Rozenbaum et al., 1990; Evans et al., 1991).

It is noteworthy that these early examples of IFN-induced KS regression were achieved in the absence of *any* concomitant antiretroviral therapy, as HIV was not discovered until several years after the first clinical trials of IFN-α, and the first HIV nucleoside reverse transcriptase inhibitors were not widely available until the late 1980s.

Several of these studies explored a range of IFN-α doses and demonstrated the therapeutic superiority of high, often toxic, doses of IFN-α, generally at least 20 million U/m^2 body surface area administered on a daily or thrice-weekly schedule, over lower doses. On average, the higher doses of IFN-α induced objective (complete or partial) tumor responses in about 30% of patients (Krown et al., 1983; Groopman et al., 1984; Gelmann et al., 1985; Rios et al., 1985; Real et al., 1986; Fischl et al., 1987; Volberding et al., 1987; Lane et al., 1988; deWit et al., 1998; Evans et al., 1991), whereas less than 10% of patients who received IFN-α doses of up to 7.5 million U/m^2 body surface area showed an objective response (Groopman et al., 1984; Gelmann et al., 1985; Real et al., 1986). This suggested to some that direct antiproliferative effects of IFN-α were primarily responsible for its anti-KS activity.

Interferon-α with antiretroviral therapy: Following the discovery of HIV and the development and introduction into clinical practice of a variety of drugs that inhibit its replication, studies were conducted to evaluate combination therapy with antiretroviral drugs and IFN-α. The basis for combining these classes of agents was, at least in part, the finding that HIV replication was synergistically suppressed in vitro when IFN-α was combined with various HIV nucleoside reverse transcriptase inhibitors (NRTIs) (Ho et al., 1985; Hartshorn et al., 1987; Vogt et al., 1988; Johnson et al., 1991). Because of its unique mechanisms of action against HIV (Pitha, 1994), it was also hypothesized that IFN-α might influence NRTI resistance. In fact, Johnson and colleagues (1991) demonstrated synergistic inhibition of zidovudine-resistant HIV isolates in vitro when IFN-α was combined with zidovudine or didanosine. Additionally, although the precise link between HIV infection and the development and control of KS/AIDS was not clear, administration of NRTIs had been shown to favorably influence certain immune function markers associated with KS response to IFN-α (Jacobson et al., 1991; Mildvan et al., 1992). Although it is now known that combination antiretroviral therapy in itself can lead to KS regression in some cases (Krown, 2004), single drug anti-HIV therapy alone has not resulted in clinically significant KS regression (deWit et al., 1989; Lane et al., 1989b).

The combination of IFN-α with zidovudine was intensively studied in clinical trials performed in the late 1980s and early 1990s (Kovacs et al., 1989; Krown et al., 1990; Stadler et al., 1990; Baumann et al., 1991; deWit et al., 1991; Fischl et al., 1991; Podzamczer et al., 1993; Mauss and Jablonowski, 1995; Fischl et al., 1996;

Shepherd et al., 1998). Most of these trials documented objective KS responses in 40% or more of patients, sometimes at IFN-α doses considerably lower than those shown to be active when IFN-α was administered alone. In addition, whereas KS regression was generally observed only in patients with relatively high baseline CD4 counts when high-dose IFN-α was administered as a single agent, some of the combination regimens induced responses in up to 30% of subjects with baseline CD4 T-lymphocyte counts below 200 cells/μl (Krown et al., 1990; Stadler et al., 1990; Podzamczer et al., 1993; Fischl et al., 1996) and considerably higher response rates than those previously reported for IFN-α monotherapy among those with higher CD4 counts.

Hematologic toxicity, especially neutropenia, was a major dose-limiting side effect of the IFN-α and zidovudine combination and limited the amount of IFN-α that could be safely administered. Neutropenia could be prevented or reversed by the co-administration of recombinant granulocyte-macrophage colony-stimulating factor (Davey et al., 1991; Scadden et al., 1991; Krown et al., 1992), but this did not lead to a significant increase in the maximum tolerated dose of IFN-α or in tumor response rates because non-hematologic toxicities developed. It should be noted that at the time these and earlier studies of the IFN-α and zidovudine combination were conducted, the recommended dose of zidovudine monotherapy, 1,200 mg/day, was twice the dose currently recommended when zidovudine is administered as part of combination antiretroviral therapy, so adverse events associated with combination therapy need to be viewed in that context. Another potential strategy to reduce the hematologic toxicity of combination therapy arose when less myelosuppressive NNRTIs than zidovudine became available. One such drug, didanosine, was combined with either of two daily doses of IFN-α, 1 million units or 10 million units, in a randomized trial (Krown et al., 2002). Whereas no significant differences in response rates were observed between the two treatment arms, the lower IFN-α dose, which is some 30-fold lower than that shown effective as monotherapy, was associated with significantly better tolerance and greater increases in CD4 counts than the higher dose (Krown et al., 2002).

All of the previously cited studies were conducted before the introduction into routine clinical practice of effective combinations of antiretroviral drugs that included HIV protease inhibitors and/or non-nucleoside reverse transcriptase inhibitors. Thus, the conclusions reached regarding optimal doses of these drugs in combination have limited application to patients on currently recommended antiretroviral regimens. To our knowledge, there has been only one study that investigated IFN-α with combination antiretroviral therapy for KS/AIDS. In this phase I study, which enrolled patients between 1997 and 1998, the majority of patients received ritonavir as the sole protease inhibitor in the combination, and zidovudine was not permitted (Krown et al., 2006). The study defined a maximum tolerated daily IFN-α dose of 5 million IU and identified neutropenia as the most common dose-limiting toxicity.

Interferon-α with chemotherapy: The combination of IFN-α with chemotherapy was explored early on in an attempt to increase response rates. Combinations with vinblastine (Krown et al., 1986; Rios et al., 1985; Fischl et al., 1987; Evans

et al., 1991), etoposide (Krigel et al., 1988), and the combination of actinomycin D, bleomycin, and vincristine (Shepherd et al., 1988) showed little evidence for increased efficacy against KS, but hematologic and constitutional toxicities were increased. Similarly, a trial of IFN-α combined with systemically administered all-*trans*-retinoic acid showed little anti-KS activity, but increased toxicity was associated with the retinoid (Bailey et al., 1995).

Other interferons: Although most studies in KS/AIDS have been conducted with IFN-α, several have explored the therapeutic potential of other IFN species. Interferon beta (IFN-β), which shares the same cellular receptor and has 30% amino acid homology with IFN-α, differs significantly from IFN-α in its absorption, pharmacokinetics, and hematologic toxicities. As a single agent, a serine-substituted recombinant IFN-β preparation induced KS regression in 6 of 39 patients and was associated with disease stabilization in another 16 patients (Miles et al., 1990). A phase I trial of the combination of recombinant IFN-β with zidovudine was associated with local skin necrosis at IFN-β injection sites and malaise as the major toxicities, and a response rate of only 13% was documented (Miles et al., 1998).

Interferon gamma (IFN-γ) is distinct from IFNs α and β in its structure, receptor binding, and biologic activities. Because of its unique immunomodulatory properties and its ability to enhance macrophage-mediated cytotoxicity against intracellular pathogens (Murray et al., 1985), it was considered a prime candidate for trials in KS/AIDS. Despite enthusiasm about the therapeutic potential of IFN-γ in KS, the results of several small clinical trials performed in the 1980s provided little evidence for antitumor activity, and severe adverse reactions were reported in some cases (Krigel et al., 1985; Ganser et al., 1986; Miles et al., 1986; Vadhan-Raj et al., 1986; Lane et al., 1989a). In fact, acceleration of tumor growth during IFN-γ treatment was suggested in some studies (Krigel et al., 1985; Ganser et al., 1986). These clinical findings are consistent with the observation that IFN-γ induces lytic KSHV replication in latently infected primary effusion lymphoma (PEL) cells in vitro (Chang et al., 1999; Blackbourn et al., 2000; Mercader et al., 2000), but are difficult to reconcile with the reported therapeutic success of recombinant interleukin (IL)-12, an inducer of IFN-γ, in the treatment of AIDS-related KS (Little et al., 2006). It has been suggested, however, that upregulation of KSHV lytic replication in vivo might be opposed by augmented cytotoxic T-lymphocyte killing of infected cells, resulting in the observed antitumor effects of IL-12 (Chang et al., 1999). In addition, in contrast to its ability to induce lytic reactivation in PEL cells, IFN-γ treatment has been reported to decrease lytic gene expression in KSHV-infected microvascular endothelial cells in vitro (Milligan et al., 2004), so the effects of various cytokines in vivo may be cell type specific.

Correlates of response: A variety of clinical and laboratory features have been analyzed for their association with tumor response to IFN-α treatment, with the thought that these might help in selecting patients most likely to benefit and possibly provide clues to the mechanisms underlying IFN-α activity against KS/AIDS. The features consistently associated with KS regression are those that reflect the

degree of impairment of host immune function and activation of the endogenous interferon system. Some of the earliest studies showed that HIV-infected individuals with a history of opportunistic infections that reflect severely impaired cell-mediated immune function or who had systemic symptoms (unexplained fever, night sweats, weight loss, diarrhea) indicative of advanced HIV disease were unlikely to show KS regression during IFN-α treatment (Mitsuyasu et al., 1986; Real et al., 1986). Even in the absence of these clinical signs of advanced HIV infection, individuals with high CD4 T-lymphocyte counts were significantly more likely than those with low counts to show IFN-α-induced KS regression. For example, Evans and colleagues, in a meta-analysis of multiple studies of recombinant IFN-α2a in KS, described a progressive increase in tumor response rates with increasing CD4 count among individuals with KS/AIDS who lacked symptoms of advanced HIV disease. Response rates increased from only 7.2% for those with CD4 counts below 200 cells/μL to 27.5% among those with 200–400 cells/μL and 45.4% among those with over 400 cells/μL (Evans et al., 1991). Although studies in which IFN-α was combined with other antiretroviral agents yielded increased response rates among subjects with lower CD4 counts, differential effects favoring subjects with higher CD4 counts were maintained (Krown et al., 1990; Fischl et al., 1991; Scadden et al., 1991; Podzamczer et al., 1993; Mauss and Jablonowski, 1995; Fischl et al., 1996; Shepherd et al., 1998; Krown et al., 2002). Other correlates of tumor response associated with preservation of host immune function have included the presence of intact delayed hypersensitivity skin test reactions and relatively high in vitro lymphocyte proliferative responses to mitogens and microbial antigens (Mitsuyasu et al., 1986; Vadhan-Raj et al., 1986). In contrast, neither tumor stage nor involvement of the gastrointestinal tract with KS was associated with a reduced incidence of IFN-induced tumor response (Mitsuyasu et al., 1986; Real et al., 1986).

The levels of various markers that may reflect the state of activation of the endogenous IFN system have also been shown to correlate with the response of AIDS-associated KS to IFN-α treatment. Some HIV-infected patients were shown to have detectable serum levels of an unusual form of acid-labile IFN-α, the presence of which correlated with elevated levels of the IFN-inducible products, β_2-microglobulin and neopterin. Although the elevated levels of neopterin in HIV-infected individuals have been ascribed to induction by IFN-γ, which appears to be the physiologic stimulus for neopterin production by macrophages (Huber et al., 1984), and have been considered to reflect a state of immune activation associated with HIV infection (Mildvan et al., 2005), IFN-α has also been shown to induce neopterin in vivo (Lang, Niederwieser and Huber, 1984). Detection of IFN-α or elevated levels of IFN-inducible products have been associated in several studies with a decreased likelihood of KS response to exogenous IFN-α treatment (Gelmann et al., 1985; Preble et al., 1985; Vadhan-Raj et al., 1986; Krown et al., 1991). These findings are consistent with the idea that prior activation of the IFN system may render patients refractory to further IFN-induced antitumor effects and with the observation that endogenous IFN-α downregulates IFN-α receptors on the lymphocytes of HIV-infected individuals (Lau et al., 1988).

The Interferon System in HIV Infection

As noted above, perturbations in the IFN system in HIV-infected people, with and without KS, were described in the early 1980s, long before the initial description of KSHV by Chang et al. (1994). The demonstration of decreased in vitro production of IFNs-α and -γ by the PBMCs of HIV-infected individuals in response to viral and antigenic stimuli suggested that deficient IFN generating capacity contributed to the development in AIDS patients of disseminated opportunistic infections (OIs) with viral and intracellular pathogens (Lopez et al., 1983; Murray et al., 1984). Subsequent studies showed that concurrent deficits in IFN-α generation and CD4 counts were required for the development of OIs (Siegel et al., 1986) and that effective antiretroviral therapy resulted in a rapid increase of in vitro IFN-α generating capacity that preceded CD4 count recovery and was associated with a reduced OI risk (Siegel et al., 2001). The decreased ability to produce IFN-α in HIV-infected individuals has been linked to depletion of plasmacytoid dendritic cells, the major source of type 1 IFNs (i.e., IFNs-α and -β) in antiviral innate immune responses (Feldman et al., 2001; Soumelis et al., 2001). Interestingly, whereas the development of OIs apparently requires impairment of both the innate (IFN) and adaptive (CD4 cells) immune systems, one study described three patients with active KS who had normal CD4+ T-cell counts but low numbers of IFN-producing cells (Soumelis et al., 2001). It remains to be seen whether this defect is present in other KS patients with normal CD4+ T-cell numbers (Maurer et al., 2007; Krown et al., 2008).

At the same time that deficiencies in IFN generation were described, many HIV-infected individuals were also found to have elevated serum levels of IFN. The IFN detected in serum was most often characterized as acid-labile IFN-α (in contrast to the typically acid-stable form), but was otherwise typical of IFN-α in its antiviral activity on bovine cells and its neutralization by IFN-α antibodies (DeStefano et al., 1982; Buimovici-Klein et al., 1983; Eyster et al., 1983;Vadhan-Raj et al., 1986). Less often IFN-γ was detected (Buimovici-Klein et al., 1983; Fuchs et al., 1989). Detection of serum IFN activity in HIV-infected individuals has been associated with an increased risk of developing AIDS in hemophiliacs (Eyster et al., 1983), clinical progression of HIV disease in individuals receiving antiretroviral therapy (Mildvan et al., 2005), and shortened survival among patients with AIDS-KS (Vadhan-Raj et al., 1986); sera from HIV-infected patients containing IFN-α can induce excess production of tumor necrosis factor (TNF) and upregulate its receptors in vitro (Lau and Livesy, 1989).

KSHV-Encoded Interferon Regulatory Factors

The production of type I IFNs by virus-infected cells constitutes the primary innate immune response against viral infection and is regulated through IFN-regulatory factors (IRFs), a family of transcription factors that may function as transcriptional activators or repressors (Pitha et al., 1998). KSHV has developed a unique

mechanism for subverting responses to cellular IFN-mediated antiviral activity by incorporating viral homologs of cellular IRFs (reviewed in Rezaee et al., 2006; Offermann, 2007). Four vIRFs have been identified, vIRF1–4, of which the fist three have been shown variously to alter responses to cellular IRFs and to IFNs, to block apoptosis and activation of the double-stranded RNA-activated protein kinase (PKR) and the NF-κB pathway, and to alter responses to transforming growth factor β and the tumor suppressor protein, p53. In addition, another KSHV protein unrelated to the vIRFs, the immediate-early protein ORF45, has been shown to bind to a cellular IRF, IRF-7, disrupting its function and blocking type I IFN induction during KSHV infection (Zhu et al., 2002), whereas the viral RTA protein inhibits IRF-7 by targeting it for proteasome-mediated degradation (Yu et al., 2005).

The foregoing indicates that KSHV has evolved multiple ways to evade innate and acquired cellular antiviral defenses, but it is not clear to what extent these mechanisms are important in the context of IFN treatment for KS. While the available evidence suggests that these IFN-regulatory viral proteins, which are expressed during the viral lytic cycle, function to enhance the production of infectious virus, the mere fact of their expression has not prevented cells supporting lytic replication from responding to exogenous IFNs (Monini et al., 1999; Krug et al., 2004; Pozharskaya et al., 2004a, b). In addition, the viral transcripts detected in most KS lesional cells are those associated with viral latency, whereas only a small proportion of the cells are undergoing lytic replication (Staskus et al., 1997). Although this latter cell population is required for production of infectious virus and expresses viral proteins that provide paracrine signals that contribute to the angiogenesis and inflammatory response that characterize KS lesions, it is not known if their expression could attenuate the therapeutic response to IFN.

Potential Mechanisms of Interferon Action in Kaposi's Sarcoma

Many of the pleiotropic activities of IFNs have been considered candidate mediators of its anti-KS activity, but it is not known which of these is most important in inducing tumor regression nor is it known how non-responding tumors evade IFN action. A direct cytotoxic or cytostatic effect of IFN-α was suggested by early clinical trials that linked higher IFN-α doses to superior therapeutic effects, but later studies showing that low-dose IFN-α was effective when combined with antiretroviral drugs suggested that less direct mechanisms were involved. In fact, only modest inhibition of KS-derived spindle cell growth in vitro was induced by exogenous IFN-α (Reiter et al., 1992; Lebbe et al., 1997).

Because both cytotoxic T-lymphocyte (CTL) and NK cell responses against KSHV-infected cells may be reduced in patients with AIDS-associated KS (Brander et al., 2000; Sirianni et al., 2002), it has been suggested that IFN-α may control KS by augmenting these responses. Although not systematically studied in clinical trials of IFN-α in KS, in vitro studies have shown that KS-derived cells show increased sensitivity to lysis by IFN-treated NK cells (Reiter et al., 1992; Lebbe

et al., 1997). Although to our knowledge not specifically tested, IFN-α treatment would be expected to lead to increased HLA class I surface expression and enhanced susceptibility of KS cells to CTL-mediated lysis and might counteract specific downregulation of MHC-I by KSHV (Coscoy and Ganem, 2000; Ishido et al., 2000; Lagos et al., 2007).

In addition to its inhibitory effects on immune effector cells that may be important in controlling KS growth, HIV may have more direct effects on KS that could be influenced by IFN treatment. Specifically, the HIV gene product, Tat, has been shown to bind to the VEGF-R2, the receptor for VEGF-A (Albini et al., 1996), and to stimulate a number of signal transduction pathways associated with cell growth and migration (Ganju et al., 1998). Because Tat can be released by infected cells during poorly controlled HIV infection, it may play a paracrine role in KS pathogenesis, and control of HIV infection and Tat release might inhibit KS growth. IFNs-α and -β have shown potent inhibitory activity on endothelial cell migration in vitro and on angiogenesis induced by HIV Tat in vivo (Marchisone et al., 1999). However, despite IFN-α's in vitro activity against HIV and evidence for antiviral synergy when administered together with zidovudine in HIV-infected subjects (Mildvan et al., 1996), one study failed to show a correlation between its activity against HIV and its clinical anti-KS activity (Frissen et al., 1997).

Recently developed insights into KSHV-induced sarcomagenesis have focused attention on potential inhibitory effects of IFN on KSHV replication and on KSHV-stimulated signaling pathways that contribute to dysregulated angiogenesis and cell growth. Numerous studies show that type I IFNs inhibit KSHV lytic cycle induction in latently infected cells in culture (D'Agostino et al., 1999; Monini et al., 1999; Chang et al., 2000; Pozharskaya et al., 2004a). Because lytic reactivation of KSHV is required for viral spread and because several lytic viral gene products can stimulate angiogenesis, control of lytic activation by IFN-α could influence KS progression. As noted by Chang et al. (2000), however, significant inhibition of KSHV induction in vitro was observed principally at high IFN concentrations, whereas at lower concentrations inhibition was weak and may have been opposed by viral IRFs. Culture of PBMCs from KS patients with IFN-α resulted in a reduction of KSHV viral load (Monini et al., 1999), but this was achieved only at high IFN concentrations (100–500 IU/ml) that are not reliably achieved or sustained at IFN doses used clinically. Although IFN-α treatment was reported to induce KS regression that was accompanied by clearance of KSHV from the circulation, increased CD4+ T-lymphocyte counts, and enhanced NK cell lysis of K562 targets in a single patient with extensive classic KS (Monini et al., 2001), others have reported failure to clear KSHV from PBMC or plasma of KS patients whose tumors regressed on IFN-α treatment (Deichmann et al., 1998; Krown et al., 2006).

Finally, there is substantial evidence that IFN-α can affect signaling pathways activated by KSHV. KSHV infection results in induction of hypoxia-inducible factor (HIF)-1α, a subunit of HIF-1. HIF-1 is a heterodimeric transcription factor that regulates tissue response to hypoxia and causes induction of target genes including VEGF-A and VEGF-C that are implicated in KS development (Carroll et al., 2006; Montaner, 2007). A novel mechanism that involves an interaction between

vIRF3 and HIF-1α, leading to HIF-1α stabilization and transcriptional activation and resulting in VEGF expression, has been described recently (Shin et al., 2008). IFN-α has been shown to inhibit this process in various cell types by a number of mechanisms, including inhibition of PI3kinase and MAP kinase signaling (Wu et al., 2005) and inhibition of VEGF transcription through inhibition of the VEGF promoter (von Marschall et al., 2003). In addition, Kaur et al. (2007) have recently described the existence of an IFN-regulated mechanism that diverts mTOR and its effectors from the transmission of mitogenic signals and redirects cap-dependent translation to proteins that exert opposing biological effects. Given the evidence that the PI3kinase/Akt/mTOR pathway is highly activated in KSHV-associated tumors and the reported susceptibility of KS to mTOR inhibitors (Tomlinson and Damania, 2004; Stallone et al., 2005; Montaner, 2007; Wang and Damania, 2008), IFN effects on this pathway in KS tumors could account for its antitumor effects. However, formal proof that any of these mechanisms accounts for the antitumor activity of IFN in clinical situations is currently lacking.

Future Potential for Interferons in Kaposi's Sarcoma

The use of IFN-α to treat AIDS-associated KS has declined in recent years. This is at least partly a consequence of characteristic IFN toxicities and the need for frequent self-injection, the declining incidence of KS where effective combination antiretroviral regimens are available, and the availability of other forms of treatment, in particular the liposomal anthracyclines, that are easier to administer and associated with a low incidence of the types of constitutional and laboratory toxicities that limit IFN use in the clinic. In addition, the very wide range of IFN activities that may mediate its anti-KS effects have made it difficult to understand IFN's primary mechanism(s) of action and make it challenging to design studies to optimize its therapeutic activity. Virtually all of the large clinical trials of IFN-α in KS were conducted prior to the introduction of modern antiretroviral therapy, the discovery and characterization of KSHV, the elucidation of IFN's many potentially relevant activities, and the technical capacity to study these as part of clinical trials. Thus, despite the fact that IFN-α has been one of the more active agents against KS/AIDS and was able to induce major and long-lasting tumor regression in some patients even before the availability of effective HIV therapy, the prospects for its future development in this disease are relatively poor.

It is possible that an optimal role for IFN will be found in combination with other pathogenesis-directed agents currently under investigation in KS. For example, in chronic myelogenous leukemia (CML) which like KS responds to treatment with both IFN-α and imatinib (Koon et al., 2005), IFN-α was shown to induce phosphorylation of mTOR and downstream phosphorylation of p70S6 kinase, whereas imatinib induced the opposite effect on the mTOR/p70S6 kinase pathway (Parmar et al., 2005). Nonetheless, both agents inhibited proliferation of leukemic progenitor cells, and this effect was enhanced by the addition of rapamycin (sirolimus),

an inhibitor of mTOR, to either IFN-α or imatinib (Parmar et al., 2005). Given the growing evidence that activation of the Akt/mTOR pathway is involved in KS pathogenesis (Montaner, 2007; Wang and Damania, 2008) and the antitumor activity of rapamycin against KS arising in the setting of post-transplant immunosuppression (Campistol et al., 2004; Stallone et al., 2005), therapeutic trials of IFN-α combined with rapamycin appear warranted. Although the seemingly paradoxical effects in CML cells illustrate the difficulties involved in dissecting out the roles of different pathways involved in the many biological activities of IFN, they provide a possible rationale for similar investigations in KS.

Another potential combination has been suggested by the finding that IFN-α increases apoptosis of cells with lytic cycle virus while protecting uninfected cells from KSHV infection (Krug et al., 2004; Pozharskaya et al., 2004b). In tissue culture experiments with KSHV latently infected microvascular endothelial cells, when IFN-α was present when lytic infection was induced, apoptosis occurred prior to production of infectious virus, whereas more infectious virus was produced if lytic infection was induced prior to treatment with IFN (Pozharskaya et al., 2004b). This has suggested the potential strategy of first treating patients with IFN-α followed by agents that could induce lytic viral replication (Klass and Offermann, 2005). Commercially available therapeutic agents such as histone deacetylase inhibitors (HDACs) or bortezomib have been shown capable of inducing lytic reactivation (Shaw et al., 2000; Klass et al., 2005; Fu et al., 2007, 2008) and could be candidates for combination with IFN-α, although it has been suggested that HDACs may induce apoptotic cell death with only minimal lytic reactivation (Niedermeier et al., 2006).

Newer formulations of IFN-α may also prove better tolerated than conventional IFN-α, at least insofar as decreasing the need for frequent self-injection. For example, pegylated IFN-α preparations that require only once-weekly injection are already in use for treatment of chronic hepatitis B and C, and other preparations that may exert long-lasting effects with even less frequent administration are being developed (De Leede et al., 2008; Subramanian et al., 2007).

The ultimate question is whether IFN can be rationally developed to selectively inhibit targets that are abnormal in KS compared with normal tissues. This will require a better understanding of how IFN affects proximal events in signaling pathways that drive KS growth.

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Metronomic Therapy for HIV-Associated Malignancies

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Abstract Therapeutic complications following maximum tolerated dose cytotoxic chemotherapy are a reality of current cancer care treatment regimens and protocols. These complications include bone marrow suppression with anemia, bleeding, and neutropenia and without appropriate supportive care, result in unacceptably high morbidity and mortality rates. Over 30 million people worldwide are infected with HIV, and of these, the vast majority live in sub-Saharan Africa. Many patients with AIDS are also malnourished and so are especially vulnerable to the mucositis and weight loss that often accompanies chemotherapy further compounding the inherent risks of myelosuppressive chemotherapy. In addition, HIV infection is thought to cause abnormalities in hematopoiesis, compounding the myelotoxicity of cytotoxic therapy. Thus, myelotoxicity is a major complication following the delivery of cytotoxic therapy at maximum tolerated doses to treat AIDS-associated malignancies in developing countries. Alternative approaches to the management of AIDS-associated malignancies are needed in resource-poor countries. In this chapter, we will describe a new approach for the delivery of cytotoxic drugs – metronomic therapy – that reduces the toxicity associated with the delivery of chemotherapeutic drugs at maximum tolerated doses.

Introduction

Therapeutic complications following maximum tolerated dose cytotoxic chemotherapy are a reality of current cancer care treatment regimens and protocols. These complications include bone marrow suppression with anemia, bleeding, and neutropenia and without appropriate supportive care, result in unacceptably high morbidity and mortality rates (Hesseling et al., 2003; Orem et al., 2004; Otieno et al.,

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2002). At the end of 2007, there were over 30 million people worldwide infected with HIV, and of these, the vast majority live in sub-Saharan Africa. In most African countries, due to the increase in HIV/AIDS, blood and blood products for transfusions are not readily available and platelets for prevention of bleeding are rarely available (Hesseling et al., 2003). Appropriate antibiotic therapy is difficult due to limited diagnostic capabilities and therapeutic armamentarium. Many patients with AIDS are also malnourished secondary to the cancer and so are especially vulnerable to the mucositis and weight loss that often accompanies chemotherapy, further compounding the inherent risks of myelosuppressive chemotherapy (Hesseling et al., 2003). Finally, HIV infection is thought to cause abnormalities in hematopoiesis, compounding the problems associated with myelotoxicity of cytotoxic therapy (Bower, 2002). Thus, myelotoxicity is a major complication following the delivery of cytotoxic therapy at maximum tolerated doses to treat AIDS-associated malignancies in developing countries. Published mortality rates for treating AIDS malignancies in resource-constrained settings range between 20 and 60% (Orem et al., 2005) and underscore the clinical challenges of cancer treatment in this setting. Alternative approaches to the management of AIDS-associated malignancies are needed in resource-poor countries. In this chapter, we will describe a new approach for the delivery of cytotoxic drugs – metronomic therapy – that reduces the toxicity associated with the delivery of chemotherapeutic drugs at maximum tolerated doses.

Angiogenesis

The importance of angiogenesis in tumor development was first recognized by Folkman (1971). He proposed that tumors lay dormant in situ for months to years, rarely growing beyond 2–3 mm^3 in maximum size until the development of neovascularization. When a tumor becomes vascularized, a subgroup of cells "switch" to an angiogenic phenotype with the emergence of markedly increased tumor growth, tumor cell invasion, and, ultimately, dissemination. In the last two decades, a wealth of research has supported and expanded this original concept and demonstrated that a variety of solid and hematologic malignancies are indeed dependent on angiogenesis to maintain tumor growth and survival (Kerbel, 2000). Not surprisingly then, targeting the angiogenic process has received considerable attention as a potential therapeutic approach for treatment of cancer. Relative to HIV-associated malignancies, angiogenesis has been demonstrated in cervical cancer, Hodgkin's disease, non-Hodgkin's lymphoma including Burkitt's lymphoma (BL), and Kaposi's sarcoma (Aldenhoven et al., 2006; del Campo et al., 2008; Jorgensen et al., 2007; Randall et al., 2008; Ruddell et al., 2003; Wang and Damania, 2008).

Maximum Tolerated Dose for Delivery of Chemotherapy

Traditional chemotherapy relies on giving cytotoxic drugs at the maximum tolerated dose (MTD) usually in a bolus dose fashion at 3–4 week intervals. The conventional

wisdom is that by effectively saturating the patient with the drug, more tumor cells will be targeted and enhance the efficacy of tumor killing. However, cytotoxic drugs given at the maximally tolerated dose cause significant toxicity to the host including anemia, neutropenia, and mucositis.

Because of the significant toxicity associated with cytotoxic drugs given at maximum tolerated doses, patients typically are given breaks between dosing usually of 3–4 weeks duration allowing the patient to recover from toxic side effects. There is evidence to suggest that this "break" in cytotoxic drugs allows for and promotes emergence of drug-resistant tumor cell clones. Moreover, the toxicity from cytotoxic drugs given at the maximum tolerated dose results in higher costs to the patient both physically and financially. This toxicity also comes at a higher risk for AIDS patients who already have compromised bone marrow function.

Metronomic Therapy

Conventional cytotoxic drugs can function both by inducing apoptosis of the cancer cell and by indirect effects on the tumor vasculature (Miller et al., 2001). However, for maximal antiangiogenic activity, lower doses of cytotoxic drugs chronically administered are required. This approach to cytotoxic drug delivery was coined "metronomic therapy" (Gasparini, 2001; Hahnfeldt et al., 2003; Hanahan et al., 2000). In this approach, the dose of cytotoxic drugs is significantly lower than the maximum tolerated dose and the dosing can be daily with no prolonged drug-free breaks. This approach is counterintuitive if the goal is to directly kill tumor cells but as will be described below, both preclinical and clinical studies are proving the efficacy of metronomic therapy for a number of malignancies. Importantly, the reduced dosing also minimizes the side effects associated with cytotoxic therapy given at MTDs. An obvious advantage of this approach, especially for implementation in developing countries, is that it does not require the use of expensive new drugs but rather uses a different dose and scheduling of drugs that have already been clinically tested, are readily available, and are relatively inexpensive.

How might metronomic therapy work? One possibility is that scheduling more frequent and lower dose cytotoxic drug delivery may have preferential "cytotoxic" effects on replicating endothelial cells in newly forming blood vessels in contrast to normal, quiescent endothelial cells in normal tissues [reviewed in Kerbel and Kamen (2004)]. This "collateral damage" to tumor vasculature or endothelium could provide the enhanced therapeutic index of very low-dose cytotoxic chemotherapy. Laboratory evidence that the endothelial cell is the target of cytotoxic therapy in drug-resistant Lewis lung carcinoma is supported by observations of more pronounced antiangiogenic response in p53-null mice when compared to $p53^{+/+}$ mice (Browder et al., 2000). An additional direct effect could be through action on the bone marrow environment to suppress the mobilization of endothelial progenitor cells that are needed for tumor angiogenesis to be maintained (Shaked et al., 2005c). An indirect effect of metronomic therapy has also been proposed whereby

the cytotoxic drugs induce thrombospondin 1, an endogenous inhibitor of angiogenesis (Bocci et al., 2003; Hamano et al., 2004). An additional target of metronomic therapy was found from a clinical study of end-stage cancer patients (Ghiringhelli et al., 2007). Ghiringhelli and colleagues observed that cyclophosphamide given orally resulted in a selective reduction of regulatory T cells, suggesting metronomic chemotherapy could be immunomodulatory as well as antiangiogenic. More research is clearly needed to establish the mechanism of action of metronomic therapy on tumor vasculature and immune function.

Preclinical Models of Metronomic Therapy

Preclinical models are an important step in determining both the mechanism and the efficacy of metronomic therapy. To date, the preclinical models used either engraftment of syngeneic mouse tumors or engraftment of human tumors into SCID mice. Browder and colleagues were the first to demonstrate that a metronomic schedule of cyclophosphamide given to mice harboring Lewis lung carcinoma and L1210 leukemia avoided drug resistance and was able to eradicate the tumor, an outcome not possible with a conventional dosing schedule (Browder et al., 2000). The addition of an angiogenesis inhibitor, TNP-470, potentiated this response as well. Following this seminal study, Klement and colleagues observed that very low, non-toxic doses of vinblastine in combination with VEGFR-2 antibody induced sustained tumor regression in SCID mouse xenografts of two independent neuroblastoma cell lines (Klement et al., 2000). In a human colorectal cancer xenograft model, treatment with irinotecan in combination with the tyrosine kinase inhibitor semaxanib was shown to inhibit tumor growth. Both a decrease in microvessel density and increases in thrombospondin-1 gene expression were observed, suggesting a direct antiangiogenic effect (Bocci et al., 2008). Other preclinical models have also demonstrated the efficacy of metronomic therapy with or without additional angiogenesis inhibitors (Bello et al., 2001; Blansfield et al., 2008; Hamano et al., 2004; Klement et al., 2002; Ma and Waxman, 2008; Man et al., 2002; Zhang et al., 2002). In general, the observations from these studies are that single-agent metronomic scheduling is not as efficacious as multi-agent scheduling but there is a significantly reduced toxicity following metronomic scheduling compared to MTD.

Metronomic dosing with cyclophosphamide was also used in SCID mice engrafted with the Namalwa BL cell line. Increased endothelial cellular precursors were observed and tumor growth was delayed (Bertolini et al., 2003; Rochford et al., 2006). No long-term analysis of treatment survival or measurement of drug toxicity was done but these data support the hypothesis that metronomic dosing of cytotoxic drugs could be effective in the treatment of AIDS-BL. We have also tested whether metronomic therapy would be amenable to treatment of HIV-associated BL (Rochford et al., 2006). To do this, we developed a xenograft model using NOD/SCID mice engrafted with a short-term cell line (BL-7) derived from the BL biopsy of an AIDS patient (Kurokawa et al., 2005). After 27 days, four groups were treated: control (saline only), metronomic i.p. [cyclophosphamide i.p. every 6 days],

metronomic oral (cyclophosphamide in drinking water), and maximum tolerated dose (cyclophosphamide i.p. 3 times per week followed by 2 weeks rest, repeated thrice). The average time before control mice were sacrificed due to tumor growth was 56.8 days. When the last control mouse succumbed to tumor growth, treatment was terminated on the remaining mice. Mice treated with the maximum tolerated dose of cyclophosphamide survived until 204 days. Sixty percent of mice treated with the metronomic dose of cyclophosphamide i.p. also survived until 204 days without evidence of tumors. Mice that received cyclophosphamide in their drinking water succumbed to tumors, but not until 92 days, approximately double the time of the control mice, indicating that even minimal levels of cyclophosphamide in the drinking water had an antitumor effect. These data indicate that metronomic scheduling of cyclophosphamide given i.p. significantly enhanced the survival of SCID mice engrafted with an AIDS-BL tumor, was comparable to cyclophosphamide given at the maximum tolerated dose, and was associated with reduced weight loss indicating reduced toxicity.

Clinical Trials of Metronomic Therapy

The first reported use of metronomic therapy in the clinic was by Colleoni and colleagues (Colleoni et al., 2006, 2002). Both low-dose cyclophosphamide and methotrexate were given and found to have minimal toxicity and be effective in the treatment of advanced stage breast cancer. Since this first clinical report, over 15 pilot, phase I, or phase II clinical trials using cytotoxic drugs given on a metronomic schedule alone or in combination with molecularly targeted drugs (e.g., specific angiogenesis inhibitors such as cyclooxygenase-2 inhibitors, glitazones, thalidomide, and bevacizumab) have been tested on a number of different malignancies (Bottini et al., 2006; Buckstein et al., 2006; Coleman et al., 2008a, b; Dellapasqua et al., 2008; Garcia et al., 2008; Glode et al., 2003; Krzyzanowska et al., 2007; Munoz et al., 2006; Orlando et al., 2006a, b; Sanborn et al., 2008; Spieth et al., 2003; Steinbild et al., 2007; Sterba et al., 2006; Vogt et al., 2003; Young et al., 2006). In general, clinical results from this approach have been promising in patients with advanced melanoma, children and adults with solid tumors, malignant vascular tumors, advanced breast cancer, ovarian cancer, prostate cancer, and refractory lymphoma. Metronomic therapy was found to be well tolerated in renal cell carcinoma patients but not effective (Krzyzanowska et al., 2007). Metronomic therapy using existing cytotoxic drugs has also been used for palliative therapy in advanced breast cancer patients (Bocci et al., 2005). Selected trials are briefly summarized in Table 1.

Although there are no clinical studies to date in which metronomic therapy was directly tested on patients with AIDS-associated malignancies, two papers have direct relevance for HIV-related non-Hodgkin's lymphoma. In the first, a low dose of cyclophosphamide with metronomic scheduling in combination with COX-2 was effective for treatment of advanced non-Hodgkin's lymphoma, suggesting

Table 1 Selected early-phase feasibility and pilot trials of metronomic therapy

Author (year)	Agent(s)	Disease target	Comment(s)
Colleoni et al.(2006, 2002)	Methotrexate, cyclophosphamide, ± thalidomide	Breast cancer	Response rate 19% and clinical benefit rate 32%
Bottini et al.(2006)	Letrozole ± cyclophosphamide	Breast cancer in elderly	Response rate 88% metronomic schedule
Coleman et al. (2008a, b)	Prednisone, etoposide, procarbazine, and cyclophosphamide	Non-Hodgkin's lymphoma	Highly active regimen in low-grade histology and mantle cell lymphoma
Garcia et al. (2008)	Bevacizumab and cyclophosphamide	Ovarian cancer	Partial response rate 24%
Sanborn et al. (2008)	Docetaxel and thalidomide	Solid tumors	Demonstrable activity in prostate cancer, melanoma, and small cell lung cancer. Described "metronomic" tolerable dose
Dellapasqua et al. (2008)	Cyclophosphamide, capecitabine, and bevacizumab	Breast cancer	Objective response rate 48%

this regimen could also be efficacious for HIV-associated NHL (Buckstein et al., 2006). A second study by Coleman et al. (2008a) reported a 16-year retrospective analysis of the use of oral combination chemotherapy for the treatment of refractory lymphoma. They demonstrated that administration of low-dose oral agents (e.g., cyclophosphamide, prednisone, etoposide, and procarbazine) given with minimal drug-free intervals was well tolerated and a 69% objective response rate was achieved.

Minimal grade 3 or 4 toxicities have been observed using metronomic scheduling in phase II clinical trials (Steinbild et al., 2007; Young et al., 2006). For example, in a phase II clinical trial of cyclophosphamide, vinblastine, and rofecoxib in patients with advanced solid tumors, the incidence of grade 3 and 4 toxicities was low and no patients developed grade 3 or 4 nausea, vomiting, mucositis, or alopecia (Young et al., 2006).

An important feature emerging from these studies is that drugs given at the maximally tolerated dose early in treatment did not preclude the same drug being efficacious when given at metronomic dosing (Browder et al., 2000; Buckstein et al., 2006). Thus, there is the potential to provide additional benefit to patients with advanced stage diseases who have been heavily pretreated with cytotoxics. Phase II clinical trials have been initiated for some malignancies but there is still the need to extend these studies to larger randomized clinical trials.

Challenges in Metronomic Therapy

To determine the maximum tolerated dose of conventional cytotoxic drugs, phase I trials are done by escalating the dose of the drug and determining both toxic and

therapeutic doses (Graham and Workman, 1992). The paradigm in clinical oncology has been "more is better" and this paradigm also permeates clinical trial design (Sleijfer and Wiemer, 2008). However, if metronomic therapy is to be of clinical value, new statistical methods are needed to define the optimal metronomic dosing schedule of conventional cytotoxic drugs (Sanborn et al., 2008). This is already being done for new anticancer agents that are based on molecularly designed targets (Seymour, 2002). In addition, another key challenge will be to identify biomarkers that can be used as surrogates for the efficacy of metronomic therapy (Brown et al., 2008). Preclinical studies have demonstrated the efficacy of using mobilized $CD34^+$ $CD133^+$ VEGFR-2^+ endothelial progenitor cells as a surrogate pharmacodynamic marker (Shaked et al., 2005a, b, c). A promising alternative marker demonstrated in both preclinical and clinical settings is based on the measurement of apoptotic circulating endothelial cells which are thought to be derived from tumor vasculature (Mancuso et al., 2006).

Applications of Metronomic Therapy to HIV-Associated Malignancies

Doxorubicin-based combination chemotherapy [triplet regimen comprised of doxorubicin plus bleomycin plus vinblastine or vincristine (ABV)] has been a time-honored therapeutic approach to patients with advanced AIDS-related Kaposi's sarcoma (Gill et al., 1991; Laubenstein et al., 1984). Clinicians who have treated Kaposi's sarcoma not infrequently reduce the dose and schedule of the agents comprising the regimen to a weekly or biweekly schedule in order to minimize toxicity yet maintain palliative benefit and/or tumor remission. The newer liposomal anthracyclines, which are regarded as frontline agents, have an improved therapeutic index with markedly less toxicity. Given the pharmacokinetic profiles of these agents, they are administered on 2–3 week schedules at low dose (Gill et al., 1996; Northfelt et al., 1997, 1998; Stewart et al., 1998). Similarly, paclitaxel has been shown to have demonstrable activity in the second-line setting of this disease and in many instances the drug is administered on a weekly or biweekly schedule below the traditional maximum tolerated dose (Gill et al., 1999; Park and Levitt, 1993; Saville et al., 1995; Welles et al., 1998). These observations substantiate the feasibility of using metronomic therapeutic principles in the management of AIDS-associated Kaposi's sarcoma in contemporary practice.

Given the efficacy and reduced toxicity of metronomic therapy for treatment of a number of different malignancies including AIDS-associated Kaposi's sarcoma, the promise of this approach for the treatment of HIV-associated malignancies is warranted. Optimism for building on these therapeutic principles, specifically tailoring even lower and more chronic dosing schedules for the management of Kaposi's sarcoma with these more common and other cytotoxic or novel agents, especially in the context of resource-poor countries, is needed. In the resource-constrained setting where many patients present with tumors at late stage and have underlying

malnutrition, it is imperative to develop non-myelotoxic therapies (Orem et al., 2004, 2006). An advantage of metronomic therapy in resource-limited settings is the cost associated with this therapeutic approach. Typically cytotoxic drugs given on a metronomic schedule are low cost and readily available, and the associated reduced toxicity minimizes the expense of the supportive care frequently needed when cytotoxics are given at the maximum tolerated dose. Clinical trials are needed to establish whether metronomic therapy will be one answer to treatment of HIV-associated malignancies especially in the context of resource-poor regions of the world where the impact of HIV is the highest.

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Targeting EGFR in HPV-Associated Cancer

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Abstract Squamous cell carcinoma of the cervix, anal canal, and hypopharynx is known to be associated with human papilloma virus (HPV) infection. HPV is a double-stranded DNA virus that encodes oncogenic proteins, including E5, E6, and E7. E5 activates epidermal growth factor receptor (EGFR) by binding to its 16 kDa subunit of protein pump ATPase, thereby promoting proto-oncogene expression, inhibits the expression of the tumor suppressor gene p21, and amplifies mitogenic EGFR signals. In addition, E6 and E6 proteins bind to p53 and pRb, respectively, resulting in reduced levels and function of p53 and pRb within the cell, thereby inhibiting apoptosis and promoting genomic instability. EGFR-directed therapies are effective treatments for squamous cell carcinoma of the head and neck and are also being evaluated in other HPV-associated tumors.

Introduction

Human papilloma virus (HPV) has been implicated in the pathogenesis of squamous cell carcinoma of the cervix, oropharynx, and anogenital tract of both men and women. Some of the key features of HPV are summarized in Table 1 and described in this chapter. HPV infections of the anogenital tract and oral cavity are sexually transmitted and endemic worldwide (Palefsky, 1994). There is strong epidemiologic, histological, and molecular evidence indicating that HPV infection is the primary initiating event in these cancer types. HPV deoxyribonucleic acid (DNA) has been identified in nearly all cervical squamous cell carcinomas (Einstein and Goldberg, 2002; Schiffman et al., 1993), in most anogenital cancers (Palefsky,

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Table 1 Characteristics of HPV infection

Structure	Double-stranded, circular DNA with icosahedral protein capsid
Subtypes	More than 100 subtypes – most common oncogenic subtypes include HPV 16, 18, 31, 33, and 45
Transmission	Sexually transmitted
Viral infection	Episomal form – genital/plantar warts Viral integration – precancerous and cancerous lesion
Sites of infection	Squamous epithelium of the skin, oral cavity, and anogenital tract
Early gene/protein functions	E1: DNA replication E2: Regulates E6 and E7 expression E4: Disrupts cytokeratin network E5: Transformation via EGFR activation, proto-oncogene expression, and inhibition of p21 tumor suppressor gene E6: Transformation via binding to p53, telomerase activation E7: Transformation via binding to pRB
Associated cancers	Squamous cell carcinoma of cervix, anal canal, oropharynx, vulva, penis, urethra

2008; Frisch et al., 1997), and about one-third of head/neck cancers, usually those involving the oropharynx (tonsils) (Agrawal et al., 2008; Giuliano et al., 2008).

HPV Virus Infection and Vaccination

HPV is a non-enveloped, double-stranded circular DNA virus that is enclosed by an icosahedral protein capsid that is a member of the papovavirus family. Its genome is about 7,900 base pairs in length with its DNA usually in the supercoiled form. There are three regions that are illustrated schematically in Fig. 1, including (1) the early region, which encodes proteins involved in DNA replication, (2) the late region, which encodes the major and minor capsid proteins, and (3) the long control region, which regulates viral gene expression (Chong et al., 1991). In addition, viral open reading frames may be present in the early or late region of the genome based on the time of their protein expression within the infected host cells. HPV DNA can either remain in an episomal form, as is common in genital or plantar warts, or integrate into host DNA, as seen in most cancers (Dell and Gaston, 2001; Einstein et al., 2002). An electron micrograph of HPV virus-like particles is shown in Fig. 2.

HPV is the most common sexually transmitted infection worldwide, with a cumulative incidence in young women approaching 60% (Ho et al., 1998; Winer et al., 2003) and a cross-sectional prevalence of 27% in young to middle-aged US women (Dunne et al., 2007). The highest prevalence in one study was among females aged 14–24 years (34%), corresponding to 7.5 million infected young women (Dunne et al., 2007). HPV prevalence is nearly as high in males (Dunne et al., 2006; Partridge and Koutsky, 2006).

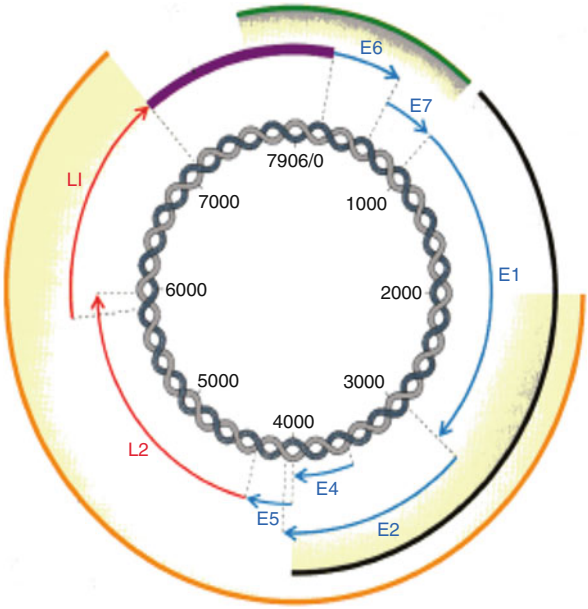

Fig. 1 Schematic representation of the organization of HPV genome denoting (1) the early region (E1–7 shown in *blue*) which encodes proteins involved in DNA replication, (2) the late region (L1–2 shown in *pink*) which encodes the major and minor capsid proteins, (3) the long control region (shown in *purple*) which regulates viral gene expression in early and late regions, (4) double-stranded DNA (shown in *gray*), and (5) the capsid proteins (shown in *orange*, *green*, and *black*) (Adopted with permission from Burk, 1999)

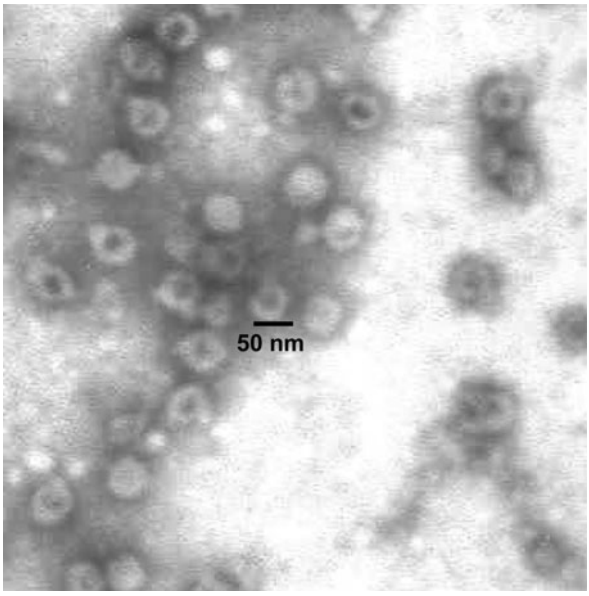

Fig. 2 Electron micrograph of HPV virus-like particles (VLPs) measuring 50 nm in size

HPV Infection and Host Immunity

HPV infection can be modulated by the host immune system. Poor immune responses and/or lack of a sustained natural immune response to the virus may also facilitate progression of mucosal disease (Stern, 2005; Ahmed et al., 2006). Several aspects of the HPV life cycle facilitate avoiding immune surveillance and enhancing survival of the virus. By infecting only the basal layer of the cervical epithelium, HPV is isolated from circulating immune cells. Also, non-lytic replication limits innate immune responses that would normally occur in response to cell death. High levels of viral protein required for identification by the immune system are not expressed until the later stages of epithelial differentiation (and the viral life cycle). Since HPV infection is not blood borne, it is not readily exposed to the systemic immune system. Unlike blood-borne pathogens, such as hepatitis B virus, an immune response against HPV must be generated in the anogenital tract. The limited innate immune response, low levels of viral gene expression in the lower layers of the epithelium, and lack of cytopathic effects all contribute to a delayed adaptive immune response to initial infection, allowing HPV to establish persistent infection within the cervical epithelium and other infected sites.

HPV Vaccination

A quadrivalent vaccine targeting HPV types 6, 11, 16, and 18 was recently approved for the prevention of primary HPV infection after it was shown to be highly effective in preventing anogenital warts and intraepithelial neoplasia of the cervix, vagina, and vulva (Siddiqui and Perry, 2006; Garland et al., 2007). The ability of HPV vaccination to reduce HPV-associated cancer rates is dependent on the population selected for vaccination, vaccination uptake in that population, the proportion of cancers that are attributed to high-risk HPV subtypes included in the vaccine, and host immune response. Although HPV types 16 and 18 are associated with ~70% of cervical cancer cases, they were found to have a prevalence of only 1.5 and 0.8%, respectively, among US women and did not rank within the 10 most prevalent types (Dunne et al., 2007).

The US Federal Advisory Committee on Immunization Practices (ACIP) recommends that adolescents (or young adults up to the age of 26) should be vaccinated before the onset of sexual activity and exposure to HPV infection (Saslow et al., 2007). There appears to be limited efficacy in women with prior HPV 16 and 18 exposure (Garland et al., 2007). The Centers for Disease Control (CDC) reported an uptake rate of <10% in 19- to 26-year-old women in 2007 and only 1% uptake among Hispanic patients in this age group (http://www.cdc.gov/nchs/data/hestat/vaccine_coverage.pdf). High cost and the absence of public health mandates are barriers to widespread HPV vaccination in the United States (Segraves, 2007). Vaccination in developing nations

with high cervical cancer rates faces even greater challenges. Reductions in cervical cancer rates are not likely to occur unless there is widespread vaccination and may take decades to appear due to the prolonged natural history of cervical neoplasia (Schiffman et al., 1993; Schiffman and Brinton, 1995). Given the challenges in implementing widespread vaccination and the absence of effective antiviral agents against HPV, there is a need to identify new therapeutic approaches for HPV-associated cancers.

HPV Subtypes and Cancer Risk

There are over 100 HPV subtypes that differ in tissue tropism (mucosal or cutaneous) and oncogenicity (high and low risk). Most cases of dysplasia or cancer are associated with at least 18 high-risk HPV subtypes such as 16 or less frequently 18, 31, 33, 45. Oncogenic HPV types produce E6 and E7 proteins that bind to p53 and pRb, respectively, resulting in reduced levels and function of p53 and pRb within the cell; the alterations inhibit apoptosis and promote genomic instability (Einstein and Goldberg, 2002). E5 protein is also carcinogenic and appears to mediate some of these effects via processes that are dependent on the epidermal growth factor receptor (EGFR) pathway (Tsai and Chen, 2003). The EGFR, whose ligands include EGF and transforming growth factor alpha, is a member of the ErbB family of receptors that is broadly expressed in epithelial cancers, including squamous cell carcinoma of the anogenital tract and oropharynx. EGFR expression has been associated with prognosis and response to EGFR-directed therapies (Palefsky et al., 1991; Dziadziuszko et al., 2006; Linardou et al., 2008).

Epidemiology of HPV Infection and HPV-Associated Cancers

HPV is widely accepted to be the central etiologic agent in cervical tumorigenesis and also contributes to tumorigenesis in other HPV-associated cancers (Lorincz et al., 1992; Schiffman et al., 1993; Bosch and de Sanjose, 2003; Einstein and Burk, 2001). Nearly all cervical cancers are associated with high-risk, oncogenic HPV subtypes (Ault, 2006), and cervical cancer does not develop in the absence of persistent HPV infection (Bosch et al., 2002; Franco and Harper, 2005). HPV is also associated with about 80–90% of all anal cancers (Schiffman and Kjaer, 2003; Daling et al., 2004; Saslow et al., 2007), 40% of vulvar cancers (Trimble et al., 1996; Munoz et al., 2006), and penile (Rubin et al., 2001), vaginal (Daling et al., 2002), head and neck (Forastiere et al., 2001; Herrero et al., 2003; Kreimer et al., 2005), and urethral cancers (Cupp et al., 1996) in varying proportions depending on the study and HPV primer sets used for detection. Oropharyngeal cancer has been associated with sexual practices, including a higher lifetime number of sexual partners engaging in both vaginal and oral sex (D′Souza et al., 2007; Gillison et al., 2008).

Table 2 Incidence of HPV-associated cancers

Cancer	Annual worldwide cases	Annual worldwide deaths	Annual US cases	Annual US deaths	US S.I.R. (per 100,000) general population	US S.I.R (per 100,000) HIV-infected population
Cervix	483,000	278,000	11,700	3,880	11.4	134.5
Head/neck cancers associated with HPV	275,000	127,000	35,310	7,590	11.7	36.9
Anal canal	Not reported	Not reported	5,070	680	1.3	78.2

Note: Statistics shown for worldwide estimates are for 2002 (Parkin et al., 2005) and for the United States are for 2008 (Jemal et al., 2008). For head/neck cancers, worldwide statistics include nasopharynx and other pharynx (with oral cavity and larynx excluded due to their relation to tobacco exposure) and US statistics include pharynx and other oral cavity sites (with tongue, mouth, and larynx excluded). Standardized incidence rates (S.I.R.) per 100,000 using SEER (Surveillance Epidemiology, End Results) data for the United States for 2000–2003 (Patel et al., 2008); for head/neck cancers associated with HPV, the US S.I.R. data provided were for oropharyngeal cancer

The incidence of HPV-associated cancers worldwide (Parkin et al., 2005) and in the United States (Jemal et al., 2008) is shown in Table 2, including projections for standardized incidence rates in the United States based on Surveillance, Epidemiology, and End Results (SEER) data in the general population and HIV-infected individuals (Patel et al., 2008). The most HPV-associated cancers and cancer deaths worldwide include carcinomas of the cervix, head and neck, and anal canal. These cancers are considerably less common in the United States, especially cervical cancer, because of cytological screening. Among nations outside the United States, there is a 4.9-fold higher incidence of cervical cancer in developing nations compared with developed nations and a 5.9-fold higher death rate (Parkin et al., 2005).

The incidence of some HPV-associated cancers has been increasing. According to SEER data, cervical and oropharyngeal cancer rates have not significantly changed, although the proportion of oropharyngeal cancers attributed to HPV appears to be rising (Chaturvedi et al., 2008). SEER data have also shown a four-fold increase in the risk of anal cancer (Patel et al., 2008). Other studies have shown similar findings which have been attributed to changing sexual behavior (Beckmann et al., 1989). Surrogates for homosexual behavior in men (i.e., never having been married) were shown to correlate with an increased risk of anal cancer nearly two decades ago (Daling et al., 1982, 1987). Even before HIV became widely epidemic, the incidence of anal cancer in men with a history of receptive anal intercourse exceeded the incidence of cervical cancer prior to the introduction of Pap smear screening (Melbye et al., 1994).

HPV-associated cancers are more common in individuals who are also infected with the human immunodeficiency virus (HIV). For example, there is about a 3-fold

higher risk of head/neck cancer in HIV-infected individuals in the United States compared with the general population, a 12-fold higher rate of cervical cancer, and a 60-fold higher rate of anal cancer (Table 2) (Patel et al., 2008). HPV-associated cancers associated with HIV infection also tend to be associated with a more aggressive clinical course and poorer tolerance to therapy. For example, cervical intraepithelial neoplasia (CIN) is associated with higher recurrence rates after local therapy, and cervical cancer is associated with poor tolerance to radiation and higher recurrence in HIV-infected subjects (Gilles et al., 2005; Shrivastava et al., 2005). Head and neck cancer associated with HIV infection also appears to be associated with a worse outcome (Haigentz, 2005). For anal cancer, retrospective reports have indicated 60% 2-year disease-free survival rates and better outcomes for patients with high CD4 counts (Chadha et al., 1994; Holland and Swift, 1994; Hocht et al., 1997; Peddada et al., 1997; Hoffman et al., 1999; Cleator et al., 2000; Kim et al., 2001; Place et al., 2001). A case–control study compared 40 consecutive HIV-positive patients with 81 consecutive HIV-negative subjects treated at four institutions over a 10-year period. HIV-positive patients were younger (mean age, 48 vs. 62 years; $p < 0.0005$), more likely to be male (93% vs. 25%; $p < 0.0005$), and more likely to present with early-stage disease (25% vs. 14% stage 0–I, $p = 0.06$) and large-cell histology (90% vs. 67%, $p < 0.005$) (Oehler-Janne et al., 2008). Although 5-year overall survival rates were similar (61% vs. 65%), local control rates were significantly inferior in the HIV-positive group (38% vs. 87%, $p = 0.008$). HIV-positive subjects experience more grade 3–4 toxicity (48% vs. 31%, $p = 0.10$), including significantly more acute skin reactions (35% vs. 17%, $p = 0.04$), and more hematologic toxicity in mitomycin-C-treated patients (33% vs. 12%, $p = 0.08$).

Mechanisms of HPV-Induced Oncogenesis

With persistent infection, viral DNA integrates into the host genome. HPV 16 has been shown to integrate into the host genome via nonhomologous recombination (Einstein et al., 2002). Viral integration has been shown to be associated with deletion of cellular sequences and alterations of proto-oncogenes that confer a growth advantage to infected epithelial cells (Durst et al., 1987; Lazo et al., 1989).

E6 and E7 Proteins and Carcinogenesis

E6 and E7 proteins are more abundant in high-grade precancerous lesions compared to low-grade lesions, and maintained expression of these proteins promotes progression to cervical cancer (zur Hausen, 2000; Einstein and Burk, 2001). E6 and E7 induce oncogenesis by three major mechanisms (Table 1) (Bosch et al., 2002; Wentzensen et al., 2004). First, E6 and E7 can have direct effects on tumor suppressor proteins p53 and pRB, respectively (zur Hausen, 2000). Binding of E7 to pRB promotes host cell and viral DNA synthesis, whereas E6 blocks the ability of p53 to

inhibit the genetic damage that ultimately leads to random mutations that can cause malignant transformation. Second, viral oncoproteins induce genomic instability by increasing the number of chromosomes and structural damage to the host DNA (Duensing and Munger, 2001, 2002). As a third mechanism, E6 activates telomerase, which leads to a disruption of normal replicative senescence (Klingelhutz et al., 1996; James et al., 2006).

Relationship Between E5, E6, E7 Proteins and EGFR Signaling

Immortalized keratinocyte cell lines generated by cotransfection with HPV 16 E6 and E7 show upregulation of EGFR, decreased membrane E-cadherin expression, and redistribution of alpha-, beta-, and gamma-catenin from the undercoat membrane to the cytoplasm, indicating a functional interaction between growth regulatory factors and adhesion molecules (Wilding et al., 1996). In addition, p53 transactivates EGFR in an E6-dependent manner (Deb et al., 1994). E5 protein is also known to be oncogenic, mediating some of these effects via the EGFR pathway (Tsai and Chen, 2003). The E5 protein is an 83 amino acid protein that is very hydrophobic and therefore associated with membranes of the Golgi apparatus, endoplasmic reticulum, and nucleus. E5 activates EGFR via binding to the 16 kDa subunit of protein pump ATPase (Tsai and Chen, 2003). EGFR activation leads to overexpression of a variety of proto-oncogenes and stimulates cellular proliferation. E5 also inhibits the expression of the tumor suppressor gene p21 (WafI/SdiI/CipI), thereby impairing cell cycle control (Tsai and Chen, 2003). E5 has also been shown specifically to amplify mitogenic signals from the EGFR (Pim et al., 1992). E5 protein promotes transformation of kerantinocyte cell lines via an EGFR-dependent process and also modulates late viral functions through activation of proliferative capacity in differentiated cells (Fehrmann et al., 2003).

Anti-EGFR-Directed Therapies

Several agents targeting the EGFR pathway are available, including monoclonal antibodies (cetuximab and panitumumab) and small-molecule tyrosine kinase inhibitors (gefitinib and erlotinib) (Table 3). Cetuximab is a chimeric human and mouse monoclonal antibody which tightly binds to EGFR, preventing binding of its ligands and resulting in internalization and downregulation of the receptor (Blick and Scott, 2007). Panitumumab is a fully human immunoglobulin G2 monoclonal antibody directed against EGFR that produces similar effects (Hoy and Wagstaff, 2006). Both gefitinib and erlotinib are small-molecule, orally bioavailable inhibitors of the EGFR tyrosine kinase (Frampton and Easthope, 2004; Tang et al., 2006). Cetuximab is approved for the treatment of adenocarcinoma of the colon and rectum that is refractory to standard chemotherapy, and in combination with radiation

Table 3 EGFR-directed agents

Class/agent	Dose/schedule	Indication
Antibody		
Cetuximab	400 mg/m^2 loading dose, then 250 mg/m^2 weekly	Metastatic colorectal cancer • As a single agent in EGFR-expressing disease after failure of both irinotecan- and oxaliplatin-based regimens or in patients who are intolerant to irinotecan-based regimens • In combination with irinotecan in EGFR-expressing disease refractory to irinotecan-based chemotherapy Squamous cell carcinoma of the head/neck • Locally or regionally advanced disease in combination with radiation therapy • In combination with platinum-based therapy
Panitumumab	6 mg/kg every 2 weeks	Metastatic colorectal cancer • As a single agent after disease progression following fluoropyrimidine-, oxaliplatin-, and irinotecan-containing chemotherapy regimens
Tyrosine kinase inhibitors		
Erlotinib	150 mg daily (lung)	Non-small cell lung cancer • As a single agent after failure of at least one prior chemotherapy regimen
	100 mg daily (pancreas)	Pancreatic cancer • In combination with gemcitabine
Gefitinib	250 mg daily	Non-small cell lung cancer

or chemotherapy for head/neck cancer, whereas panitumumab is approved for colorectal cancer. Erlotinib is approved for non-small cell lung cancer and also in combination with gemcitabine for pancreatic cancer (Senderowicz et al., 2007). Gefitinib was originally approved for non-small cell lung cancer based on a phase II trial (Kris et al., 2003), but then lost its approval status when a phase III trial failed to confirm survival benefit when compared with supportive care alone (Thatcher et al., 2005).

The most common toxicities of anti-EGFR-directed therapies include diarrhea and papulopustular ("acneiform") skin rash, the latter being a biomarker that correlates with response (Perez-Soler et al., 2004; Perez-Soler and Saltz, 2005). Several studies with both antibodies and tyrosine kinase inhibitors have

demonstrated an association between skin rash and improved clinical outcomes in different diseases, including colorectal, head/neck, and non-small cell lung cancer (Cunningham et al., 2004; Burtness et al., 2005; Shepherd et al., 2005). Suppression of phosphorylated (activated) EGFR in skin biopsies during treatment may be associated with improved patient outcomes with these agents (Agulnik et al., 2007). Cetuximab has also been associated with hypomagnesemia, which may also be a biomarker predictive of therapeutic benefit (Fakih, 2007; Tejpar et al., 2007; Vincenzi et al., 2008). Interstitial pneumonitis has also been described with EGFR-directed small-molecule inhibitors and antibodies, which may be difficult to distinguish in some circumstances from disease progression and which may respond to interruption of therapy and corticosteroids (Yoneda et al., 2007).

EGFR Expression or Mutations and Prognosis or Response to Therapy

A relationship between EGFR expression and clinical outcomes has been seen in a variety of cancer types and in studies evaluating its role as a prognostic factor as well as a predictive factor for response to EGFR-directed therapies. Most studies have focused on diseases that are responsive to EGFR-directed therapies (e.g., non-small cell lung, colorectal, and head/neck cancers). Methods for evaluating EGFR expression have included evaluation of protein expression (by immunohistochemistry [IHC]), gene amplification (by fluorescent in situ hybridization [FISH]), and gene mutations (in the *EGFR* or *k-RAS* genes). EGFR expression by IHC may not be optimal or sufficiently standardized to permit clinical application (Cunningham et al., 2004; Ciardiello and Tortora, 2008).

EGFR and k-RAS Gene Mutations

In non-small cell lung cancer, activating somatic mutations in the tyrosine kinase domain of the *EGFR* gene were found to be predictive of response to gefitinib and erlotinib (Lynch et al., 2004; Pao et al., 2004). Mutations are found in approximately 10% of Caucasians and 30% of Asians with non-small cell lung cancer and have also been associated with certain clinical characteristics, including female gender, non-smokers, and bronchioloalveolar histology. Approximately 90% of *EGFR* mutations affect small regions of the gene within exons 18–24 that code for the *EGFR* tyrosine kinase domain (Shigematsu et al., 2005; Sequist et al., 2007). Such mutations appear to be uncommon in other cancer types.

Mutations in the *k-RAS* gene appear to be more common, especially among cancers of the colon, rectum, and pancreas (Rowinsky et al., 1999). *k-RAS* gene mutations occur in about 40% of adenocarcinomas of the colon and rectum (Karapetis et al., 2008) and about 20% of adenocarcinomas of the lung, but are uncommon in squamous cell carcinoma of the lung (Zhu et al., 2008), head/neck

(Weber et al., 2003), cervix (Pappa et al., 2006), and anal canal (Hiorns et al., 1990). *k-RAS* gene mutations have been associated with resistance to panitumumab and cetuximab (Benvenuti et al., 2007; Karapetis et al., 2008), but do not predict benefit from EGFR tyrosine kinase inhibitors (Zhu et al., 2008).

EGFR Gene or Protein Expression

EGFR expression has been associated with a poor outcome in a variety of cancer types including colorectal (Moroni et al., 2005), non-small cell lung (Zhu et al., 2008), oropharyngeal (Ang et al., 2002; Kumar et al., 2007), and cervical cancers (Kersemaekers et al., 1999). In addition to being a poor prognostic marker in patients treated with standard chemotherapy or untreated patients, some studies have shown that high EGFR expression also predicts benefit from EGFR-directed therapies (Zhu et al., 2008). In metastatic colorectal cancer, higher EGFR protein expression (by IHC) was not associated with benefit from cetuximab, although other studies found that higher *EGFR* gene expression (by FISH) was associated with greater benefit from panitumumab (Moroni et al., 2005).

Relationship Between EGFR Expression and Mutation Status

Some studies have evaluated *EGFR* gene expression and *EGFR* mutation status in the same population. For example, in non-small cell lung cancer, high *EGFR* gene copy number and *EGFR* mutations were associated with prognosis and response to therapy in a randomized trial of erlotinib compared with placebo (Zhu et al., 2008). Thirty-eight percent had a high *EGFR* gene copy by FISH and 17% had an *EGFR* mutation (exon 19 deletion or exon 21 L858R mutations). Response rates were higher in those with mutant *EGFR* (27% vs. 7%; $p = 0.03$) and *EGFR*-amplified disease (21% vs. 5%; $p = 0.02$). Although a significant survival benefit from EGFR-directed therapy was observed for patients with wild-type *k-RAS* (hazard ratio 0.69, $p = 0.03$) and *EGFR* FISH positivity (hazard ratio 0.43, $p = 0.004$), *EGFR* FISH-positive status was prognostic for poorer survival in multivariate analysis and was also predictive of differential survival benefit from anti-EGFR-directed therapy.

EGFR Expression and Mutation Status in HPV-Associated Cancers

The association between *EGFR* gene mutations with HPV and clinical outcomes was evaluated in 108 patients with head and neck carcinoma (Na et al., 2007). Sixteen percent had *EGFR* mutations and 9% were HPV positive; none of the HPV-positive cases had *EGFR* mutations. HPV was more common in tonsillar cancers than other sites (26% vs. 0%, $p < 0.001$). HPV infection was associated with

significantly improved survival in both univariate ($p = 0.025$) and multivariate ($p = 0.007$) analyses, but *EGFR* mutations were not ($p = 0.746$). In another study, *EGFR* and *BRAF* genes were evaluated in 90 patients with oropharyngeal cancer treated with surgery and were matched for high-risk HPV DNA and the p53 status (Perrone et al., 2006). The authors identified four distinct groups that exhibited similar histology but different molecular/cytogenetic patterns. These included (1) 19% of cases that were HPV positive and lacked gene alterations, (2) 37% that were HPV negative and had p53 mutations, (3) 34% that were HPV negative with wild-type p53 and loss of 9p21 (p16INK4a and p15INK4b) and/or cyclin D1 overexpression, and (4) 10% that were HPV negative and lacked tumor suppressor genes and cell cycle alterations. The second, third, and fourth groups showed an increased copy number of *EGFR* and chromosome 7 (43%), suggesting that these patterns may be useful to select subjects for EGFR inhibitors.

Management of HPV-Associated Cancers

The principles of management for HPV-associated cancer include surgical excision for minimal localized disease or combined modality therapy consisting of chemotherapy and radiation for localized or locally advanced disease. Anti-EGFR-directed therapies have an established role in head and neck carcinoma and are currently being evaluated in other HPV-associated cancer types.

Carcinoma of the Head and Neck

Carcinoma of the head and neck is a heterogeneous group of malignancies arising from mucosal surfaces of the oral cavity, oropharynx, larynx, hypopharynx, and other anatomic sites within the head and neck. Most are also of squamous cell origin and histology and will therefore be referred to as squamous cell carcinoma of the head and neck (SCCHN). There is a well-established association of SCCHN with tobacco and alcohol consumption that was established more than 40 years ago (Flamant et al., 1964). Death rates from oropharyngeal and laryngeal cancer in the United States have declined over 15 years by 29 and 25%, respectively, reflecting reduced incidence rates due to declining tobacco use (Jemal et al., 2008).

More recently, evidence suggests an association with HPV infection and SCCHN in about 35% of cases (Termine et al., 2008), with a particular association with tonsillar cancer (Mork et al., 2001; Herrero et al., 2003; D′Souza et al., 2007). HPV 16 integration occurs in tonsillar crypts, followed by cell cycle abnormalities including p16 overexpression (Kim et al., 2007). HPV-associated cancers are also associated with tumor overexpression of p16 (Perrone et al., 2006).

SCCHN is a potentially curable disease. Organ preservation is a goal of therapy, but may not be appropriate for all individuals (Forastiere et al., 2001, 2006). Treatment with either surgery or radiotherapy may be adequate for appropriately

selected patients with early-stage disease localized to the primary tumor site. For those who present with regionally advanced, non-metastatic disease, multimodality therapy consisting of chemotherapy, radiotherapy, biological therapy, and sometimes surgery is also potentially curative, but may result in substantial acute- and chronic-term toxicity and organ dysfunction. The driving force of clinical research and clinical care is finding the balance between therapeutic approaches that maximize anticancer activity while minimizing toxicity. To this end, considerable attention has been directed to biological therapies which offer the prospect of specifically targeting tumor cells and thereby limiting the systemic toxicities associated with cytotoxic chemotherapies. Since HPV-associated SCCHN is known to be associated with more favorable outcomes, this has presented a unique opportunity for efforts to reduce treatment morbidity (Fakhry et al., 2008). The favorable prognosis may be attributable in part to a lower incidence of p53 mutations in HPV-associated SCCHN compared with tobacco-associated SCCHN (Smith et al., 2008).

Several trials have evaluated the role of anti-EGFR-directed therapies in SCCHN (Table 4). When used as a single agent in patients with cisplatin-refractory disease, cetuximab produced a response rate of 13% (Vermorken et al., 2007), and other trials have demonstrated encouraging activity when cetuximab was combined with chemotherapy (Baselga et al., 2005; Herbst et al., 2005; Bourhis et al., 2006). Bonner et al. reported the results of a phase III trial in which 424 patients with SCCHN were randomized to receive irradiation alone or in combination with cetuximab (400 mg/m^2 in week 1, then 250 mg/m^2 weekly in weeks 2–8) (Bonner et al., 2006). Physicians could select the radiation fractionation schedule (including 70 Gy in 35 daily fractions over 7 weeks, 72–76.8 Gy in 60–64 fractions twice daily over 6–6½ weeks, or 72 Gy given in 42 fractions over 6 weeks). After a median follow-up of 54 months, cetuximab administration was associated with significantly prolonged progression-free survival (hazard ratio 0.70; $p = 0.006$). The median duration of locoregional control was 24.4 months among patients treated

Table 4 Phase III trials of cetuximab in squamous cell carcinoma of the head and neck

Study	Population	Arms	No. of patients	Median PFS	Median OS
Bonner et al.	Locally advanced disease at initial presentation	RT alone	212	12.4 mo	28 mo
		RT + cetuximab	211	17.1 mo ($p = 0.006$)	58 mo ($p = 0.02$)
Burtness et al.	Metastatic disease or locally recurrent after prior RT	CDDP + placebo	60	2.7 mo	8.0 mo
		CDDP + cetuximab	63	4.2 mo ($p = 0.09$)	9.2 mo ($p = 0.21$)
Vermorken et al.	Metastatic or locally recurrent disease	CDDP/5-FU	220	3.3 mo	7.4 mo
		CDDP/5-FU+ cetuximab	222	5.6 mo ($p < 0.001$)	10.4 mo ($p = 0.04$)

Abbreviations/notes: CDDP – cisplatin (Vermoken study allowed carboplatin instead of cisplatin); OS – overall survival; PFS – progression-free survival

with cetuximab plus radiotherapy and 14.9 months among those given radiotherapy alone (hazard ratio 0.68; $p = 0.005$). The median duration of overall survival was 49.0 months among patients treated with combined therapy and 29.3 months among those treated with radiotherapy alone (hazard ratio 0.74; $p = 0.03$). With the exception of acneiform rash and infusion reactions, the incidence of grade 3 or greater toxic effects, including mucositis, did not differ significantly between the two groups. The subset of patients with oropharyngeal cancer (representing approximately 60% of the study population) appeared to experience the greatest benefit from cetuximab (hazard ratio 0.61 for locoregional control; 0.62 for overall survival). Therefore, although the HPV status of tumors was not examined, it appears that cetuximab is particularly effective as a radiosensitizer against tumors typically associated with HPV infection. Cetuximab therapy was not associated with increased in-field toxic effects associated with curative radiotherapy doses to the head and neck (e.g., mucositis, xerostomia, dysphagia, and radiation dermatitis). Additionally, patient quality of life was not adversely affected with the addition of cetuximab (Curran et al., 2007). In another trial, Burtness reported the results of a phase III trial in 117 patients with recurrent/metastatic SCCHN who were randomly assigned to receive cisplatin every 4 weeks plus cetuximab or a placebo. The objective response rate was significantly higher in the cetuximab arm (26% vs. 10%; $p = 0.03$), although median progression-free survival (4.2 vs. 2.7 months; $p = 0.09$) and median overall survival (9.2 vs. 8.0 months; $p = 0.21$) were not significantly different. Skin rash was associated with improved survival in cetuximab-treated patients (hazard ratio 0.42, $p < 0.05$). Benefits seemed to be greater in patients with lower EGFR expression levels by immunohistochemistry. Finally, 442 patients with untreated or recurrent or metastatic SCCHN received a platinum agent (physician's choice of cisplatin, 100 mg/m^2, or carboplatin, AUC 5) plus 5-fluorouracil ([5-FU], 1,000 mg/m^2/day for 4 days) every 3 weeks for a maximum of 6 cycles, or the same chemotherapy plus cetuximab (400 mg/m^2 initially as a 2-h intravenous infusion, then 250 mg/m^2 as a 1-h intravenous infusion weekly) (Vermorken et al., 2008). Adding cetuximab significantly prolonged the median overall survival (10.1 vs. 7.4 months: hazard ratio 0.80, $p = 0.04$), median progression-free survival (5.6 vs. 3.3 months; hazard ratio 0.54; $p < 0.001$), and response rate (36% vs. 20%; $p < 0.001$). There were more episodes of sepsis in the cetuximab arm (9 vs. 1 patient, $p = 0.02$). Taken together, these trials demonstrate a beneficial role for cetuximab when combined with radiation for localized disease or with chemotherapy in patients with recurrent or metastatic disease.

Carcinoma of the Anal Canal

About 90% of carcinomas of the anal canal are also of squamous cell histology. Standard therapy consists of chemotherapy and radiation therapy, which results in about 60–70% disease-free survival and local failure rates of about 30–40% (Esiashvili et al., 2002). Randomized trials have clearly established that combined modality therapy (CMT) consisting of mitomycin-C, 5-FU, and irradiation results

in improved local control and disease-free survival compared with irradiation alone (UKCCCR Working Party 1996; Flam et al., 1996; Bartelink et al., 1997). More recently, the Radiation Therapy Oncology Group (RTOG) reported the results of a phase III trial comparing standard mitomycin/5-FU plus irradiation to concurrent cisplatin/5-FU preceded by induction cisplatin/5-FU before beginning concurrent chemoradiotherapy (Ajani et al., 2008). Six hundred eighty two patients were randomly assigned to receive a standard regimen of two cycles of 5-FU and mitomycin plus radiotherapy, or 5-FU plus cisplatin given for four cycles plus radiotherapy, with two chemotherapy cycles given before and two cycles given concurrently with irradiation. All patients received an initial radiation dose of 30.6 Gy in seventeen 1.8 Gy fractions to a large pelvic field (superior border at L5-S1), followed by a field reduction (reduce superior border to inferior level of S1) for an additional 14.4 Gy in eight 1.8 Gy fractions. For patients with high-risk disease (T3, T4, or N+ lesions and T2 lesions with residual cancer after 45 Gy), an additional 10–14 Gy (2 Gy/fraction) was delivered to gross primary or nodal disease plus a 2–2.5 cm margin for a total dose of 55–59 Gy within the boost field. The median age for the study population was 55 years, 69% were women, 27% had a tumor diameter greater than 5 cm, and 26% had clinically positive nodes. After a median follow-up of 2.5 years, the 5-year disease-free survival rate (the primary trial endpoint) was 60% in the group that received mitomycin-based therapy compared with 54% in the group that received cisplatin-based treatment ($p = 0.17$), and the 5-year overall survival rates were 75 and 70%, respectively ($p = 0.10$). Five-year local–regional recurrence and distant metastasis rates were 25 and 15%, respectively, for mitomycin group, compared with 33 and 19%, respectively, for the cisplatin group. The cumulative rate of colostomy was significantly higher in the cisplatin arm (10% vs. 19%; $p = 0.02$), although severe hematologic toxicity was worse in the mitomycin arm ($p < 0.001$). The results of this study favor continued use of mitomycin/5-FU in combination with irradiation as the standard of care. One ongoing study (ACTII – http://www.ucl.ac.uk/cancertrials/trials/actii/index.htm) that will include 950 patients with anal carcinoma is directly comparing mitomycin/5-FU with cisplatin/5-FU given without induction chemotherapy and is also evaluating the role of "maintenance" mitomycin/5-FU or cisplatin/5-FU after concurrent chemoradiotherapy.

There are anecdotal reports of activity of cetuximab in anal cancer (Phan and Hoff, 2007). There are currently two phase II trials evaluating cetuximab with cisplatin/5-FU and irradiation in patients with HIV infection (ClinicalTrials.Gov identifier NCT00324415) and patients without HIV infection (NCT00316888). These trials should help define whether there is a role for EGFR-directed therapy in this condition.

Carcinoma of the Cervix

Cervical cancer is a potentially curable disease. Surgery and radiation are the standard treatments for early-stage disease (CIN to stage IA), whereas combined

modality therapy including platinum-containing chemotherapy and irradiation is the standard for patients with localized or regionally advanced disease (stage IB–IVA) (Thomas, 1999). For patients with metastatic disease, single-agent cisplatin has been considered the drug of choice, although several cisplatin-containing two-drug regimens have been associated with improved response and PFS, and administration of the combination of cisplatin and topotecan was associated with improved overall survival (Long, 2007). There is limited information regarding EGFR-directed therapies in cervical cancer. One study demonstrated the feasibility of combining erlotinib with cisplatin and radiation therapy in stage IIB–IIIB disease (Nogueira-Rodrigues et al., 2008). Clinical trials are in progress to evaluate the role of cetuximab in recurrent cervical cancer (NCT00499031) and in combination with cisplatin and irradiation in stage IB–IVA disease (NCT00104910) and locally advanced disease (NCT00292955).

Conclusions

HPV is a double-stranded DNA virus that encodes oncogenic proteins that contribute to the pathogenesis of squamous cell carcinoma of the cervix, oropharynx, anal canal, and other genital tract sites. Individuals co-infected with HIV and HPV have a substantially increased risk of these cancers. Oncogenic HPV types produce E6 and E7 proteins that bind to p53 and pRb, respectively, inhibiting apoptosis and promoting genomic instability. Oncogenic E5 protein is also carcinogenic and appears to mediate some of these effects via EGFR-dependent processes (Tsai and Chen, 2003). EGFR-directed therapies are effective treatments for SCCHN, including the 35% of tumors that are typically oropharyngeal cancers associated with HPV infection. EGFR-directed therapies are currently being evaluated in other HPV-associated cancers.

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Cidofovir Against Human Papillomavirus-Associated Diseases

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Abstract HPV-associated conditions including condyloma, high-grade squamous intraepithelial lesions of the cervix, anus, perianal skin, and vulva are commonly diagnosed and difficult to successfully treat in HIV-infected individuals. Effective, novel, non-surgical treatment modalities would be a welcome addition to the current treatment options. Cidofovir, an antiviral agent, appears to have efficacy with these diseases.

Introduction

To understand the proposed mechanism of action of cidofovir, we first briefly review the current knowledge of HPV infections and their manifestations in HIV-infected patients and then discuss what is known about cidofovir as treatment for various HPV-associated diseases.

Human Papillomavirus Infections and Natural History

It is now well established that human papillomaviruses (HPVs) are the etiologic agent of many benign and malignant lesions arising from epithelial cells. The HPVs comprise more than 130 genotypes with 30–40 HPV genotypes infecting the genital tract. The HPV genotypes are classified as low-risk types associated with benign warts and high-risk types associated with malignant disease. The two most common low-risk types in the genital tract are HPV-6 and HPV-11, whereas of the 15 high-risk or oncogenic HPVs infecting the genital tract, the two most important are HPV-16 and HPV-18 (Stanley, 2008). HPV-associated malignancies include cervical cancer (Bosch et al., 1995; Palefsky and Holly, 1995; Walboomers et al., 1999) and its precursor, high-grade cervical intraepithelial neoplasia (CIN) (Palefsky and

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Holly, 1995; Koutsky et al., 1992), anal cancer (Zaki et al., 1992; Frisch et al., 1997) and its putative precursor, high-grade anal intraepithelial neoplasia (AIN) (Palefsky et al., 1997, 1998a, b, c), as well as some oropharyngeal cancers (Gillison et al., 2008). HIV-infected women and men have increased rates of these HPV-associated cancers and precursors compared to immunocompetent women and men. In addition, HIV infection appears to confer an increased risk of persistent HPV infections as well as an increased likelihood of co-infection with multiple HPV types.

HPV infections are completely intraepithelial. HPV is a small non-enveloped virus with a double-stranded circular DNA genome within an icosahedral capsid. Its genome codes for 8–10 genes, including *E6* and *E7*. HPV is thought to be transmitted through micro-abrasions of the epithelium, allowing the virus to infect the primitive basal keratinocyte. Replication of HPV depends on the DNA synthetic machinery of the host epithelial cell and occurs as the epithelial cells move up from the basement membrane to become mature keratinocytes. When the infected keratinocyte enters the differentiating compartment exiting the cell cycle, there may be upregulation of viral gene expression, which permits viral DNA replication to occur. Expression of viral E6 and E7 in squamous cells inhibits apoptosis and delays the differentiation program of the infected keratinocyte, creating an environment permissive for viral DNA replication. High-risk HPV replication may then deregulate growth control in the infected cells causing dysplasia or cancer (Gillison et al., 2008; Stanley, 2008). HPV DNA replication and virus assembly occur in a cell that will terminally differentiate and die by natural causes. There is no blood-borne or viremic phase of the HPV life cycle, and only minimal amounts of the replicating virus are exposed to the host immune system (Stanley, 2008).

Despite the ability of HPV to evade host immune response, the average duration of HPV infection is only 8 months and about 90% of cervical HPV infections in healthy young women clear at 2 years (Ho et al., 1998). Giuliano et al. have found that immunocompetent men have high acquisition rates and clearance rates of genital HPV, with 75% of infections clearing at 1 year. Unlike women, however, acquisition rates of genital HPV in men appear to be independent of age (Giuliano et al., 2008). Although HPV persistence greatly increases the risk of developing high-grade squamous intraepithelial lesion (HSIL) and cancer of the cervix, the majority of HPV-infected women will never develop these conditions. A combination of viral, environmental, and host factors appears to interact in the development of HSIL and cancer (Gagnon et al., 2007).

HPV-Associated Diseases and the HIV-Infected Patient

The HIV/AIDS Cancer Match Study found that cervical cancer incidence among HIV-infected women in the United States has not significantly changed since the introduction of highly active antiretroviral therapy (HAART). The pre-HAART rate was 64.2 cases per 100,000 person-years (1990–1995) compared with 86.5 cases per 100,000 person-years since the widespread introduction of HAART (1996–2002) [relative risk (RR) 1.41, 95% confidence interval (CI) 0.81–2.46] (Biggar et al.,

2007). In this same time period, however, anal cancer rates appear to be increasing. Cohort studies have shown that the anal cancer incidence in men with HIV has climbed from 35–49 cases per 100,000 person-years in the pre-HAART era to 92–144 cases per 100,000 person-years (Bower et al., 2004; Diamond et al., 2005). Compared to the general population, cancer risk in HIV-infected individuals has been estimated to be as much as 29 times increased for anal cancer, 4 times for penile cancer, 6 times for vulvar and vaginal cancer as well as cervical cancer, and 2.3 times increased for oropharyngeal cancer (Grulich et al., 2007). As the increased incidence of these HPV-associated cancers in HIV-infected persons persists despite apparent immune reconstitution with HAART, the need to optimize screening, prevention, and treatment of these diseases becomes even more important.

Similarly, HAART has not been associated with a decreased incidence or persistence of either human papillomavirus (HPV) infection or high-grade squamous intraepithelial lesions (HSILs) of the cervix (Lillo et al., 2001; Minkoff et al., 2001; Robinson et al., 2001; Heard et al., 2002; Schuman et al., 2003; Ahdieh et al., 2004; Del Mistro et al., 2004) or the anus (Palefsky et al., 2001; Fox et al., 2003; Piketty et al., 2004; Abramowitz et al., 2007).

Available Treatments

HSIL of the cervix is typically treated with excision or ablation of the transformation zone. However, there is no standard of care for treatment of HSIL of the anus. Most frequently, focal ablative therapy with cautery, cryotherapy, or laser has been utilized. Unlike the cervix, excision or ablation of the entire transformation zone of the anus is not feasible, as this would result in sphincter loss with attendant morbidity and diminished quality of life. Similarly, there is no standard treatment for HSIL of the perianus (aka Bowen's disease) or HSIL of the vulva, especially in HIV-infected patients. HIV-infected patients treated for HSIL of the cervix or the anus have much higher recurrence rates than do immune-competent patients. A variety of therapeutic modalities utilizing office procedures or medical treatment for anal dysplasia are being assessed. A pilot study to examine the role of infrared coagulation (used for treatment of hemorrhoids) in the treatment of focal high-grade dysplastic lesions in HIV-infected individuals showed a complete response to treatment in 10 of 16 patients followed for 1 year (62.5%) (Stier et al., 2008). There were no significant adverse events associated with the procedure. A larger study to better assess the efficacy of this approach is in development. Imiquimod, a topical, immune-modulating agent, appears to decrease HPV viral load and clear perianal warts and mild dysplasia in HIV-infected men (Wieland et al., 2006; Kreuter et al., 2007; Sanclemente et al., 2007). Larger studies with long-term follow-up and analysis will be necessary to assess the durability of these novel treatment approaches in anal HPV-related disease.

Chemoradiation is a standard treatment for invasive anal cancer of all stages, oropharyngeal cancers, as well as locally advanced cervical cancer. However, treatment-associated morbidity especially in HIV-infected patients may be significant (Salama et al., 2007).

Taken together, these data indicate that HPV-associated intraepithelial neoplasms are likely to be a continuing therapeutic challenge in HIV-infected individuals and that currently available standard therapeutic options for diffuse and/or multifocal pre-invasive lesions of the anus, perianus, and vulva frequently fail to control the disease. Unlike the cervix, for which removal of the entire transitional zone is possible without excessive morbidity, the anal canal, the perianus, and the vulva are anatomic areas that cannot be fully excised or ablated without major morbidity. The accessibility of the latter two areas provides the opportunity to test the efficacy of agents, including antiviral drugs, that can be applied locally to a wide field and that may not only induce local regression of intraepithelial neoplasia but also be cytotoxic to HPV-infected cells.

Cidofovir

Cidofovir is a cytidine nucleotide analogue with in vitro and in vivo activity against a broad spectrum of herpesviruses as well as adenoviruses, human papillomaviruses (HPVs), polyomaviruses, and human poxviruses (Safrin et al., 1997). It is currently licensed as an intravenous treatment for cytomegalovirus (CMV) retinitis in people with AIDS. Its broad-spectrum antiviral activity has prompted investigations in a variety of other virally induced diseases, including those associated with HPV. Unlike CMV infection, which is usually systemic, the HPVs are strictly epitheliotropic, i.e., they infect only cutaneous or mucosal surfaces. Thus, the use of locally injected or topically applied antiviral agents to treat HPV-associated lesions is a rational approach that could deliver high concentrations of the agent to infected cells while avoiding the dose-limiting nephrotoxicity associated with systemic administration.

Cidofovir for Treatment of HIV-Positive Subjects with HPV-Associated Diseases

A number of investigators have explored the local use of cidofovir for HPV-associated lesions in subjects with or without concurrent immunosuppression, including HIV infection. By far, the most common condition studied has been genital and/or perianal warts (condylomas), using cidofovir formulated as a 1% gel or cream (Douglas et al., 1997; Orlando et al., 1999; Martinelli et al., 2001; Matteeli et al., 2001; Snoeck et al., 2001a; Orlando et al., 2002; Stragier et al., 2002; Coremans et al., 2003). Although application times and treatment schedules varied from study to study, the literature indicates that this approach is highly effective, as measured by wart regression and recurrence rates, and generally well tolerated. One report compared sequential groups of HIV-positive and HIV-negative subjects treated with surgical electrocoagulation (20 subjects) or with cidofovir 1% gel applied 5 days a week for 5 h, every other week for up to 18 weeks (27 subjects)

(Coremans et al., 2003). A 92% overall response rate (complete and partial) was reported for cidofovir. The relapse rate was only 3.7% for cidofovir-treated lesions (vs. 55% for electrocoagulation), and there was significantly less pain associated with cidofovir treatment than with surgery. In a randomized comparison of cidofovir 1% gel and placebo gel in HIV-negative subjects (applied 5 days a week every other week, for at least 4 h or overnight, for up to six cycles), Snoeck et al. (2001a) observed significant differences in the rates of complete response (9/19 vs. 0/11), overall response (16/19 vs. 2/11), and progression (0/19 vs. 5/11) for the cidofovir and placebo arms, respectively. Moreover, only one of nine complete responders showed a recurrence (at 120 days) after a median follow-up of 168 days. Local reactions were similar in the cidofovir and placebo groups, and consisted of reversible pain, pruritus, rash, erosions, or ulcerations at the application site. In another small, placebo-controlled study in HIV-positive subjects, however, Matteeli et al. (2001) reported erosions and itching only in subjects who received cidofovir 1% cream. In the largest randomized study reported to date, Orlando et al. (2002) compared electrocautery alone (29 subjects); 1% cidofovir gel alone 5 days a week for 1–2 h, for up to 6 weeks (26 subjects); and electrocautery followed, within 1 month, by cidofovir 5 days a week for 2 weeks (19 subjects) in HIV-positive subjects with genital warts. Complete response rates for the three arms were 93, 76, and 100%, respectively ($p = 0.003$). Relapse rates (74, 35, and 27%, respectively) favored the two cidofovir treatment arms ($p = 0.018$), as did time to relapse (66 days for electrocautery alone, not reached for either of the cidofovir arms). Relapse was independent of baseline CD4 count, antiretroviral treatment, or the presence of high-risk HPV types, which were present in 25 of 52 subjects tested at baseline. Clearance of high-risk HPV DNA was studied after treatment in a small subset of subjects and was 0, 57 and 25%, respectively, for the three treatment groups.

Several additional studies or case reports have appeared in the literature documenting the activity of cidofovir in other HPV-associated diseases, including some associated with high-risk HPV types. Among lesions reported to regress were laryngeal papillomas (Snoeck et al., 1998; Wilson et al., 2000), bowenoid papulosis of the penis (Snoeck et al., 2001b), verruca vulgaris (Zabawski et al., 1997; Stragier et al., 2002), which, like genital and perianal warts, are generally associated with low-risk HPV types, vulvar intraepithelial neoplasia III (VIN III) (Koonsaeng et al., 2001), squamous papilloma of the hypopharynx–esophagus (Van Cusem et al., 1995), recurrent Bowen's disease (Toro et al., 2003), and cervical intraepithelial neoplasia III (CIN III) (Snoeck et al., 2000), which are associated with high-risk HPV types.

Several studies and case reports have noted excellent responses to topical cidofovir for treatment of non-genital warts in immunosuppressed patients. Calista (2000) treated seven HIV-infected patients with resistant warts (i.e., persistent despite ablative therapies) on non-genital areas including hands, face, and lips with 1% topical cidofovir cream and noted no relapses in six of the seven patients at an average follow-up of 24 months. Similarly positive results were noted for 1% cidofovir cream in a man with HIV and Hodgkin's lymphoma who had cutaneous warts affecting the hands and feet (De Socio et al., 2008). Successful treatment of

refractoryL oral/gingival warts with topical cidofovir in HIV-infected patients has also been reported (DeRossi and Laudenbach, 2004; Husak et al., 2005).

Capaccio et al. (2008) described the successful treatment of an HIV- and an HCV-infected patient with recurrent nasal squamous papillomatosis (HPV-11 detected from the histologic specimen) with a combination of repeated surgical excision, intralesional injection, and topical application of 0.5% cidofovir solution. The patient was reported to be relapse-free 30 months following the third treatment.

In Vitro Studies Evaluating Mechanism of Action and Possible Efficacy for Treatment of HPV-Associated Cancers

The mechanism by which cidofovir leads to regression of HPV-associated lesions is being actively investigated (Andrei et al., 1998a, b; Johnson and Gangemi, 1999; Andrei et al., 2001). Cidofovir's active metabolite, cidofovir diphosphate, inhibits CMV DNA polymerase at concentrations about 8- to 600-fold lower than those needed to inhibit human DNA polymerases α, β, and γ. HPV, unlike CMV, does not encode its own DNA polymerase. The mechanism of action against HPVs differs from that of CMV since HPV utilizes the host cell DNA polymerase and not a virally encoded polymerase to replicate its genome. The available data indicate that HPV-infected cells accumulate diphosphorylated cidofovir, whereas normal cells accumulate cidofovir in the form of a choline adduct, indicating modulation of cellular kinases in the virus-infected cells. This may account for the selective cytotoxic effect of cidofovir on HPV-infected cells, with accumulation of cells in late S phase, an effect that occurs at concentrations that do not affect normal cell proliferation (Johnson and Gangemi, 1999).

It is unclear if the activity of cidofovir against HPV is more an antiproliferative or an antiviral effect. The strong relation between the virus and the different cellular mechanisms responsible for cell replication suggests that the activity of cidofovir could be related to its interference with the viral control of cell replication.

Treatment of HPV-positive cell lines with cidofovir causes a concentration- and time-dependent inhibition of cell proliferation (Andrei et al., 1998a), selectively inhibits proliferation of HPV-infected cells compared with uninfected primary human keratinocytes (Andrei et al., 1998a), and potently inhibits the growth of HPV-infected cells grown as xenografts in nude mice (Andrei et al., 1998b). By studying various indicators of apoptosis, Andrei et al. (2001) have concluded that the mechanism of cell death following cidofovir treatment of HPV-infected cells is based on induction of apoptosis. Additionally, cidofovir induced a concentration- and time-dependent accumulation of cells in the S phase of the cell cycle and increased levels of the tumor suppressor protein p53 and the cyclin-dependent kinase inhibitor p21/WAF-1. High-risk-type HPV-infected cells may have very low levels of p53 and p21 as a result of inactivation and proteasomal degradation of p53 mediated by the HPV E6 oncoprotein, whereas the HPV E7 protein interferes with the function of pRb and can prevent p21 from inhibiting CDK2/cyclin E activity and PCNA-dependent replication. Abdulkarim et al. (2002) have shown that cidofovir

reduced E6 and E7 expression in Me180 cervical carcinoma and HEP2 head and neck squamous cell carcinoma cells at the transcriptional level, increased levels of active p53 and pRb in these cell lines, and enhanced the radiation sensitivity of HPV-infected cells.

Sirianni et al. (2005) demonstrated that cidofovir may also increase radiosensitivity of HPV-associated squamous cell carcinoma of the head and neck (SCCHN). Treatment of a naturally occurring HPV-16-infected squamous cell carcinoma of the head and neck cell line (SCC90) with cidofovir resulted in increased radiosensitivity compared with cidofovir treatment of an HPV-negative cell line of head and neck origin. The HPV-infected cell line treated with CDV showed enhanced p53 expression (perhaps by inhibiting E6 expression) and as p53 mediates pro-apoptotic effects of XRT, this may explain the mechanism of radiosensitization.

A major limitation of systemic cidofovir has been its nephrotoxicity and the need for intravenous administration. Hostetler et al. (2006) have synthesized a series of orally bioavailable cidofovir analogues that show more than 100 times greater potency than cidofovir against CMV, HSV, adenovirus, and poxviruses in vitro. These highly active cidofovir analogues were shown to be several logs more active than unmodified cidofovir against four cervical cancer cell lines, including HPV-negative C33A cervical cancer cells as well as the CaSki, Me-180, and HeLa cervical cancer lines, which contain the high-risk HPV types 16, 68, and 18 respectively. As the analogues were effective against HPV-negative as well as HPV-positive cancer cell lines, it is likely that the mechanism of action may involve more than inhibition of E6 and E7. An oral chemotherapeutic treatment for cervical cancer and its precursor lesions would be a great step forward and has significant implications for treatment in resource-limited settings where the bulk of cervical cancer is diagnosed (Hostetler et al., 2006).

Future Directions

Cidofovir may prove to be a viable treatment option for resistant or diffuse HPV-associated premalignant conditions in HIV-infected patients. We are currently studying the role of topical cidofovir in HIV-positive subjects with perianal and vulvar high-grade dysplasia and are also performing correlative laboratory studies to better understand its mechanism of action.

As studies of topical cidofovir and oral analogues of cidofovir mature, we may see significant changes in the available options for treatment of not only condyloma and high-grade dysplasia but also invasive cancers of the anus, the cervix, and the oropharynx.

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Note: The letters 'f' and 't' following the locators refer to figures and tables respectively.

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