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Characterization of Two Kaposi's Sarcoma Cell Lines and Their Response to Chemotherapeutic Agents

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Abstract

Kaposi's sarcoma (KS) is a multifocal proliferative disorder that is commonly associated with individuals who suffer some degree of reduction in immune competence. Therapy for this disorder varies and is determined by the seriousness of KS disease in the patient as well as the patient's ability to tolerate the different treatments. A better understanding of how KS arises would allow investigators to tailor therapies that would be more specific to KS and less detrimental to the individual.

In this work, cell lines were established from skin biopsies taken from KS lesions of individuals with AIDS. The KS1 cell line presented as a combination of adherent and non-adherent cells that proliferated aggressively without senescence. The KS4 cell line presented as the typical spindle cell population that proliferated aggressively but eventually senesced. Both cell lines were positive for the presence of Factor VIII-related antigen (FVIII) and the production of IL-6 and basic Fibroblastic Growth Factor (bFGF). When the association with Human Herpesvirus 8 (HHV8) was assessed, the virus proved undetectable in both cell lines.

The response of the KS cell lines to Doxorubicin, Etoposide, and ALX40-4C was assessed, and proliferation was effectively inhibited for both cell lines by all three agents. As expected, inhibition of proliferation in response to Doxorubicin and Etoposide was also observed in two control cell lines, a human endothelial cell line and a human fibroblast cell line. With respect to ALX40-4C, the dose dependent inhibition of the KS cell lines occurred at concentrations that did not affect the proliferation of control cell lines; the IC_{50}s were 5.4 uM and 47.1 uM for the KS4 and KS1 cell lines respectively, and 70 uM for the control cell lines. Subsequently, the production of bFGF was assessed in response

to ALX40-4C exposure and was found to decrease in a dose dependent fashion akin to proliferation. ALX40-4C may be interfering with the autocrine growth mechanism of KS growth.

In conclusion, I have demonstrated that the cells dealt with in this work are indeed KS cells and that it may be possible to inhibit their proliferation by interfering with their autocrine mechanism of growth.

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List of abbreviations

AIDS	Acquired Immune Deficiency Syndrome
bFGF	Basic Fibroblastic Growth Factor
CD	Cluster Designation
CMV	Cytomegalovirus
DAB	3,3'-Diaminobenzine Tetrachloride
DMSO	Dimethyl Sulfoxide
EBV	Epstein Barr virus
ECGS	Endothelial Cell Growth Supplement
ELISA	Enzyme Linked Immunosorbant Assay
FBS	Fetal Bovine Serum
g	Gravitational constant
HBV	Hepatitis B Virus
HHV6	Human Herpesvirus 6
HHV8	Human Herpesvirus 8
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HPV	Human Papillomavirus
HSV	Herpes Simplex Virus
HTLV	Human T cell Leukemia Virus
IC_{50}	Inhibitory Concentration 50%

IL-1	Intherleukin 1
IL-6	Interleukin 6
IRF	Interferon Regulatory Factor
KS	Kaposi's Sarcoma
KSGM	Kaposi's Sarcoma Growth Medium
KSHV	Kaposi's Sarcoma Associated Herpes Virus
LDH	Lactate dehydrogenase
MIP	Macrophage Inflammatory Chemokine
PCR	Polymerase Chain Reaction
PBL	Peripheral Blood Leukocytes
RT-PCR	Reverse Transcriptase - Polymerase Chain Reaction
UEA - 1	Ulex Europaeus Agglutinin Type-1
UV	Ultra Violet

Introduction

Kaposi's sarcoma (KS) is a multifocal, proliferative disorder commonly seen in individuals who suffer from some degree of reduced immune competence. Initially described by Moritz Kaposi in 1872, KS has gone from being a rare occurrence to becoming the most frequent malignancy seen in individuals who are infected with the human immunodeficiency virus (HIV) and who develop acquired immune deficiency syndrome (AIDS).

Currently there are three different forms of KS identified in the literature; Classic KS, African or endemic KS, and immunosuppressed KS (Wahman et al. 1991, Beral 1991). The classification is based on the population affected and the course of disease. Immunosuppressed KS can be further subdivided into iatrogenic immunosuppression-associated KS and AIDS-associated KS. The incidence of KS has been attributed to a number of epidemiological factors ranging from geographic and ethnic background to types of sexual behavior. The two factors that appear to be common across all affected groups are the alteration of immune competence and the association with an infectious agent.

The disease ranges from the relatively indolent to the aggressive and its outcome depends on the population involved. The relative status of an individual's immune system seems to correlate directly with the extent of disease development and rate of disease progression (Lasoued et al. 1991, Buchbinder and Friedman-Kien 1992, Hermans et al. 1994, Krown 1997). KS is more often associated with a high degree of morbidity, but in some forms it may also be associated with mortality (Wahman et al. 1991).

The trigger for KS development remains elusive. However, observations suggest that an inflammatory environment, rich in pro-inflammatory cytokines and angiogenic growth factors, may be a predisposing factor that enhances the subsequent development and maintenance of KS (Mitsuyasu 1993, Hermans and Clumeck 1995, Wang et al. 1995, Krown 1997).

Currently, treatment for KS remains palliative at best. Common chemotherapeutic agents and anti-neoplastic treatments are used singly or in combination in an effort to control clinical manifestations or disease progression (Krown 1997). The type of therapy selected for any given individual depends highly on the extent of KS disease and the individual's ability to tolerate treatment. Disease management can range from doing nothing, to local therapy for cosmetic or comfort reasons, to systemic therapy for more advanced and aggressive forms of KS (Buchbinder and Friedman-Kien 1992, Hermans and Clumeck 1995, Krown 1997).

An understanding of the mechanisms of KS development is required for the development of novel therapies that will be more specific for the treatment of KS, thereby reducing the unpleasant side effects so commonly associated with standard anti-neoplastic therapies.

Epidemiology of KS

Classic KS is the original form of KS first described by Moritz Kaposi in 1872 (Wahman et al. 1991, Beral 1991). It affects predominantly males of southern Mediterranean and Middle Eastern origin, in their later decades of life. Its occurrence is rare and published

rates of incidence are hard to find. The lesions are generally indolent and isolated to the skin of the lower extremities. The disease is rarely fatal and patient mortality is usually due to unrelated circumstances (Wahman et al. 1991, Beral 1991).

African KS was found, a few decades later, to be the most common form of KS at the time (Wahman et al. 1991, Beral 1991, Buchbinder and Friedman-Kien 1992). In some areas of Africa it represented 10% of total malignancies observed in hospitals (Oettle 1962, Hutt 1984). It affects a wide range of individuals in all age groups, and the skewing towards males appears to be maintained, however the ratio is lower then that of Classic KS (Wahman et al. 1991). The severity of disease seems to vary with age, with children developing a more aggressive form of the disease involving the lymphatics and internal organs, and adults developing a more indolent form of the disease which resembles Classic KS (Oettle 1962). With more aggressive forms of African KS, mortality is not an uncommon association (Wahman et al. 1991).

Finally, immunosuppressed KS is the most recent form of KS described to date, and has quickly become the most common (Whitby et al. 1995). Like the aggressive form of its African counterpart, this form of KS develops very rapidly and often involves internal organs (Wahman et al. 1991, Beral 1991). Afflicted individuals suffer a high degree of morbidity and occasionally die from complications.

As renal transplantation began to increase in the 1970's, patients receiving immunosuppressive therapy to prevent organ rejection began to develop KS lesions. Interestingly, when many organ recipients were taken off their immunosuppressive therapy,

their KS lesions resolved (Wahman et al. 1991, Beral 1991), a fact that demonstrated a possible link between immune competence and the development of KS

In the early 1980s, KS became one of the first defining conditions of a new disease that seemed to be isolated to young homosexual males. Those who developed this disease underwent a gradual but complete decline in immune competence. This new disease was subsequently identified as Acquired Immune Deficiency Syndrome-AIDS, and has become one of the most important medical emergencies of the twentieth century. It is estimated that in the US, 15 to 20% of homosexual and bisexual males with HIV infection are also affected by KS (Krown 1997). Currently, KS remains the most frequent malignancy associated with the development of AIDS (Krown 1997). In an effort to understand the occurrence of KS in different populations, investigators began to address factors that were associated with KS development.

Initially, KS was described to occur predominantly in males; Moritz Kaposi recorded a male to female ratio of 15:1 (Buchbinder and Friedman-Kien 1992). As time passed however, many reports began to reflect ratios that were closer to 2-4:1 (Wahman et al. 1991). As suggested by Wahman et al. (1991), this decrease may have been due to the possibility that women were becoming more susceptible to KS over time. It is equally possible that the decrease simply reflected a more efficient process of data acquisition and analysis. Regardless, for classic as well as African endemic KS, incidence remains higher in males than females (Wahman et al. 1991, Beral 1991, Buchbinder and Friedman-Kien 1992).

Hormonal factors in women could somehow be protective (Masood et al. 1993, Hermans and Clumeck 1995). Observations that KS cells implanted into pregnant mice failed

to establish tumors, and that human chorionic gonadotropin was able to inhibit KS growth *in vitro* supported this idea (Lunardi-Iskandar et al. 1995). Some unpublished observations suggested that in some women, KS resolved during pregnancy. However, observations by others that KS resolution did not occur in HIV infected African women who were pregnant challenged the hormonal theory (Rabkin et al. 1995).

In AIDS cases in Africa where HIV transmission is almost exclusively heterosexual, as well as in immunosuppressive therapy cases where HIV infection is absent, the expressed sex ratio for KS development is very close to 1:1 (Beral 1991, Biggar and Rabkins 1992, Ebrahim et al. 1993). This suggests that the segregation of KS development may be more related to contact with an afflicted individual than the gender of the parties involved. This in turn could be indicative of a possible transmissible agent playing a role in KS incidence. In light of these observations, the preferential development of KS in the males of certain populations remains a mystery.

The incidence of KS also appeared to vary according to geography. The majority of Classic KS diagnosed in the world can be traced back, either in origin or ancestry, to southern European countries or the Middle East (Wahman et al. 1991, Beral 1991, Buchbinder and Friedman-Kien 1992). For African or endemic KS cases, there is a definite over representation in the sub-Saharan regions of Africa, where KS represented approximately 9% of all malignancies recorded (Wahman et al. 1991, Beral 1991, Buchbinder and Friedman-Kien 1992). Saudi Arabia has the highest transplant-associated KS rate in the world, at 9% (Qunibi et al 1988). Following the arrival of the AIDS epidemic in North America, the incidence of KS in a given population seemed to vary with the geographical position of that population. For

example KS was more prominent in areas like New York and California as compared to Kansas (Beral 1991). The variation of geographic associations with KS does not seem to support the evolution of KS from a particular part of the globe. However, if KS development is associated with an infectious agent, the geographic information could give clues to the dynamics of transmission of this agent on a global scale.

Prior to the AIDS epidemic, KS diagnosis was more often associated with individuals of Jewish or Mediterranean descent or with individuals of African descent (Ross et al. 1985, Wahman et al. 1991, Beral 1991, Buchbinder et al. 1992). Even following the arrival of the AIDS epidemic in North America, it was suggested that Haitian-born heterosexual AIDS cases presented with a higher incidence of KS as compared to heterosexuals of other origins (Selik et al. 1987). However, Beral et al. (1991), found that the risk of AIDS-associated KS correlated more with geography, as previously mentioned, than with race or ethnicity (Beral et al. 1990).

An association of KS development with human leukocyte antigens (HLA) haplotypes has also been suggested (Friedman-Kien et al. 1982, Pollock et al. 1983, Prince et al. 1984, Brunson et al. 1990, Wahman et al. 1991, Beral 1991, Buchbiner and Friedma-Kien 1992). Many of the groups affected by KS demonstrated a higher prevalence of HLA-DR5, including individuals from the early part of the AIDS epidemic. When transplant recipients who developed KS were HLA typed, there was an increased frequency of HLA-B5, -B8, -B18, and -DR5 (Buchbiner and Friedma-Kien 1992); all of which are found to be prevalent in patients of Italian, Greek, Jewish, and Arabic descent. However, the HLA association has never been confirmed and interest in this potential genetic association is dissipating (Krown 1997).

Initially, KS was described in elderly men in the last decades of life, and it was accepted at the time that an increased risk for the development of KS was associated with increased age. However, a closer look at Kaposi's original report reveals that some of the individuals were under fifty years of age (Wahman et al. 1991, Beral 1991); Kaposi also noted that in the younger individuals, the course of the disease was often more aggressive.

In the African endemic form of KS, it was observed that the disease could affect a wide range of ages (Wahman et al. 1991), and that KS was not simply a disease of the elderly. Unfortunately, as the age of onset decreased, the severity of the disease appeared to increase. Individuals under the age of 25 usually developed a more aggressive form of KS that often led to internal involvement of organs and lymphatics, whereas adults more often developed a more indolent form of KS that was similar to Classic KS.

With the arrival of the AIDS epidemic KS began to affect young homosexual males in North America. The risk of KS development in this population, contrary to what is seen in Classical KS, seems to decrease with age; probably due to the differences in sexual behavior between the younger and the older population (Beral 1991).

An increased risk of KS development was observed in HIV infected children born of infected mothers (Beral 1991, Orlow et al. 1993, Hermans and Clumeck 1995). Like the aggressive form of African KS, disease development in these children tends to be more advanced (Wahman et al. 1991, Beral 1991, Wang et al. 1995). The variation in the ages of the afflicted would suggest that age may not play a role in determining who will develop KS. However, it may be an important factor in relation to the severity of KS that an individual may develop.

With the development of the AIDS epidemic in North America, the association of KS with sexual practices also became a target of investigation. As the AIDS epidemic progressed and currently continues, homosexual and bisexual men with AIDS remain the highest risk group for the development of KS among the populations afflicted with AIDS (Wahman et al. 1991, Beral 1991, Huang et al, 1992, Bernstein and Hamilton 1993, Herman and Clumeck 1995, Centers for Disease Control and Prevention 1995). Within this group, KS development seems to be associated with those individuals who are more sexually active and, who are more likely to perform certain types of sexual behavior (Beral 1991). In North America, the incidence of KS is higher in foci of the AIDS epidemic where certain types of sexual practices are more likely to occur (Beral 1991, Buchbinder and Friedman-Kien 1992, Bernstein and Hamilton 1993, Hermans and Clumeck 1995, Wang et al. 1995). This would explain the geographic distribution of KS in North America previously described.

The incidence of AIDS-associated KS in North American women is reported as rare, and HIV-infected women who do present with KS are usually documented as having sexual contact with bisexual men who are HIV positive (Selik et al. 1987, Biggar et al. 1989, Beral et al. 1990, Beral 1991). In Africa, where the primary route of HIV spread is through heterosexual contact, AIDS associated KS is as common in women as in men (Beral 1991, Ebrahim et al. 1993).

The apparent segregation of AIDS-associated KS to the homosexual population of North America, and the subsequent observation that individuals who come in sexual contact with this group increase their risk of developing not only AIDS but also KS, was probably one of the strongest early indications that KS development may indeed be associated with a

sexually transmissible agent. The retrospective identification of KS development in North American homosexual men, prior to the AIDS epidemic, suggests that this sexually transmissible agent may have been present in the homosexual population prior to the arrival of HIV (Friedman-Kien et al. 1990, Kitchen et al. 1990, Muret et al. 1990, Beral 1991).

The issue of immune competence was addressed only after the development of immune suppression-associated KS. As the frequency of organ transplant increased, so did the incidence of KS associated with immunosuppressive therapy (Pen 1979, Kinlen 1982, Pen 1988, Qunibi et al 1988, Beral 1991, Hermans and Clumeck 1995). Interestingly, the KS lesions of individuals on immunosuppressive therapy often resolved themselves following a reduction in the doses of immunosuppressive drugs (Filipovich 1980, Hamilton et al. 1986, Pen 1988, Qunibi et al 1988, Beral 1991, Wahman et al. 1991, Peterman et al. 1993, Hermans and Clumeck 1995). This observation strongly suggested that there may be a close association between KS development and immune competence. Subsequently, the severity of AIDS associated KS was found to be linked with the immunological status of the afflicted (Lasoued et al. 1991, Buchbinder and Friedman-Kien 1992, Hermans et al. 1994, Krown 1997). So strong is the association between AIDS-associated KS and immune status, that AIDS-KS is often used as a prognostic marker for HIV disease.

Although a strong association between KS and immune status has not been made in the individuals afflicted with either Classic or African KS, the immune system must not be ignored in either of these situations (Friedman-Kien and Ostreicher 1984, Friedman-Kien and Saltzman 1990, Wahman et al. 1991). As we age, the immune system is thought to undergo alterations in response to multiple factors such as diet, life style, and environment, (Miller 1996). In

relation to both Classic KS and African KS, these alterations may result in a reduction in immune competence which could allow for the development and progression of KS (Wahman et al. 1991). In addition, the people of Africa often co-exist with chronic infections and constant immunological challenge, which may alter their immune status in such a way as to allow for the development and progression of KS (Wahman et al. 1991, Bentwich et al. 1995). Both scenarios represent milder forms of reductions in immune competence and are supported by the more indolent forms of the diseases that develop in these populations.

Oddly, other forms of humoral and cellular immunodeficiency, such as bone marrow transplantation or congenital immunodeficiencies have not been associated with KS development (Buchbinder and Friedman-Kien 1990).

Pathology of KS

Histological comparisons of the different forms of KS reveal few or no differences (Wahman et al 1991, Enzinger and Weiss 1995, Kaaya et al. 1995). They present as multicellular foci containing an inflammatory infiltrate, endothelial cells, fibroblasts, and a distinguishable over representation of spindle-shaped cells (Templeton 1988, Corbeil et al. 1991, Huang et al. 1993).

The spindle shaped cells are the characteristic tumor cells of KS. The origin of these cells remains unclear, however, based on the presence of Factor VIII (FVIII), CD34, and Ulex Europaeus Agglutinin Type 1(UEA-1), many investigators suggest that they may arise from either the vascular or lymphatic endothelium (Guarda et al. 1981, Nadji et al. 1981, Flotte et al. 1984, Hashimoto et al. 1987, Sankey et al. 1990, Nickoloff 1991, Gray et al. 1991,

Wahman et al 1991, Corbeil et al 1991, Taylor and Cote 1994, Jones et al. 1995). Others have demonstrated the presence of an alternate spindle shaped population in KS lesions; one that is myeloid-like as assessed by cellular markers (Parravicini et al. 1991, Kaaya et al. 1995).

The lesions do not appear to be clonal in nature, therefore, it is not likely that KS is, or begins as, a true neoplasm. However, there is evidence suggesting that there is potential for the KS lesions to develop neoplastic characteristics (Schultz and Weiss 1995, Hermans and Clumeck 1995); In some lesions, there is a presence of altered karyotypes that is often indicative of neoplastic characteristics (Hermans and Clumeck 1995).

Establishment of long-term cultures from biopsied lesions reveals an outgrowth of spindle shaped cells akin to those observed in histological samples (Corbeil et al 1991). From these *in vitro* cultures, two types of KS cell populations can be established. One that is dependent on a medium that is supplemented with growth factors, and another that is not (Hermans and Clumeck 1995). This observation supports the possibility that KS may progress to develop into a true neoplasm.

Etiology and Pathogenesis of KS

Although a reduction in immune competence has been suggested to be an important factor in the development of KS, it may not be the only factor responsible for the induction of KS development. From all the epidemiological evidence available, it appears that the incidence of KS may be related to a sexually transmissible infectious agent. Some of the possible culprits that have been investigated include: Human Cytomegalovirus (HCMV), Human papalomavirus

(HPV), Human herpesvirus 6 (HHV6), Hepatitis B Virus (HBV), and Human Immunodeficiency Virus (HIV). However, the true agent remains elusive.

Chang et al. described unique sequences that were present in 90% of KS tissue and 15% of non KS tissue in HIV infected individuals (Chang et al 1994). These sequences displayed some homology to members of the gamma-Herpesviridae family, and led them to hypothesize that the presence of a novel herpesvirus may be responsible for the development of KS in immunocompromised individuals.

The existence of these newly identified sequences, which at this point were being proposed to derive from a virus termed Kaposi's Sarcoma associated herpesvirus (KSHV), was confirmed by others; not only were the KSHV sequences present in samples from HIV-infected individuals with KS, but were also present in all other groups of individuals afflicted by KS. The virus soon was referred to as Human herpesvirus 8 (HHV8) (Moore and Chang 1995, Dupin et al. 1995, Huang et al. 1995, Whitby et al. 1995).

Closer examination using *in situ* PCR and RT-PCR techniques revealed that the HHV8 DNA sequences were associated with the endothelial and spindle cells present in the KS lesions and that these cells supported HHV8 viral transcription (Boshoff et al. 1995, Li et al. 1996, Huang et al. 1996, Foreman et al. 1997, Staskus et al. 1997). Not only does this suggest a causal association of HHV8 with KS, but it also supports the theory that the cell of origin for KS may be an endothelial cell progenitor (Boshoff et al. 1995, Li et al. 1996, Foreman et al. 1997, Huang et al. 1996, Staskus et al. 1997). However, the HHV8 sequences that were detectable in the cells of KS lesions were no longer detectable following early passages *in vitro* of cell lines that were established from KS lesions (Offermann 1996). This may be indicative of

episomal viral sequences that are unable to maintain replication following removal of the cells from the complex *in vivo* environment.

HHV8 sequences have also detected in the peripheral blood of many individuals with KS, and seemed to be associated with the CD19+ (B cell) population of peripheral blood leukocytes (PBL) (Ambroziak et al. 1995). Whitby et al. (1995) observed that HHV8 was also present in HIV-infected individuals who did not have KS. It was suspected that the presence of HHV8 in the PBLs of these individuals would predispose them to the development of KS, therefore, they were followed for KS development. As time progressed the proportion of these individuals that remained KS negative decreased with 50% of them developing KS within 3.5 years of HHV8 detection (Whitby et al. 1995). The indication that the presence of HHV8 in PBLs predisposes for the development of KS further supports the idea that HHV8 may play a causal role in KS development.

The detection of HHV8 in the CD19+ population was compared to the detection of EBV in the same individuals, in order to ensure specificity, and no correlations were observed (Whitby et al. 1995). However, like EBV, HHV8 detection and possibly replication appear to be related to an individual's immune status (Whitby et al. 1995).

HHV8 sequences were also found in other non-KS neoplasms; such as dysplastic and malignant skin lesions arising in organ transplant recipients, angioimmunoblastic lymphadenopathy, multicentric Castleman's disease, hyperplastic lymph nodes of patients with HIV infection, the bone marrow dendritic cells from multiple myeloma patients, and more specifically with a form of non-Hodgkin lymphoma in individuals afflicted with AIDS (Rady et al. 1995, Bigoni et al. 1995, Soulier et al. 1995, Corbellino et al. 1995, Cesarman et al. 1995,

Rettig et al. 1997). The HHV8 DNA in the non-Hodgkin lymphomas was found to be episomal in nature and in substantially higher amounts than in KS tissue (Cesarman et al. 1995). Although these findings do not disprove a causal role for HHV8 in KS development, they do raise the possibility that HHV8 is more ubiquitous than originally thought and may simply be a passenger virus found in many different environments (Krown 1997). The frequency of lymphoid disorders associated with HHV8 does support the possibility that, like EBV, HHV8 is maintained in a lymphoid reservoir.

Establishment of *in vitro* cultures from the non-Hodgkin B-Lymphomas, has allowed further study of this agent. Purified virus was able to infect a CD19+ enriched population but not a CD19+ depleted population. Infection was dependent on biologically active virus because it was inhibited by UV irradiation and the anti-herpesvirus drug foscarnet (Mesri et al. 1996). These findings further support the suggestion that HHV8 is lymphotropic specifically for the B cell and identifies the B cell as the possible vehicle for viral dissemination throughout the body. The isolation of purified virus also allowed investigators to sequence the HHV8 genome. Along with all the regulatory and structural genes that are so common among viruses, HHV8 also coded for many interesting cellular homologues, such as macrophage inflammatory chemokines (MIP), IL-6, interferon regulatory factor (IRF), and G-protein coupled receptors (Russo et al. 1996).

Immunohistochemical staining revealed that AIDS-associated and Classical KS lesions express significant levels of cytoplasmic BCL-2, a proto-oncogene known to prevent apoptosis (Morris et al. 1996). Sequence analysis reveals that the HHV8 genome codes for a functional BCL-2-like protein that is capable of inhibiting Bax-mediated apoptosis (Russo et al. 1996).

This may account for the characteristic non-neoplastic nature of the majority of KS lesions. A latent HHV8 presence in KS lesions could lead to the limited transcription of virally coded genes which may work to create an environment which induces and maintains aggressive proliferation.

In light of the association of HHV8 with conditions other than KS, HHV8 may simply be a passenger virus. However, with all the evidence in support of a causal role for HHV8, it is difficult to disregard the possibility that HHV8 may be the possible etiological agent of KS. As stated by Whitby et al. (1995), "...the ability to predict the development of KS in individuals negative for KS lesions but positive for the KSHV sequence strongly challenges the passenger theory."

In addition to the induction of disease, the status of an individual's immune system is also directly correlated to the severity of KS development. As previously mentioned, individuals who suffer from greater degrees of immune suppression, such as organ transplant recipients and AIDS patients, often develop more aggressive forms of the disease. AIDS patients who develop opportunistic infections frequently suffer from a dramatic increase in KS progression in response to the induction of inflammation (Lasoued et al. 1991, Buchbinder and Friedman-Kien 1992, Hermans and Clumeck 1995).

The KS lesions themselves are usually associated with an inflammatory infiltrate, and KS like cells, *in vitro*, have been demonstrated to be positively responsive to pro-inflammatory cytokines and growth factors (Masood et al 1993, Wang et al. 1995, Hermans and Clumeck 1995, Bailer et al. 1995, Krown 1997), substances that can act as powerful inducers/enhancers of angiogenesis which is one of the hallmarks of KS (Hermans and Clumeck 1995). Together,

this information suggests that in an environment of reduced immune competence, inflammation may act as a secondary stimulus that enhances the development of KS resulting in increased severity of disease (Mitsuyasu 1993, Hermans and Clumeck 1995, Wang et al. 1995, Krown 1997).

Most *in vitro* KS cultures require supplementation with growth factors in the medium in order to sustain proliferation (Krown 1997), and in some cases, a supernatant taken from human T cell leukemia virus (HTLV) infected T cells is used. This further underlines the requirement of KS cells for an environment rich in cytokines and growth factors. In addition, many investigators have demonstrated that KS cells *in vitro* were capable of producing cytokines, such as interleukin one (IL-1), interleukin six (IL-6), tumor necrosis factor alpha (TNF-α), and basic fibroblastic growth factor (bFGF), which in turn could influence KS proliferation in an autocrine manner (Ensoli et al. 1989, Ensoli et al. 1992). Therefore, not only does an inflammatory environment enhance the development of KS, but it may also influence the production autocrine growth factors which may play a crucial role in the development of KS.

How the presence of HHV8 influences the cellular environment remains unclear. Based on serologic evidence it can be concluded that HHV8 is indeed visible to the immune system (Andre et al. 1997). HHV8 specific antibodies have been detected up to a year prior to KS diagnosis (Andre et al. 1997). Since HHV8 is capable of stimulating an immune response in an environment of reduced immune competence, immune stimulation would be favorable to KS development. What is clear is that the virus does code for gene products that may influence the production of and/or response to autocrine growth factors associated with KS.

With respect to individuals with HIV infection, there is an additional concern. HIV infection itself induces many alterations to the immune environment. People infected with HIV have been shown to secrete higher levels of many cytokines, particularly TNF, IL-1, and IL-6, which has been shown to be growth prmoting to KS cells *in vitro* (Barillari et al. 1992, Buonagaro et al. 1992, Krown 1997).

The HIV tat gene product has been shown to be released from infected cells in a biologically active form (Ensoli et al. 1993). The tat protein is able to transactivate the TNF promoter, inducing cells to secrete TNF. TNF can induce the production of IL-1, and IL-6, therefore an increase in the amount of TNF will result in an increased level of IL-1 and IL-6 in the circulation of HIV+ individuals (Buonaguro et al. 1992). *In vitro*, the HIV tat protein has been observed to act synergistically, in association with the other implicated growth factors in the stimulation of KS cells (Ensoli et al. 1990, Ensoli et al. 1994). The apparent association of the HIV tat gene product with KS development in HIV positive individuals, perhaps explains KS aggressiveness in these individuals.

Clinical presentation of KS

In all forms of KS, the most common clinical manifestation is the development of cutaneous lesions. These lesions can range in colour from pink to brown and may have a flat to raised appearance; they can vary in size and number depending on the extent of involvement (Krown 1997). The indolent forms of KS, such as Classic KS and African KS in adults, are associated with lesions that are confined to the lower extremities (Wahman et al. 1991, Beral 1991).

Clinical manifestations of the more aggressive forms of KS, such as African KS in children and immunosuppression-associated KS, are not as localized. KS characteristically begins as cutaneous lesions, which may encompass the entire body, and frequently progresses to the viscera and mucosal surfaces (WahmanBuchbinder and Friedman-Kien 1992, Mitsuhasu 1993, Masood et al. 1993, Wang et al 1995, Krown 1995). Oral, gastrointestinal, and pulmonary involvement is not uncommon in these more aggressive forms of KS. As previously stated, the extent of disease progression correlates directly with the immune status of the individual (Lasoued et al. 1991, Buchbinder and Friedman-Kien 1992, Hermans et al. 1994, Krown 1997). Disease associated mortality is rare and restricted to the more serious cases where lesion development interferes with life sustaining functions, as in the case pulmonary KS (Mitsuyasu 1993, Hermans and Clumeck 1995). Morbidity, on the other hand, can be quite common (Mitsuyasu 1993, Krown 1997); KS is often complicated by severe edema which may result in ulceration and secondary bacterial infection. Depending on the location of the lesions, the patient may suffer any number of debilitating effects, such as the inability to walk, talk, or eat.

KS Therapy

Therapy for KS generally varies and should be tailored to each individual case. In situations where the number of lesions are limited and involvement minor, the best therapy may be no therapy at all. In the more indolent forms of KS where lesion progression is slow and complications minimal it is best not to interfere until a possible problem presents itself (Buchbinder and Friedman-Kien 1992).

In the event that lesion development creates isolated problems, such as cosmetic abnormalities, the suggested mode of therapy is local treatment (Hermans and Clumeck 1995, Krown 1997). The most widely used agents for local therapy are liquid nitrogen cryotherapy, intralesional vinblastin, and radiation therapy (Krown 1997). For the more aggressive forms of KS development, where systemic involvement is suspected or diagnosed, systemic chemotherapy becomes the next viable option (Mitsuyasu 1993, Masood et al. 1993. Wang et al. 1995). However, the more aggressive forms of KS are often associated with reduced immune competence, which becomes an important issue when determining how to proceed (Masood et al. 1993).

Currently, many of the standard chemotherapeutic agents are used either alone or in combination with one another (Krown 1997). Topoisomerase inhibitors such as Etoposide and alkalating agents such as Doxorubicin have limited success and are often associated with detrimental side affects such as neutropenia and secondary neoplasms. When used in combination with one another or with other agents, such as vinca alkaloids, better response rates have been achieved but the effects are transient and short lived.

Chemotherapeutic treatment often results in an assault on the immune system, thus monitoring for opportunistic infection is crucial. In many patients with advanced KS their immune systems are already impaired; chemotherapy may result in an enhanced environment for opportunistic agents to develop. Often patients are given additional agents in association with chemotherapy for the prophylaxis of opportunistic infections and or for the stimulation of the hematopoetic system for the maintenance of immune cellularity (Krown 1997).

In general, patient response and survival depends on the combination of immune system and disease status (Wang et al. 1995). Individuals with relatively strong immune systems and relatively limited disease will respond best to therapy and have the longest lasting effects. In patients who fall into the above mentioned category, interferon therapy may also be an option. In AIDS patients with stable immunity and no history of opportunistic infections, IFN α was able to induce tumor regression in a high percentage of cases and duration of beneficial effects ranged from six months to two years (Krown 1997).

Two of the major problems associated with chemotherapy are the bio-availability of the agents and their affect on non-neoplastic tissue. To solve these problems, investigators have encapsulated agents, such as Doxorubicin, into liposomes. They have been able to demonstrate an increased bio-availability in involved tissue and a reduced cytotoxicity in non-involved tissue (Wanget al. 1995, Krown 1997). These observations may lead to viable alternatives for the treatment of malignancies.

ALX40-4C is a compound that was studied previously because of its ability to inhibit HIV replication. It is a nine amino acid peptide made exclusively of D-arginines. The proposed mode of action of ALX40-4C is the competitive inhibition of the interaction of the HIV tat protein with the HIV mRNA TAR element (O'Brien et al. 1996). This interaction results in transactivation of transcription and translation of the HIV gene products. ALX40-4C mimics an arginine rich region of the HIV tat protein that is known to be the contact site for HIV tat-TAR interaction. In inhibiting this interaction, ALX40-4C decreases the efficiency of HIV transcription and translation; an effect that has led to the reduction of HIV-associated cytopathology *in vitro*.

ALX40-4C could also interfere with early events of viral replication and may act via an uncharacterized mechanism involving the HIV gp120 protein (O'Brien et al. 1996). In addition to its anti-HIV effects, ALX40-4C has also demonstrated the ability to inhibit the replication of human cytomegalovirus (CMV) and human herpes simplex virus (HSV) *in vitro* (Sumner-Smith et al. 1995). Although the mechanism of inhibition is not yet understood, ALX40-4C may be inhibiting some aspect of viral replication in a manner similar to other cationic herpesvirus inhibitors (Langeland et al. 1987, Langeland et al. 1988).

Summary

KS is a multifocal proliferative disorder commonly associated with the combination of immune dysfunction and infectious disease. The status of the immune system often determines the form of KS that an individual develops and the rate at which it progresses (Lasoued et al. 1991, Buchbinder and Friedman-Kien 1992, Hermans et al. 1994, Krown 1997).

Closer consideration of the role that the immune system plays in the development of KS has led to the idea that immune stimulation leads to a favorable environment which further enhances the development and progression of KS (Mitsuyasu 1993, Hermans and Clumeck 1995, Wang et al. 1995, Krown 1997). The immune system also becomes an important consideration when selecting a modality of therapy (Masood et al. 1993). The strength and stability of an individual's immune system often determines their ability to respond to therapy and to tolerate associated side effects (Wang et al. 1995).

The association of a novel human herpesvirus (HHV8) with all forms of KS has been a large step forward in understanding the mechanism of KS proliferation (Moore and Chang

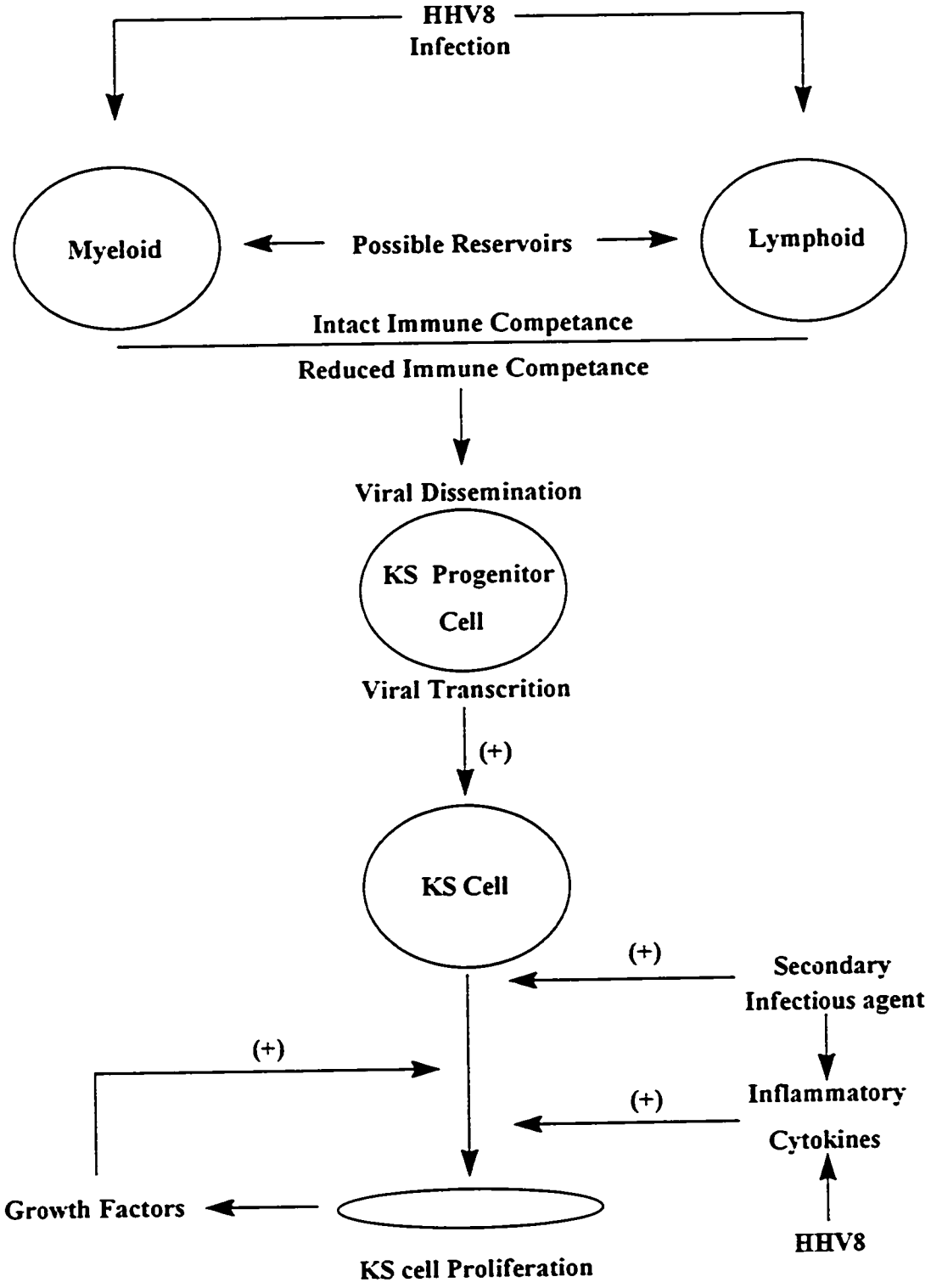
1995, Dupin et al. 1995, Q. Huang et al. 1995, Whitby et al. 1995). The virus appears to be lymphotropic in that it is often found in the B cell population of the immune system (Ambroziak et al. 1995, Whitby et al. 1995). The virus is also associated with the spindle cells the make up the KS lesions, and the endothelial cells that are frequently associated with the KS lesions (Boshoff et al. 1995, Li et al. 1996, Foreman et al. 1997).

This virus, for which almost the entire genomic sequence is known, codes for many gene products that could account for cellular immortalisation and KS pathogenesis (Russo et al. 1996). All of these observations lead to a plausible model for the development of KS (Figure 1). In situations of reduced immune competence, HHV8 escapes immunologic control, disseminates from its lymphoid reservoir throughout the body, and influences the development of KS following infection of the KS progenitor cell. The degree of immune dysfunction has a direct effect on lesion development and disease progression. The greater the reduction in immune competence, the more disseminated the virus appears to be, and the greater the possibility that non-specific chronic stimulation will occur in response to secondary infectious or opportunistic agents, resulting in an increase in the amount of pro-inflammatory and angiogenic growth factors present in the environment. These growth factors play an important secondary role by enhancing the development of KS leading to more aggressive forms of the disease.

This aspect of the model is supported by the more aggressive forms of KS that are observed in individuals who suffer from greater reductions in immune competence. Patients who respond to acquired opportunistic infections often undergo an exacerbation of their KS

disease (Lasoued et al. 1991, Buchbinder and Friedman-Kien 1992, Hermans and Clumeck 1995).

Figure 1. Model of KS development. In situations of reduced immune competence HHV8 escapes immunologic control, disseminates from its lymphoid reservoir throughout the body, and contributes to the induction of KS following infection of the KS progenitor cell. Virally coded proteins may play an important role in the induction and maintenance of KS cell proliferation. The degree of immune dysfunction has a direct effect on lesion development and disease progression. The greater the reduction in immune competance, the more disseminated the virus appears to be, and the greater the possibility that non-specific chronic stimulation will occur in response to secondary infectious or opportunistic agents, resulting in an increase in the amount of pro-inflammatory and angiogenic growth factors present in the environment. These growth factors may play an important secondary role by enhancing the development of KS leading to more aggressive forms of the disease.


HHV8
Infection
Myeloid
Possible Reservoirs
Lymphoid
Intact Immune Competance
Reduced Immune Competance
Viral Dissemination
KS Progenitor Cell
Viral Transcrition
(+)
KS Cell
(+)
Secondary Infectious agent
(+)
Inflammatory Cytokines
(+)
Growth Factors
HHV8
KS cell Proliferation

Hypothesis

Kaposi's sarcoma is a disease commonly associated with patients suffering from some degree of immune suppression. My hypothesis is that the growth of KS is mediated in part by autocrine growth factors. The production of these factors may be induced by HIV or other agents.

Patients with KS are often treated with chemotherapeutic agents. It is my hypothesis that these treatments may interfere with the autocrine growth factors of the cells.

Objectives

1. To establish and maintain long-term KS cell cultures.
2. To determine morphological and proliferative characteristics of the cells.
3. To determine which cellular markers these cell lines express
4. To determine which cytokines are produced by these cell lines.
5. To determine if HHV8 DNA is present in the KS cell cultures.
6. To assess the effect of chemotherapeutic agents on proliferation and cytokine production.

Materials and Methods

Gelatin and fibronectin coating of cell culture flasks and 24-well cell culture plates.

T-75 cell culture flasks (Corning Costar Corp, Cambridge, MA) and 24-well cell culture plates (Corning Costar) were coated with gelatin (30 mg/mL) (Bio Rad, Richmond, CA) and incubated at 37°C for 2 hours. The gelatin was removed and the containers incubated at 65°C, in a dry oven, for 2 days. On the day of use, the containers were coated with a fibronectin solution consisting of 1 mg/mL of human fibronectin (Collaborative Biomedical Products, Bedford, MA), and 20 U/mL of heparin (Organon Teknika, Toronto Ont). The containers were incubated for a minimum of 2 hours at room temperature. The fibronectin was removed and the containers seeded with cells.

Kaposi's Sarcoma growth medium (KSGM).

The medium used for the propagation and maintenance of KS cells consisted of Iscove's Modified Dubelco's Medium (IMDM) supplemented with 10% fetal bovine Serum (FBS) (Life Technologies, Grand Island, NY), 140 U/mL of heparin (Organon Teknika), 40 ug/mL human transferrin (Life Technologies), 75 ug/mL endothelial cell growth supplement (ECGS) (Collaborative Biomedical), 2 mM L-glutamine (Life Technologies), 8 ug/mL Gentamycin (Life Technologies), 5 ug/mL Amphotericin B (Sigma Chemical Co., St. Louis, MO). Medium was made fresh prior to use.

Collection of biopsy specimen and establishment of primary culture.

Punch biopsies were taken from the skin lesions of individuals that were HIV +, for the purpose of histopathological diagnosis of Kaposi's Sarcoma. Informed written consent was obtained from each patient. Portions of the skin biopsies were used for the generation of primary cultures of KS cells.

Each specimen was placed in a 100 mm plastic petri dish (Corning Costar) and rinsed twice with IMDM-EDTA (BDH Inc. Toronto, Ont). Using scalpels, normal tissue was separated from the abnormal tissue. Each of the tissues was placed in a separate 100 mm petri dishe containing IMDM-EDTA. The normal tissue served as a control culture. Both tissues were minced and treated with 15 mL of collagenase (0.1 mg/mL solution) (Sigma) in IMDM. The tissues were transferred to 50 mL polypropylene tubes (Falcon, Franklin Lakes, NJ) and incubated at 37°C for 30 minutes. The tissues were resuspended, and the larger fragments allowed to settle for 10 minutes. The supernatants were collected and washed. The cells were resuspended in 1 mL of KSGM, brought to 10 mL, and plated on T-75 cell culture flasks (Corning Costar) pre-coated with 30 mg/mL of gelatin (Bio Rad) and fibronectin at 1 mg/mL (Collaborative Biomedical).

The larger fragments of normal and abnormal tissue were washed and resuspended in 10 mL of Trypsin-EDTA (Sigma) and allowed to stand at room temperature for 20 minutes. 1 mL of FBS was added, to neutralize the enzyme, and an additional 10 mL of IMDM were added. The larger fragments were allowed to settle for 10 minutes and the supernatants collected. All fractions were washed and plated in 10 mL of KSGM.

Propagation and maintenance of established Kaposi Sarcoma cell lines.

The cells were allowed to proliferate until they reached 70 to 80% confluence. The cells were fed 2 to 3 times a week, depending on their requirements. Feedings consisted of complete removal of spent medium and replacement with fresh medium. Once 70 to 80% confluence was achieved, the flasks were rinsed with serum free IMDM and incubated with 10 mL of IMDM/30% Dispase (Collaborative Biomedical) at 37°C until the cells were lifted. The cell suspension was pelleted at 200xg for 5 minutes. The pellet was resuspended in 1 mL of KSGM, and the cell number and viability were assessed by trypan blue (Life Technologies) exclusion using a hemocytometer. The cells were utilized in one of three ways: 1) expanded into multiple T-75s at a concentration of approximately 10^4 cells/mL. 2) cryopreserved at a concentration of 10^6 cells/mL. 3) used for experimentation. Due to deteriorating culture kinetics associated with the KS4 cell line over time, cells used for experimentation were taken no later than passage 8.

Cryopreservation of cell lines.

Cultures were harvested, pelleted, resuspended in FBS and counted. The cells were brought to $2x10^6$/mL in FBS, and placed on ice. A second solution, equal in volume to the first, was made using regular KSGM/20% DMSO (BDH) and placed on ice. The solutions were gently mixed and the resulting cell concentration was $1x10^6$/mL. The cells were aliquoted at 1 mL per cryovial and frozen in a MIC 15 cryoson (Cryoson, Holland). The cells were stored in liquid nitrogen.

Other cell lines.

A human endothelial cell line ATCC # CRL-1998 (American Type Culture Collection, Rockville, MD) and a human foreskin fibroblast cell line graciously donated by Dr. D. Franks (Department of Pathology, University of Ottawa, Ottawa, Ont) were used as control cell lines for immunohistochemical staining and in ALX40-4C drug assays. Endothelial cells were propagated in medium 199 (Life Technologies) supplemented with 10% FBS, 8 ug/mL gentamycin, and 5 ug/mL amphotericin B, in T-75 cell culture flasks. Human fibroblasts were propagated in DMEM (Life Technologies) supplemented with 10% FBS, 8 ug/mL gentamycin, and 5 ug/mL amphotericin B, in T-75 cell culture flasks.

A human body cavity-based lymphoma cell line (BCBL-1), (cat# 3233, NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAD, NIH: BCBL-1 from Drs Micheal Mcgrath and Don Ganem) latently infected with HHV8, was used to supply positive control DNA for PCR (Renne et al. 1996). BCBL-1 cells were propagated in RPMI 1640 (Life Technologies) supplemented with $1x10^{-5}$ M 2-mercaptoethanol, 10% FBS, 8 ug/mL gentamycin, and 5 ug/mL amphotericin B, in T-75 cell culture flasks.

Immunohistochemical staining of cultured cells.

At time of passage, cells were seeded onto coverslips at $1x10^{4}$ cells/coverslip and allowed to propagate for one to three days. The cells were washed three times, at five minutes intervals, in PBS (Sigma) and fixed in 100% acetone (BDH) at 4°C for 2.5 minutes. The slips were allowed to dry completely and stored at -20°C until use. Cells to

be stained for Factor VIII or CD34 were brought to room temperature and treated with a 3% H_2O_2 solution for 20 minutes to eliminate any endogenous peroxidase activity.

For Factor VIII staining, cells were incubated for 20 minutes with normal swine serum (Dako Diagnostics Canada inc., Mississauga, Ont), 30 minutes with rabbit anti-FVIII antibody (Dako) diluted 1/250 in PBS, and washed for 10 minutes with PBS (pH 7.2). The cells were incubated for 20 minutes with swine anti-rabbit antibody (Dako) diluted 1/40 in PBS, washed for 10 minutes with PBS, and incubated for 20 minutes with rabbit anti-peroxidase complex (Dako) diluted 1/100 in PBS.

Cells stained for CD34 were incubated for 30 minutes with mouse anti-CD34 antibody (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) diluted 1/100 in PBS, washed for 10 minutes with PBS, incubated for 20 minutes with biotin-labeled horse anti-mouse antibody (Vector Laboratories, Burlingame CA) diluted 1/100 in PBS, washed for 10 minutes with PBS, and incubated for 20 minutes with avidin-horse radish peroxidase complex (Vector Laboratories) diluted 1/100 in PBS.

All antibody incubations were performed at room temperature. Formalin-fixed tissue sections of normal human tonsil, pre-digested with 0.4% pepsin solution (Sigma) for 20 minutes at 37°C, were used as a control for the above two stains. All samples were developed for 10 minutes in a 3,3'-diaminobenzine tetrachloride (DAB) solution, counter-stained with hemotoxaline followed by ammonium water, dehydrated, cleared, and mounted onto glass slides.

For UEA-1 staining, cells were brought to room temperature and washed three times, at five minute intervals, with PBS. The cells were incubated for 30 minutes with

UEA-1 (Dako) diluted 1/100 in PBS, washed three times at five minute intervals with PBS, incubated for 30 minutes with rabbit anti-UEA-1 (Dako) diluted 1/200 in PBS, washed three times at five minute intervals with PBS, and incubated for 30 minutes with goat anti-rabbit linked to FITC (Jackson Laboratories, West Grove, PA) diluted 1/100 in PBS. All antibody incubations were performed at room temperature. The cells were mounted onto slides with n-propylgalate mounting medium. The human endothelial cell line served as the positive control and the human fibroblastic cell line served as the negative control.

For vimentin staining, cells were brought to room temperature and washed three times, at five minute intervals, with PBS. The cells were incubated for 30 minutes with mouse anti-vimentin (Boehringer Mannheim, Laval Quebec) diluted 1/3 in PBS, washed three times at five minute intervals with PBS, and incubated 30 minutes with goat anti-mouse linked to FITC (Biosource International, Camarillo, TX) diluted 1/100 in PBS. All antibody incubations were performed at room temperature. The cells were mounted onto slides with n-propylgalate mounting medium. The human endothelial cells and human fibroblast cells served as controls for the observation of vimentin architecture. All slides were viewed using an Olympus 3MAX microscope linked to either a color or monochrome SONY charged coupled device (CCD) camera which displayed the images on a computer screen. Final magnification was 200X for tissue sections and 400X for cell lines. Image capturing was achieved using the Northern Exposure image analysis software (Empix Imaging Inc,Mississauga, Ont).

PCR screening for cell-associated HHV8.

Cells from which DNA was to be extracted were allowed to proliferate to confluence. The cells were harvested, washed in PBS, and the pellet stored at -80°C overnight. The pellet was thawed, resuspended in digestion buffer (100 mM NaCl, 10 mM Tris-Cl pH 8, 25 mM EDTA pH8, 0.5% SDS, and 0.1 mg/mL of proteinase K), and incubated overnight at 56°C. The DNA was extracted with phenol/chloroform/isoamyl alcohol until a clear aqueous phase was achieved. The extracted DNA was precipitated with 7.5 M ammonium acetate solution and 100% cold ethanol. The precipitated DNA was pelleted, rinsed with 70% ethanol and allowed to air dry. The dried pellet was resuspended in TE buffer and the DNA quantitated by spectroscopy. DNA extracted from the BCBL-1 cell line served as the positive control for the PCR. All DNAs were adjusted to a concentration of 10 ug/mL.

The PCR was performed using the PCR CORE KIT (Boehringer Mannheim, Indianapolis IN). The cycling conditions for PCR analysis were as follows: 94°C for 2 minutes (1 cycle); 94°C for 1 minute, 58°C for 1 minute, 72°C for 1 minute (35 cycles); 72°C extension for 5 minutes (1 cycle); 4°C soak. Each PCR reaction contained 0.1 ug of genomic DNA, 1 uM of each primer, 0.2 mM of dNTP mix, 1U of Taq DNA polymerase, 10 mM Tris/HCl, 1.5 mM $MgCl_2$, 50 mM KCl, in a final volume of 50 uL. Amplifications were carried out in a Perkin-Elmer Cetus Thermocycler. Amplicons were visualized on 2% agarose gels (Bio Rad). The sequence of the KSHV/HHV8 primers were as follows: 5'-AGC CGA AAG GAT TCC ACC AT-3' and 5'-TCC GTG TTG TCT ACG TCC AG-

3'. The primers were synthesized at the Biotechnology Research Institute, University of Ottawa, Ottawa, Ont.

Samples that did not display amplicons following one round of PCR were exposed to a second round of PCR. Two uL from the first reaction was combined with 8 uL of ddH_2O and placed in the reaction mixture as previously described.

***In vitro* chemotherapy sensitivity assay.**

Cells were harvested from confluent cultures and seeded onto 24-well plates at $1x10^4$ cells per well. The KS cells were seeded onto 24 well plates coated with gelatin and fibronectin. The endothelial and fibroblast cells were seeded directly onto 24 well plates. The cells were allowed to adhere overnight, washed three times with their appropriate serum-free medium, and exposed to chemotherapeutic agents.

Cells were exposed to different concentrations of Doxorubicin (Rhone-Poulenc Pharma, Montreal, PQ) and Etoposide (Bristol Laboratories, Montreal, PQ) for a period of 24 hrs. The cells were washed three times with serum-free medium and re-incubated for 72 hrs in fresh medium. Five hundred uL of the supernatant were removed and 500 uL of regular medium containing 1 uCi of H^3 thymidine were added to each well and the cells pulsed overnight.

Cells were exposed to different concentrations of ALX40-4C (Allelix Biopharmaceuticals Inc., Mississauga, Ont) initially for a period of 24 hrs, as above, and subsequently for a period of 72 hrs. Five hundred uL of the supernatant from each KS cell well were collected and stored for cytokine analysis and 500 uL of medium containing 1

uCi of H^3 thymidine were added. Five hundred uL of the supernatant was removed from each of the fibroblast and endothelial cell wells, and 500 uL of regular medium containing 1 uCi of H^3 thymidine were added

All cells were pulsed overnight. Cells were washed with serum-free medium and lifted using either 1X trypsin (Sigma) for the endothelial and fibroblast cells or IMDM/30% Dispase (Collaborative Biomedical) for the KS cells. All plates were harvested, using a Skatron cell harvester, onto filters that were allowed to dry overnight. The filters were then assessed for radioactivity using a liquid scintillation beta counter (LKB Wallac, Turku 10, Finland).

The results of the drug treatment assays were normalized and reported as % inhibition. Percent inhibition was calculated as (1-(the proliferation in the presence of drug/the proliferation in the absence of drug)) x100.

Cytokine measurements.

The supernatants from 70-80 % confluent KS culture flasks and the ALX40-4C drug assay plates were collected and stored at -80°C. When a number of samples had been collected, the supernatants were thawed and assayed for the presence of three cytokines; IL-1β, IL-6, and bFGF. Cytokine measurements were performed using the Quantikine ELISA Kits from R&D systems (R&D Systems, Minneapolis, MN). The assays were performed as per the manufacturer's directions.

Statistical analysis

Statistical analysis of raw data collected following exposure of cells to ALX40-4C for 72 hrs was performed using a repeated measures analysis of variance followed by the Dunnett's test for multiple comparisons (Glantz 1997)

Calculation of inhibitory concentration 50% (IC_{50})

The IC_{50} following exposure of cells to ALX40-4C for 72 hrs was calculated using the Spearman - Karber equation, which states that the $-LogIC_{50}$ = [-Log of the highest concentration]-[-(the sum of the % inhibitions/100 - 0.5) * Log of the dilution factor] (Hubert 1984).

Results

Establishment of long-term Kaposi's sarcoma cell lines.

Primary KS cell cultures were established from punch biopsies of cutaneous lesions taken from individuals affected by AIDS associated KS. The cultures presented as a mixed population of cells from which an outgrowth of adhered spindle shaped cells was observed. This outgrowth of cells occurred within one to two weeks of initial culturing. The remaining cells in suspension were removed and the spindle shaped cells were allowed to proliferate to 70-80% confluence.

In vitro characteristics.

KS4 cells:

The KS4 cultures proliferated as a uniform population of adherent spindle shaped cells with minimal vacuolation. Cell proliferation was relatively aggressive with cultures reaching confluence usually within one to two weeks following passage. The cells lacked contact inhibition and, if left to proliferate, would form multiple layers and sometimes foci. When the cells were seeded onto cell culture flasks, they grew in a disorganized fashion. As the cultures reached confluence, the cells would begin to arrange themselves into whorl-like patterns.

As the passage number increased, the morphological and proliferative aspects of the cultures deteriorated consistently. With each few passages the cells increased dramatically in size and in extent of vacuolation. The rate of proliferation diminished until the cultures appeared to be static by passage 12 to 14.

KS1 cells:

The KS1 cells, when originally isolated, looked very much like KS4 cells. However, since their isolation they seem to have undergone a transformation. The KS1 cultures consisted of both adherent and suspended cell populations. The adherent population consisted of cells with circular and spindle shaped morphology, both with smooth surfaces and no vacuolation. The suspended population consisted of spherical cells with a smooth appearance.

Cultures that were freshly plated consisted predominantly of adherent cells, but as the cultures proliferated a transition occurred and the suspended population would first equal and eventually surpass the adherent population, in number. The viability of the suspended population was assessed by trypan blue exclusion. The proportion of living cells was comparable to that of the adherent population. When cells in suspension were collected and re-plated, they gave rise to both populations.

The proliferation of the cultures was very aggressive and, unlike the KS4 cultures, remained constant over time. Confluence was reached well within one week following passage and was determined by assessing the cultures as a whole, since the cultures were represented by two populations. Based on their aggressive proliferation, the KS1 cultures were grown in the absence of the supplemented factors found in the KSGM, and their proliferation observed. The proliferation of the KS1 cultures was not affected by the absence of supplemented growth factors.

Table 1 displays a summary of the culture characteristics for the KS1 and KS4 cell lines.

Table 1. A summary of *in vitro* cellular characteristics as assessed by light microscopy of viable cell cultures. Cultures were grown on coated T-75 cell culture flasks and maintained with KSGM.

Table 1. *In vitro* cellular characteristics

	Cell type	
	KS4	**KS1**
Morphology	Spindle	Spindle/circular
Vacuolation	Yes	No
Adherence	Yes	Yes/No
Proliferation	Aggressive	Very aggressive
Contact inhibition	No	No
Focus formation	Yes	No
KSGM requirement	Yes	No
Finite # of passages	Yes	No

Immunohistochemical staining.

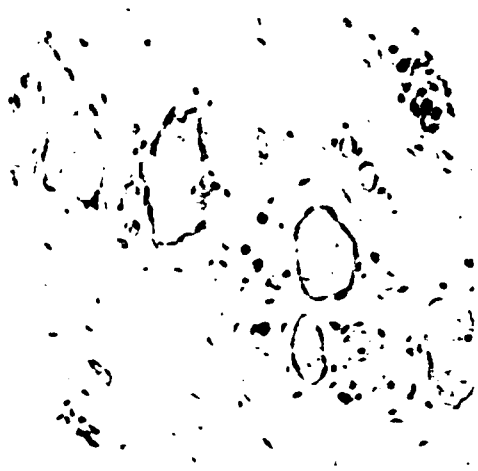
One of the major issues in the study of Kaposi's Sarcoma is the identification of the cell of origin. Immunohistochemical staining for three endothelial cell markers; Factor VIII (FVIII), CD34, and Ulex Europaeus Agglutinin Type 1(UEA-1), was performed to address the possible lineage of the KS cell lines.

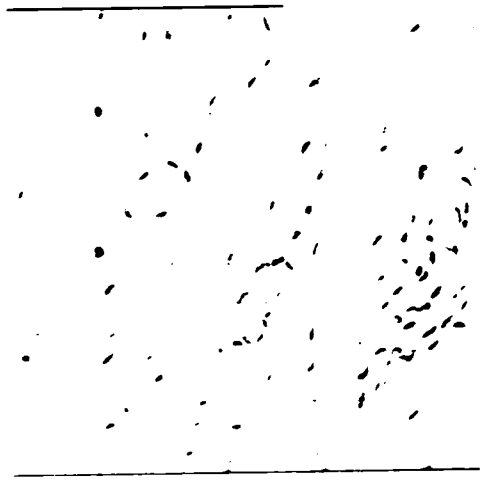
Using normal human tonsils as a control, it was demonstrated that the FVIII antibody stained the vascular endothelium specifically (Figure 2, a). Both KS cell lines demonstrated positive staining for FVIII (Figure 2, c and e). The CD34 antibody was also shown to stain the vascular endothelium specifically, using normal human tonsils as a control (Figure 3, a). However, neither of the KS cell lines stained with the CD34 antibody (Figure 3, c and e). I demonstrated that UEA-1 was specific for endothelial cells, using the a human endothelial cell line and a human fibroblasts as controls (Figure 4, b and d). The KS cells did not stain for UEA-1 (Figure 4, f and h).

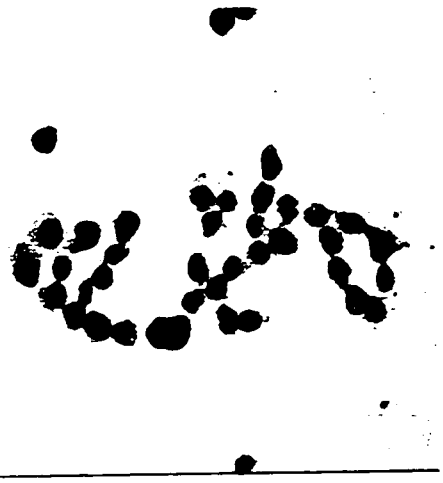
Along with identifying the cell of origin for KS there was also interest in confirming the possible association of a novel herpesvirus with KS. Prior to the development of an HHV8 specific PCR protocol, the KS cell lines and the endothelial and fibroblast cell lines were stained for the presence and organization of vimentin.

Infection with animal viruses often leads to the re-organization vimentin architecture (Liebowitz and Kieff 1989, Ferreira et al. 1994, Weclewicz et al. 1994). All cell types stained positive for vimentin (Figure 5) and each cell type expressed a uniform pattern of vimentin expression that was consistent with the literature (Ferreira et al. 1994,

Figure 2. Immunohistochemical staining for Factor VIII related antigen. The KS cell lines were grown on glass coverslips and fixed with 100% cold acetone. Formalin fixed normal human tonsilar tissue (a) was used as a positive control for antibody reactivity and specificity towards endothelial cells. Both the KS1 (c) and the KS4 cell lines (e) demonstrated positive cytoplasmic staining for Factor VIII related antigen. Plates b, d, and f represent incubations in the absence of FVIII specific antibody.







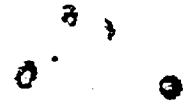
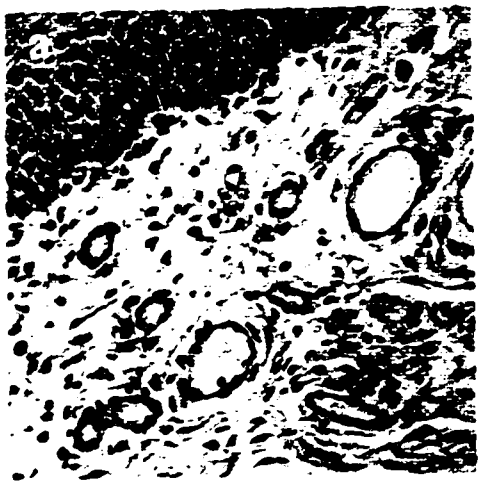


Figure 3. Immunohistochemical staining for CD34 antigen. The KS cell lines were grown on glass coverslips and fixed with 100% cold acetone. Formalin fixed Normal human tonsilar tissue (a) was used as a positive control for antibody reactivity and specificity towards endothelial cells. Neither the KS1 (c) nor the KS4 cell lines (e) demonstrated positive staining for CD34 antigen. Plates b, d, and f represent incubations in the absence of CD34 specific antibody.



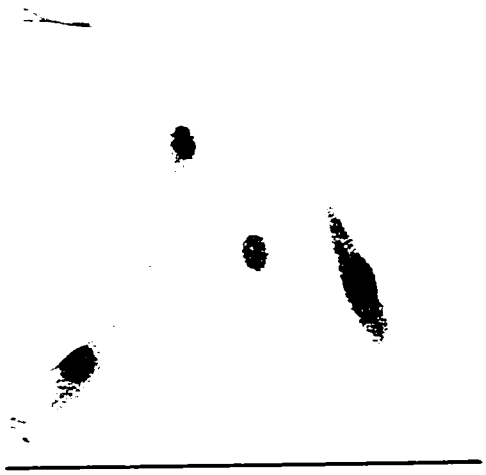


Figure 4. Immunohistochemical staining for UEA-1 antigen. The cell lines were grown on glass coverslips and fixed with 100% cold acetone. A human endothelial cell line (a and b) and a human fibroblast cell line (c and d) were used as positive and negative controls respectively. Neither the KS1 (e and f) nor the KS4 cell lines (g and h) stained positive for UEA-1 antigen. Plates a, c, e, and g are phase contrast images of the cells.

b

d

f

h

Figure 5. Immunohistochemical staining for vimentin. The cell lines were grown on glass coverslips and fixed with 100% cold acetone. A human endothelial cell line (a and b) and a human fibroblast cell line (c and d) were used for the purpose of comparison. Both the KS1 (e and f) and KS4 cell lines (g and h) stained positive for the presence of vimentin filaments. When compare the control cell lines, no apparent re-organization of vimentin filament architecture was observed. Plates a, c, e, and g are phase contrast images of the cells.

h
d
l
h

Weclewicz et al. 1994). The vimentin filaments originate from the peri-nuclear space and extend uniformly throughout the cell with no apparent re-organization or localization.

PCR-based detection of HHV8.

The possible association of HHV8 with the KS cell lines was assessed by PCR. The detection of a 233 bp fragment, that has been identified in the literature as an HHV8 specific sequence, was assayed (Chang et al 1994). The 233 bp fragment was amplified from 100 ng of positive control DNA that was isolated from the BCBL-1 cell line (Figure 6). This cell line was reported to contain episomal genomes of HHV8 (Renne et al. 1996). The 233 bp fragment was not detected in the genomic DNA that was isolated from the KS cell lines (Figure 6).

The sensitivity of the PCR protocol was assessed by diluting the BCBL-1 DNA. Amplification was achieved in as little as 0.01 ng of BCBL-1 DNA (Figure 7, lane 4). The 233 bp fragment was also not detected in the KS cell lines following a second successive round of PCR amplification (Figure 7, lanes 6 and 7).

Evaluation of cytokines associated with the KS cell lines.

A factor that has been suggested to play an important role in KS pathogenesis is the ability of KS cells to produce and release many cytokines which may act in an autocrine fashion (Ensoli et al. 1989, Ensoli et al. 1992). The KS cell lines were assayed for the presence of

Figure 6. PCR screening for HHV8 DNA. 100 ng of template DNA was screened using HHV8 specific primers in a 50 uL total reaction volume. Lane 1 BCBL-1 cell DNA, lane 2 KS4 p11, lane 3 KS4 p4, lane 4 KS1, lane 5 KS5 p1, lane 6 template negative control. A 100 bp ladder was use for the estimation of amplicon size. HHV8 was not detected in the KS cell line samples.

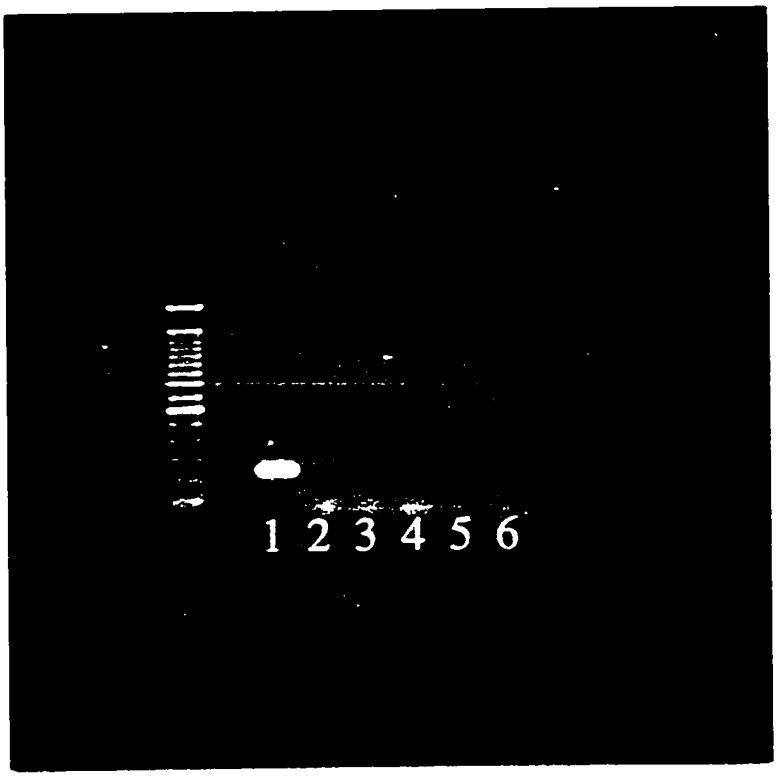
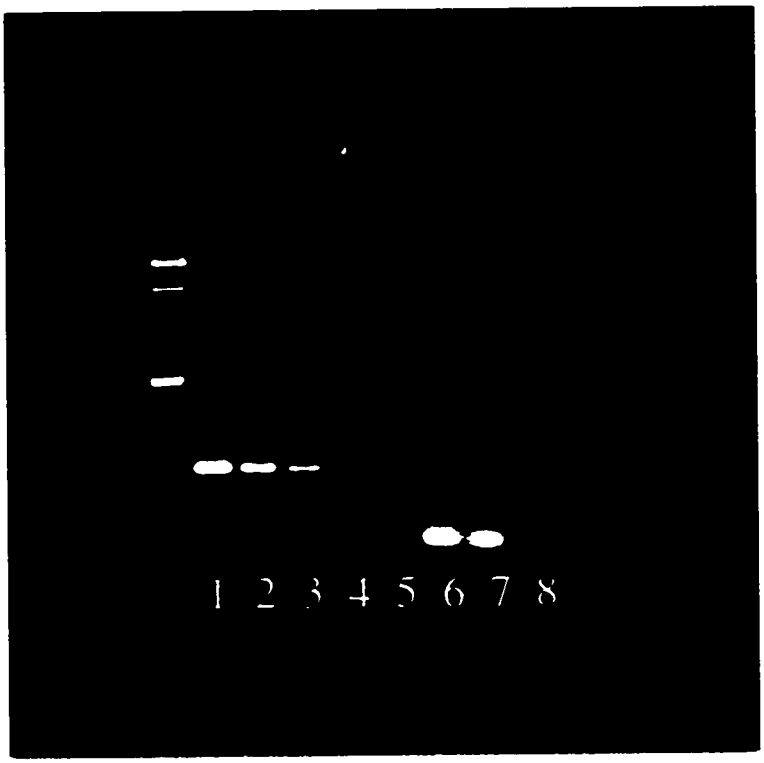

1 2 3 4 5 6

Figure 7. Sensitivity assessment of the PCR assay. To determine the sensitivity of the HHV8 PCR, BCBL-1 DNA was diluted. Products from the primary PCR were re-amplified a second time. Lane 1 100 ng of BCBL-1 DNA, lane 2 1 ng of BCBL-1 DNA, lane 3 0.1 ng of BCBL-1 DNA, lane 4 0.01 ng of BCBL-1 DNA, lane 5 0.001 ng of BCBL-1 DNA, lane 6 KS4 p1 (PCR product), lane 7 KS5 p1 (PCR product), lane 8 template negative control. A 100 bp ladder was use for the estimation of amplicon size.


1 2 3 4 5 6 7 8

cytokines using commercial ELISA kits for the quantitation of cytokines. IL-1β, IL-6, and bFGF were the cytokines chosen for measurement, based on their suspected importance in KS development as autocrine growth factors (Ensoli et al. 1989, Ensoli et al. 1992).

IL-6 and bFGF were detected in both the KS4 and KS1 cultures, but IL-1β was not detected in either of the KS cultures (Table 2).

As a control, KSGM alone was assessed for the presence of each cytokine. bFGF was listed as one of the possible components of ECGS and was present in the KSGM medium alone (Table 2). Subsequently, the KS4 cells were grown in the absence of ECGS and assessed for the presence of bFGF. bFGF was still present in the culture supernatant. (Table 2). The KS1 cells were not assessed for the presence of bFGF in the absence of ECGS since they displayed a lack of requirement for the supplements present in KSGM.

Response of KS cell lines to chemotherapeutic agents.

The treatment of KS *in vivo* with chemotherapeutic agents has yielded varying results. Tumor regression is often transient and associated with detrimental side effects. The effect of a potential therapeutic agent, the antiviral ALX40-4C, was compared to the effect of two standard chemotherapeutics already in use, Doxorubicin and Etoposide, in an effort to address how these compounds may affect the proliferation of KS cells.

A 24-hr-exposure to varying doses of Doxorubicin resulted in dose-dependent inhibition of cell proliferation in all the cell types tested, as measured by H3-thymidine

incorporation (Figure 8). A 24-hr-exposure to varying doses of Etoposide inhibited proliferation of all cell cultures by 90% or greater (Figure 9).

A 24-hr-exposure to varying doses of ALX40-4C resulted in no observable inhibition of proliferation and subsequently the exposure time was extended to 72 hrs. A dose-dependent inhibition of proliferation, restricted to KS cells, was observed (Figure 10).

Cytotoxicity was observed with each of the cell types at 100 uM of ALX40-4C, as assessed by light microscopy. KS proliferation was inhibited at lower doses of ALX40-4C in the absence of cytotoxicity, as assessed by trypan blue exclusion. The IC50, as calculated using the Spearman-Karber method, was 5.4 uM for the KS4 cell line, 47.1 for the KS1 cell line, and 70 uM for both the endothelial and fibroblast cell lines.

Supernatants from the KS cultures exposed to ALX40-4C were collected and assayed for IL-6 and bFGF to determine if drug exposure affected the cytokine levels associated with the KS cells. bFGF production displayed a dose-dependent inhibition in response to ALX40-4C that corresponded to the inhibition of proliferation (Figure 11), but for IL-6 no observable trend was observed (Figure 12).

As stated above, in the absence of ECGS the KS4 cells were capable of producing bFGF. When these cells were exposed to varying concentrations of ALX40-4C, the dose-dependent decrease of culture associated bFGF was maintained (Figure 13). In addition, it was observed that the cells grown in the absence of ECGS appeared to be more sensitive to ALX40-4C as indicated by a shift in the inhibition curve (Figure 14).

Table 2. A summary of cytokine measurements by ELISA on confluent cultures. Cells were cultured in T-75 tissue culture flasks and were allowed to achieve 70 to 80 % confluence. Supernatants were harvested and assayed for cytokines with commercial ELISA kits. The data represents an average of two measurements. ND refers to "not determined".

Table 2. Cytokine measurement of 70-80% confluent cell cultures

Cell Line	IL-1β (pg/mL)	IL6 (pg/mL)	bFGF (pg/mL)
Endothelial	ND	>300	1.28
Fibroblast	ND	0.10	0.00
KS1	0.00	2.81	25.16
KS4 with ECGS	0.00	164.31	35.10
KS4 without ECGS	ND	ND	5.94
KSGM with ECGS	0.00	0.00	40.00
KSGM without ECGS	ND	ND	0.79

Figure 8. A representitive example of the effects of Doxorubicin on cell proliferation. Cells were seeded onto 24 well tissue culture plates at a concentration of 10E4 cells per well and allowed to adhere overnight. The cells were washed and subsequently exposed for 24 hrs to Doxorubicin, in triplicate, ranging from 0 to 100 ng/mL. The cells were washed three times and re-incubated for 72 hrs with regular medium in the absence of Doxorubicin. 500 uL of supernatant were removed and replaced with 500 uL of medium containing 1 uCi of tritiated thymidine and the cells pulsed overnight. The cells were harvested onto filters and assessed for thymidine incorporation.

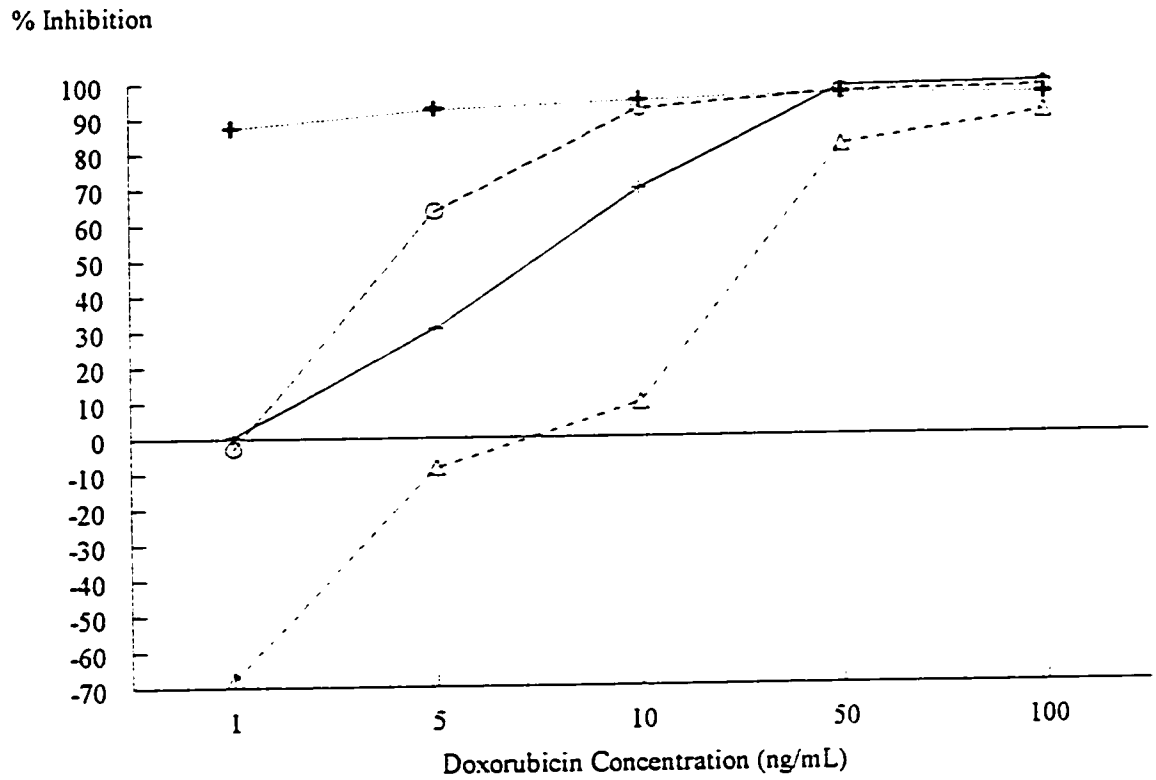
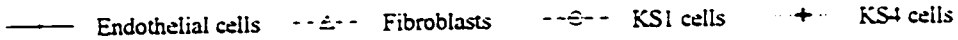

% Inhibition
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
1
5
10
50
100
Doxorubicin Concentration (ng/mL)

Figure 9. A representitive example of the effects of Etoposide on cell proliferation. Cells were seeded onto 24 well tissue culture plates at a concentration of 10E4 cells per well and allowed to adhere overnight. The cells were washed and subsequently exposed for 24 hrs to Etoposide, in triplicate, ranging from 0 to 100 ug/mL. The cells were washed three times and re-incubated for 72 hrs with regular medium in the absence of Etoposide. 500 uL of supernatant were removed and replaced with 500 uL of medium containing 1 uCi of tritiated thymidine and the cells pulsed overnight. The cells were harvested onto filters and assessed for thymidine incorporation.



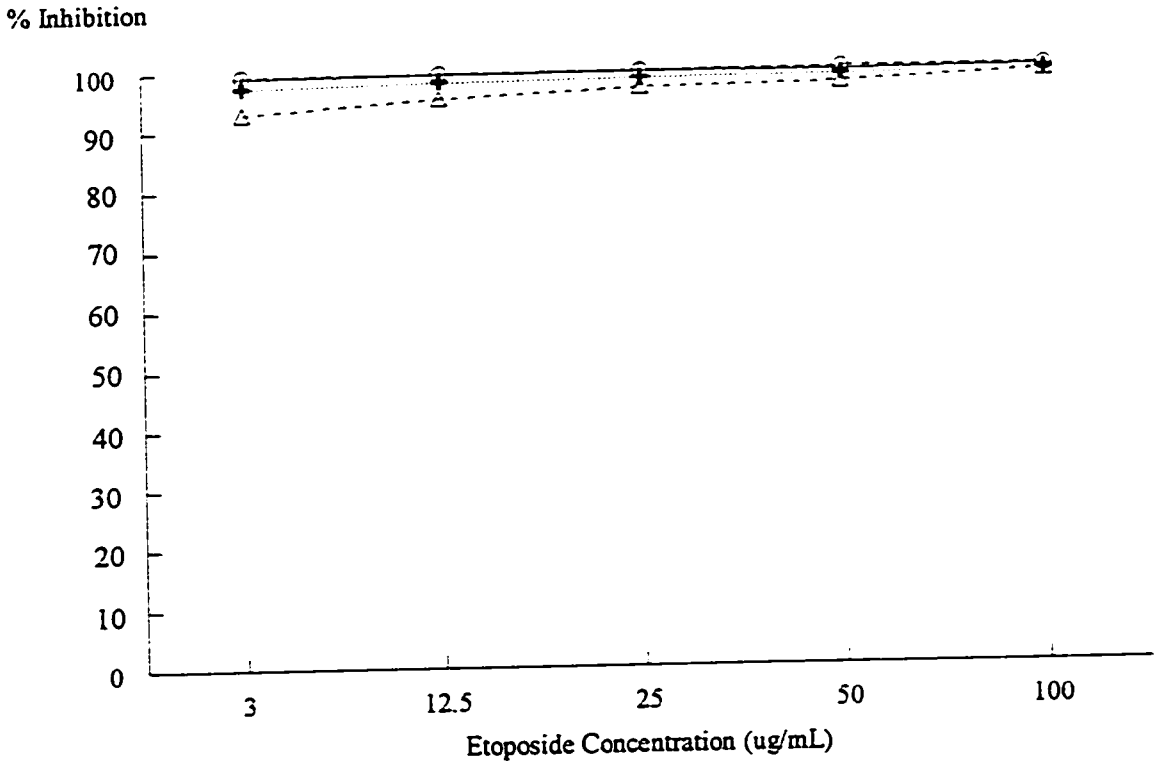
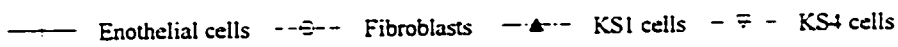

% Inhibition
100
90
80
70
60
50
40
30
20
10
0
3
12.5
25
50
100
Etoposide Concentration (ug/mL)

Figure 10. Effects of ALX40-4C on cell proliferation. Cells were seeded onto 24 well tissue culture plates at a concentration of 10E4 cells per well and allowed to adhere overnight. The cells were washed and subsequently exposed for 72 hrs to ALX40-4C, in triplicate, ranging from 0 to 100 uM. 500 uL of supernatant were collected and stored for cytokine analysis. The removed 500 uL was replaced with 500 uL of medium containing 1 uCi of tritiated thymidine and the cells pulsed overnight. The cells were harvested onto filters and assessed for thymidine incorporation. The data represent an n of 5 for the KS4 cell line and an n of 4 for the KS1, fibroblast, and endothelial cell lines. $p<0.05$ for the drug concentration of 100uM with respect to all cell lines.



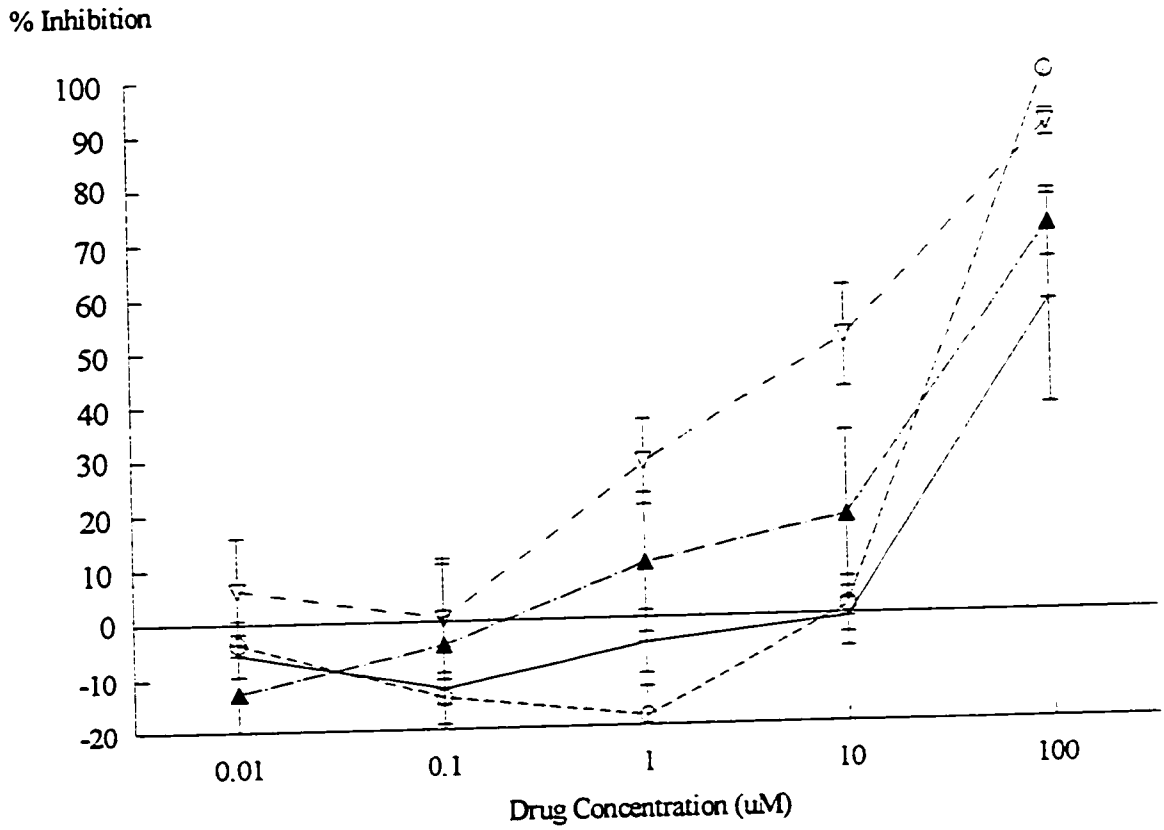
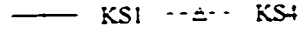

% Inhibition
100
90
80
70
60
50
40
30
20
10
0
-10
-20
0.01
0.1
1
10
100
Drug Concentration (uM)

Figure 11. A representitive example of bFGF produced by KS cultures exposed to ALX40-4C. Cells were seeded onto 24 well tissue culture plates at a concentration of 10E4 cells per well and allowed to adhere overnight. The cells were washed and subsequently exposed for 72 hrs to ALX40-4C, in triplicate, ranging from 0 to 100 uM. 500 uL of supernatant were collected and stored at -80 °C for cytokine analysis. The supernatants were thawed and assayed for bFGF using a commercially available cytokine detection ELISA.



bFGF concentration (pg/ml)

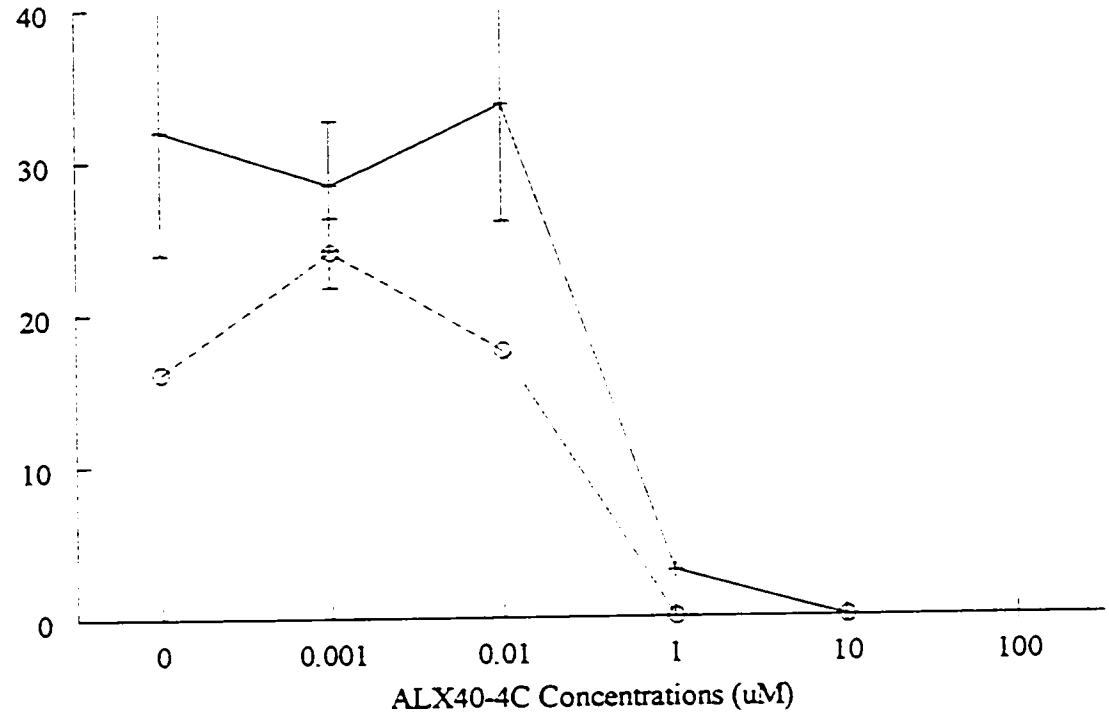
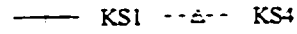


Figure 12. A representitive example of IL-6 produced by KS cultures exposed to ALX40-4C. The experiment was performed as described in the figure 11 caption.



IL-6 concentration (pg/mL)

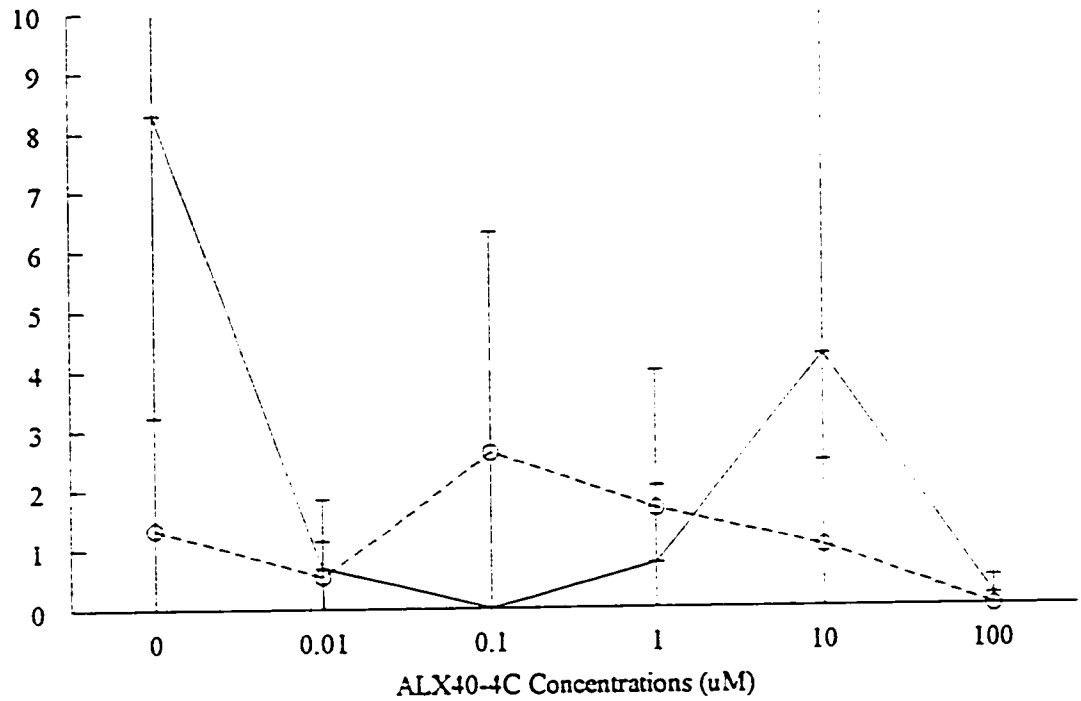


Figure 13. bFGF produced by KS4 cultures exposed to ALX40-4C in the absence of ECGS. The experiment was performed as described in the figure 11 caption.

—— without ECGS - - -▲- - - with ECGS

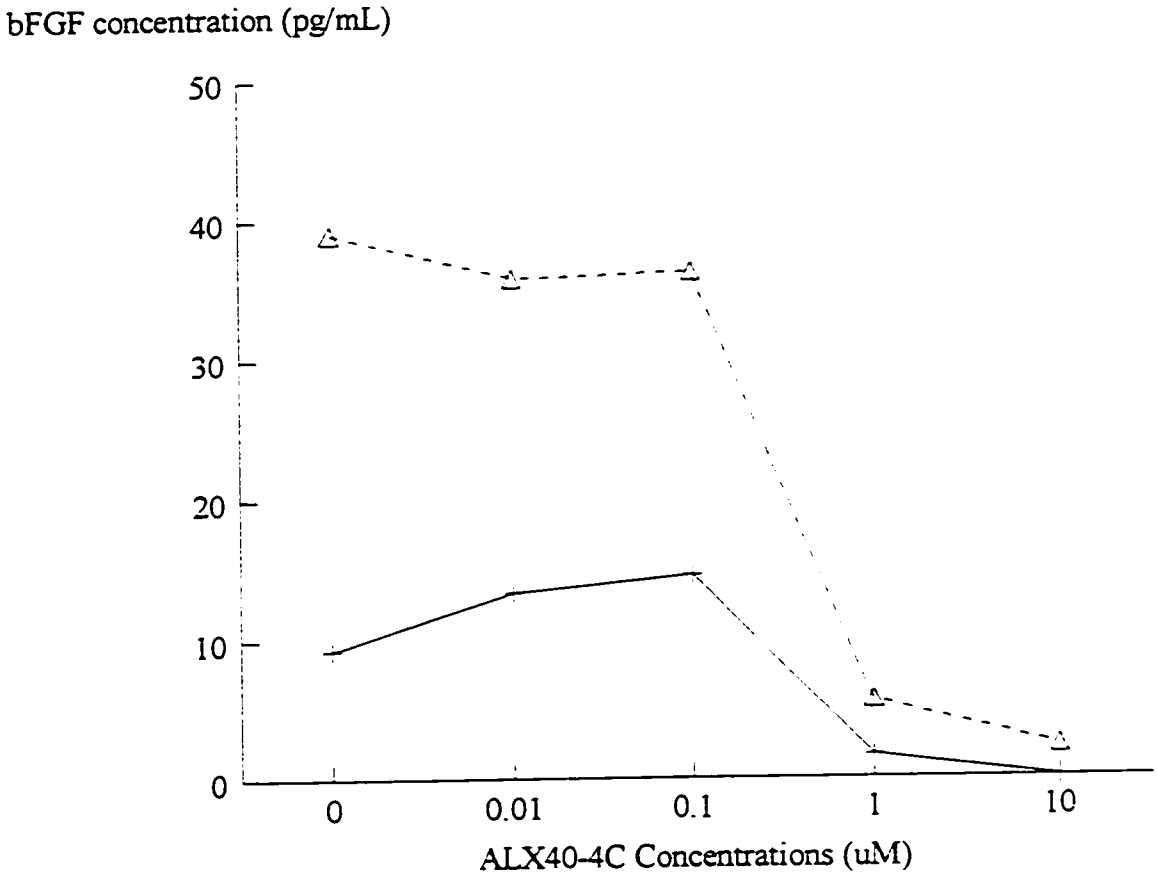
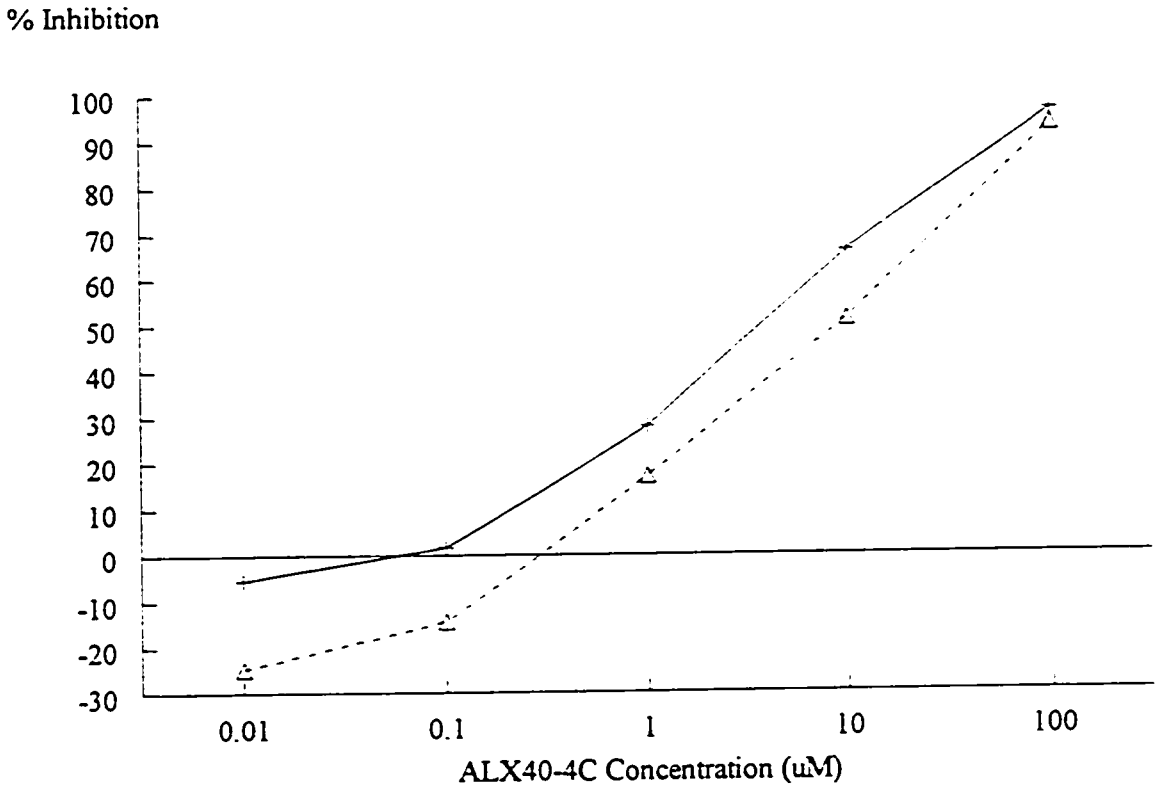

bFGF concentration (pg/mL)
50
40
30
20
10
0
0
0.01
0.1
1
10
ALX40-4C Concentrations (uM)

Figure 14. Inhibition of KS4 cell proliferation by ALX40-4C, in the absence of ECGS. KS4 cells, maintained in the presence or absence of ECGS, were seeded onto 24 well tissue culture plates at a concentration of 10E4 cells per well and allowed to adhere overnight. The cells were washed and subsequently exposed for 72 hrs to ALX40-4C, in triplicate, ranging from 0 to 100 uM. 500 uL of supernatant were collected and stored for cytokine analysis. The removed 500 uL was replaced with 500 uL of medium containing 1 uCi of tritiated thymidine and the cells pulsed overnight. The cells were harvested onto filters and assessed for thymidine incorporation.

——— without ECGS - - -▲- - - with ECGS


% Inhibition
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
0.01
0.1
1
10
100
ALX40-4C Concentration (uM)

Discussion

KS is a multifocal proliferative disorder that is commonly observed in individuals who suffer some degree of immune suppression. There is evidence to suggest that the proliferation of KS is mediated in part by an autocrine growth mechanism. KS cells *in vitro* have demonstrated an ability to produce and respond to a number of cytokines (IL-1β, IL-6, GM-CSF, TGF-β, PDGF, and bFGF) of which bFGF, IL-1β, and IL-6 appear to be the most abundant (Ensoli et al. 1989, Miles et al. 1990, Ensoli et al. 1992, Yang et al. 1994, Samaniego et al. 1995). Interference with this autocrine growth mechanism may be a possible method of inhibiting the proliferation of KS.

The objectives of this thesis included the establishment and characterization of two KS cells lines and the evaluation of chemotherapeutic agents on the proliferation of these cell lines. In addition, the presence of HHV8 was assessed in both KS cell lines due to its suggested role in the development of KS.

Establishment and maintenance of KS cell lines.

KS cultures are generally established from two sources, either punch biopsies of cutaneous lesions or from pleural and peritoneal fluids of individuals affected by KS (Corbeil et al. 1991, Ensoli et al. 1992, Yang et al. 1994). The two KS cell lines dealt with in this work were established from separate skin biopsies taken from separate individuals who were positive for HIV. Therefore these cells fell under the category of AIDS associated Kaposi's Sarcoma.

Initial culturing of biopsied material revealed an outgrowth of cells displaying spindle morphology. This spindle-shaped cell population is thought to be characteristic of KS. Its

existence is supported in the literature where the predominant cell type observed *in vivo* and *in vitro* is the spindle-shaped cell (Templeton 1988, Corbeil et al. 1991, Huang et al. 1993). As the initial culture progressed, non-adhered cells were gradually eliminated and uniform populations of adhered spindle-shaped cells emerged.

Generally, the maintenance of KS cells *in vitro* requires the presence of additional factors in the medium, although cell lines not requiring such factors have been described (Hermans and Clumeck 1995, Krown 1997). The KS cell lines in this work were maintained in medium that was supplemented with human transferrin, heparin, and endothelial cell growth supplement (ECGS). The use of these supplements for the maintenance of KS cell lines was previously established in the lab and is supported in the literature (Miles et al. 1990, Yang et al. 1994).

In addition or as an alternative to ECGS some groups supplement the KS cell growth medium with medium taken from human T cell leukemia virus (HTLV) infected T cell cultures (Nakamura et al. 1988, Miles et al. 1990, Ensoli et al. 1992, Samaniego et al. 1995). This medium from HTLV-infected cultures contains many cytokines (TNF-α and β, INF-γ, IL-1α and β, GM-CSF, and oncostatin M) that KS cells have been demonstrated to respond to (Samaniego et al. 1995). The addition of the HTLV-infected T cell conditioned medium to the culture environment may be more representative of the *in vivo* environment where many inflammatory cytokines are present. This may account for the differences observed between the groups that use this conditioned medium and those that do not (Bailer et al. 1995).

Morphological and proliferative characteristics.

The KS4 cell line presented as the classical example of an *in vitro* KS cell line (Corbeil et al. 1991). The cultures began as a population of smooth spindle-shaped cells with a strong proliferative capacity. But with time, the cells deteriorated to become large, heavily vacuolated cells with little proliferative potential. The cultures usually reached a static point after 12 to 14 passages, which is consistent with what has been reported in the literature (Corbeil et al. 1991). This gradual decline in culture stability underlines two key points about KS cell biology; the cells do not appear to be truly transformed and in the absence of the complex *in vivo* environment hyper-proliferation cannot be maintained.

The KS1 cells, when initially isolated, appeared very much like the KS4 cells. However, with continued culturing established characteristics were different becoming a mixture of highly proliferative adherent and non-adherent cells. The adherent cells presented predominantly as smooth spindle cells which gave rise to a cell population in suspension. Cells isolated from suspension were equally capable of giving rise to an adhered population. These cultures did not deteriorate over time and maintained a very aggressive rate of proliferation.

This highly proliferative cell line may have arisen from a spontaneous transformation within the culture, or is simply an outgrowth of a specific population of cells from the original biopsy. If the latter scenario is indeed the case, then this population is consistent with the theory of malignant potential for KS. Some established KS cell lines with abnormal karyotypes have demonstrated the ability to proliferate in the absence of exogenous growth factors, and the potential to cause metastatic lesions in nude mice (Schultz and Weiss 1995, Hermans and Clumeck 1995). The KS1 cell line did demonstrate the potential to proliferate in medium that

did not contain the supplemented growth factors of KSGM, suggesting that the requirement for growth factors was not as critical for this cell line. However, neither the cell karyotype nor the potential for these cells to cause metastatic lesions in mice was assessed in this study.

Although the two cell lines that were established in this work appear to be very different based on morphological and proliferative characteristics, each of their existence is supported by literature (Corbeil et al. 1991, Schultz and Weiss 1995, Hermans and Clumeck 1995). The non-transformed KS is the general form of KS observed in patients who suffer from disease. This form of the disease supports the idea that KS does not begin as a clonal neoplasm but more as a mixed population of proliferating cell types that leads to an over representation of spindle-shaped cells. However, the transformed type of KS demonstrates that this disease may have the capability of become neoplastic in nature.

Immunohistochemical staining for cellular markers

An important issue in the biology of KS that remains to be resolved is the identification of the cell or tissue of origin that leads to lesion development. There has been much controversy over this issue in the literature, with different groups debating which markers are present on which populations of cells.

KS has been characterized by many investigators as a vascular tumor which stains positive for endothelial markers such as Factor VIII-related antigen, CD34, Ulex Europaeus Agglutinin Type 1 (UEA-1) and vimentin (Guarda et al. 1981, Nadji et al. 1981, Flotte et al. 1984, Hashimoto et al. 1987, Sankey et al. 1990, Nickoloff 1991, Gray et al. 1991, Corbeil et al. 1991, Taylor and Cote 1994, Jones et al. 1995). Based on these markers the prediction is

that the tissue of origin for KS is the vascular endothelium. However, other investigators have observed slightly different results where FVIII is absent among the endothelial markers, and they suggest that the lymphatic endothelium is the more likely tissue of origin for KS (Roth et al. 1988, Beckstead et al. 1985). Still others suggest that KS arises from myeloid precursors, based on staining for such markers as Factor XIII and CD14 (Huang et al. 1993, Uccini et al. 1997). Finally, one group of investigators suggested that all forms of KS lesions contain spindle-shaped cells which are made up of a mixed population containing cells of myeloid and fibro-endothelial lineages (Kaaya et al. 1995). They also noted that when cultred, only the fibro-endothelial-like cells were present.

The presence of FVIII, CD34, and UEA-1 was assayed for in the KS cell lines. These markers were chosen based on the balance of evidence in the literature. I demonstrated that the two KS cell lines were positive for Factor VIII but negative for both CD34 and UEA-1. The absence of CD34 and UEA-1 staining in the KS cell lines was puzzling, considering the presence of FVIII would seem to predict their existence. Given the varied detection of cellular markers described above it is possible that the two KS cell lines simply do not express either CD34 or UEA-1. These KS cell lines may have lost the ability to express certain markers as a result of either differentiation or *in vitro* culturing (Green et al. 1988, Kaaya et al. 1995). Based on the positive result for Factor VIII staining, it can be concluded that these KS cells appear to be vascular endothelial in origin.

Screening for the association of HHV8 with the KS cell lines.

Although vimentin has been used as a marker for KS *in vivo*, it's use as an *in vitro* marker was not possible due to the fact that vimentin is expressed by virtually all cells when placed into culture (Colucci-Guyon et al. 1994). I did, however, employ another potential use for vimentin as an *in vitro* marker. Vimentin belongs to the family of intermediate filaments, which make up one of the three major cytoskeletal systems in the eukaryotic cell (Colucci-Guyon et al. 1994). The involvement of the cytoskeletal and nuclear matrix during animal virus infection has been well documented (Ciampor 1988). Some viral infections, including EBV (an HHV8 related γ herpesvirus), have shown to dramatically alter the expression of vimentin in infected cells (Liebowitz and Kieff 1989, Ferreira et al. 1994, Weclewicz et al. 1994). Normally, vimentin filaments originate from the peri-nuclear space and extended uniformly throughout the cell (Ferreira et al. 1994, Weclewicz et al. 1994). With some viral infections, this uniform expression is transformed to a disorganized expression with irregular distribution throughout the cytoplasm (Liebowitz and Kieff 1989, Ferreira et al. 1994, Weclewicz et al. 1994)

During the period of research for this thesis, evidence that HHV8 was associated with the cells of KS lesions was accumulating. Using PCR technology, an HHV8 specific marker sequence was identified in all forms of KS tissue (Moor and Chang 1995, Dupin et al. 1995, Huang et al. 1995, Whitby et al. 1995). In the absence of an established PCR assay, the expression of vimentin was observed in the KS cell lines to determine if there was a possible viral association. No apparent re-organization or localization could be observed, which suggests that either active viral replication may be occurring in the absence of vimentin re-

organization or that there is a lack of active replication due to viral latency, or finally that viral infection is absent in these cells.

The possible association of HHV8 with the KS cell lines was re-addressed using a PCR based assay (Chang et al. 1994). Investigators had been using KS and non-KS tissue samples to standardize their assays. The initial attempts at detecting HHV8 by PCR were inconclusive due to the absence of a positive control for the presence of HHV8.

Subsequently, the cell line BCBL-1 was made available to the public through the NIH AIDS Research and Reference Reagent Program by the group of McGrath and Ganem. This is a body cavity lymphoma cell line that was isolated from an individual positive for HIV infection; the cell line has been demonstrated to be latently infected with HHV8 (Renne et al. 1996).

Using BCBL-1 DNA as a positive control for the HHV8 specific primers the two KS cell lines were screened for amplification of the 233 bp marker of HHV8 (Chang et al. 1994). The 233 bp fragment could not be detected in 100 ng of the DNA from each of the KS cell lines. To test the sensitivity of the PCR assay, the BCBL-1 cell DNA was diluted to determine how little DNA was required for amplification. The 233 bp fragment was detected in as little as 0.01 ng of BCBL-1 cell DNA. Products from the initial PCR reaction were run through a second PCR reaction in an effort to amplify small amounts of amplicons that might have been present following the first round of amplification. The 233 bp fragment was not detected in the KS cell DNA following the second round of PCR amplification, suggesting the absence of HHV8 in the KS cell lines.

These results are supported by reports in the literature that following early passages of KS cells in culture, the 233 bp fragment can no longer be amplified (Aluigi et al. 1996, Offermann 1996, Foreman et al. 1997). This observation and the observation that HHV8 appears to be B cell tropic, suggests that that the spindle cells of KS may not be a permissive host cell for HHV8 or that a latently associate virus is not capable of maintaining its presence in the absence of its complex *in vivo* environment. The later possibility would lead to a gradual dilution of cells containing HHV8 by cells that do not. Another possibility is that cells infected with HHV8 do not survive for more then a few passages in culture.

The facts that HHV8 is actively present in the spindle and endothelial cells of KS lesions and is no longer present following a few passages *in vitro* may help to explain why the KS cultures become static over time. HHV8 has been shown to code for gene products (MIP, IL-6, IRF, BCL-2, G-protein coupled receptors) that could either directly or indirectly, via the induction of cellular transcription, play a role in KS pathogenesis (Russo et al. 1996). The presence of HHV8 within the cells may exert an influence on the proliferation of KS cells. The loss of HHV8 from the KS cells results in the cell reverting to its original state. For example, HHV8 codes for a functional BCL-2 like protein that is capable of inhibiting Bax-mediated apoptosis (Ruso et al. 1996). KS cell associated BCL-2 has shown to be highly expressed in tissue from AIDS-associated and Classic KS (Morris et al. 1996). A possible mechanism for the *in-vivo* sustaining of hyper proliferative KS cells is the inhibition of cellular apoptosis. Apoptosis, or programmed cell death, is a natural cellular defense mechanism that can be triggered following infection by viruses and microbes (Thompson 1995). The loss of HHV8

from the cultures would result in the decline in the presence of BCL-2 and subsequent reversion to normal apoptosis.

Measurement of Cytokines

A common thread to all forms of KS is an inflammatory infiltrate associated with the lesions (Masood et al 1993). Investigators have demonstrated that KS cells *in vitro* are very responsive to many cytokines and growth factors, and that maintenance of *in vitro* cultures is often dependent on medium that has been enriched with these factors (Nakamura et al. 1988, Miles et al. 1990, Ensoli et al. 1992,Yang et al. 1994, Samaniego et al. 1995 Hermans and Clumeck 1995, Bailer et al. 1995, Krown 1997). Given the proper environment, KS cells have been shown to be capable of producing a number of cytokines (IL-1β, IL-6, GM-CSF, TGF-β, PDGF, and bFGF) of which bFGF, IL-1β, and IL-6 appear to be the most abundant and important for cell proliferation (Ensoli et al. 1989, Miles et al. 1990, Ensoli et al. 1992, Yang et al. 1994, Samaniego et al. 1995). The suggested dependence of KS proliferation on autocrine growth factors makes these factors legitimate targets for the inhibition of KS.

The KS cell lines were assessed for their capability of producing IL-1β, IL-6, and bFGF. These three cytokines were chosen because of their suggested abundance in association with KS cells and because of their suggested importance in the proliferation of KS (Ensoli et al. 1989, Miles et al. 1990, Ensoli et al. 1992, Yang et al. 1994, Samaniego et al. 1995,). IL-6 and bFGF were detected in both cell lines, but IL-1β could not be detected in either cell line. The absence of IL-1β production is possibly attributable to environmental factors, since the KSGM was not supplemented with medium from HTLV-infected T cell cultures, as did the above

referenced investigators. As mentioned previously, this HTLV-infected T cell medium contains many cytokines (TNF-α and β, INF-γ, IL-1α and β, GM-CSF, and oncostatin M) that have been demonstrated to enhance KS proliferation (Samaniego et al. 1995).

The dependence of cytokine production on environmental factors was underlined by Bailer et al. (1995). They measured the production of cytokines by KS cells that were cultured in medium containing either ECGS alone or ECGS and HTLV-infected T cell-conditioned medium. They observed that the concentration and type of cytokines produced by KS cells that were increased when HTLV-infected T cell-conditioned medium was added to the culture medium. Therefore, it can only be concluded that the culture conditions were not optimal for the induction of IL-1β production by the two KS cell lines in this thesis. To determine if these cell lines have the capacity to produce IL-1β, they would have to be re-cultured in medium that was supplemented with HTLV-infected T cell-conditioned medium.

The importance of culture environment also became apparent in this work when it was realized that bFGF was present in the ECGS that was added to the culture medium. Culturing for one complete passage in KSGM lacking ECGS resulted in a decrease, but not an elimination, in the amount of bFGF detected in the KS4 cell cultures. The cells also displayed a deterioration, becoming larger and more vacuolated. This supports the idea that stimulation is required for KS cells to optimally produce cytokines.

With respect to the amounts of bFGF measured, the data does not represent the total amount of bFGF present in the KS4 cell cultures, since it was demonstrated that bFGF could be present in a membrane bound form in addition to what was present in the supernatant (Samaniego et al. 1995). There was no attempt to liberate the membrane bound form of bFGF

prior to harvest of the supernatant, therefore the bFGF measurements that resemble impute bFGF may be underestimated.

***In vitro* sensitivity to chemotherapeutic agents.**

Evidence suggests that the proliferation of KS is mediated in part by an autocrine growth mechanism (Ensoli et al. 1989, Miles et al. 1990, Ensoli et al. 1992, Yang et al. 1994, Samaniego et al. 1995). If such is the case, these autocrine growth factors would be legitimate targets for the inhibition of KS proliferation.

Miles et al. (1990) demonstrated that antisense oligonucleotides, directed at KS IL-6 mRNA, were effective at decreasing the proliferation of their KS cell culture by almost two thirds. This observation supports the idea of interference with autocrine growth factors as a method of inhibiting KS cell proliferation.

With this idea in mind, the effect of chemotherapeutic agents on the proliferation of the KS cell lines was assessed. An *in vitro* drug assay, previously established in the lab, was reproduced using three agents: Doxorubicin, Etoposide, and ALX40-4C. Low-dose exposure for a period of 24 hrs, to varying concentrations of Doxorubicin and Etoposide, efficiently inhibited the proliferation of both KS cell lines in a concentration dependent manner. A human endothelial cell line and a human fibroblast cell line were used as controls for these experiments due to the fact that endothelial cells and fibroblasts are frequently found in KS lesions. Both of the control cell lines were sensitive to the action of Doxorubicin and Etoposide. This is to be expected since the known mechanism of action of these compounds (the direct damage of DNA) does not allow for selectivity based on cell type, only on cell proliferative state. It is for

this reason that chemotherapeutic treatment is often associated with many side effects, the most serious of which is immune suppression. However, it had been previously demonstrated in our lab that the inhibition of proliferation associated with the low doses of these compounds occurred in the absence of cytotoxicity, as assessed by lactate dehydrogenase (LDH) release (Filion unpublished observation). This may be suggestive of an alternate mechanism involved in the inhibition of proliferation. This alternate mechanism may be related to published observations that Daunorubicin, an anthracycline relative of Doxorubicin, is able to inhibit HIV-1 replication in cells of monocyte lineage (Filion et al. 1993).

Assaying for the anti-proliferative capacity of ALX40-4C, the same exposure time of 24 hrs was initially used. No inhibition of proliferation was observed under these conditions. Therefore, the exposure time of the cell lines was extended to 72 hrs to determine if the extended presence of ALX40-4C would be effective. A concentration-dependent inhibition of KS cell proliferation which was absent in control cell lines, was subsequently observed. Apart from the highest concentration of 100 uM, inhibition of proliferation occurred in the absence of cytotoxicity, as assessed by trypan blue exclusion. Not only does this result support the anti-proliferative capacity of ALX40-4C, but it suggests that the compound may be acting preferentially on the KS cell lines.

ALX40-4C is a nine amino acid peptide made of exclusively D-arginines. ALX40-4C competitively inhibits the interaction of HIV tat protein with the TAR element of HIV mRNA. In *in vitro* studies it decreases the level of cytopathology observed with HIV infection, presumably by interfering with viral transcription (O'Brien et al. 1996). ALX40-4C was also suggested to interfere, via an unknown mechanism involving HIV gp120 protein, with early

events of HIV viral replication (O'Brien et al. 1996). ALX40-4C-mediated inhibition of viral replication was also extended to two human herpesviruses, CMV and HSV (Sumner-Smith et al. 1995). ALX40-4C may be acting also in a manner similar to other cationic peptides which inhibit viral replication by interfering with cellular receptor function (Langeland et al. 1987, Langelan et al. 1988). The apparent preferential inhibition of KS proliferation by ALX40-4C may be due to interactions with factors specifically involved in the mechanism of KS cell growth.

Next, the effect of chemotherapeutic agent exposure on two cytokines previously demonstrated to be present in the KS cell lines, IL-6 and bFGF, was assessed. Attention was focused on ALX40-4C due to its apparent selectivity for the KS cell lines. There was a dose dependent decline in the amount of bFGF present in the KS4 cell cultures, but no trend was observed for IL-6. The erratic nature of the IL-6 data would seem to indicate that IL-6 was not constitutively produced by the KS4 cells. This may be due to environmental conditions as supported by the demonstration that in the absence of HTLV-infected T cell-conditioned media, there was a decrease in the concentration and type of cytokines produced by KS cells (Bailer et al. 1995). The absence of constitutive production of IL-6 challenges its position as an autocrine growth factor in the KS cell lines.

bFGF has been suggested to play an important role in autocrine growth of KS (Ensoli et al. 1989, Samaniego et al. 1995). Its production is influenced by the presence of inflammatory cytokines (Samaniego et al. 1995). The interference with bFGF production or function may be a possible mechanism by which ALX40-4C inhibits KS proliferation, since the drug has demonstrated the ability to interact with RNA and proteins (Sumner-Smith et al.

1995, O'Brien et al. 1996). However, the decline in the presence of bFGF may simply be as a result of an inhibition of KS proliferation via a bFGF independent mechanism, and subsequent reduction of bFGF production.

The importance of growth factors in KS cell proliferation was underlined following the removal of ECGS from the KSGM. In the absence of ECGS the KS4 cell line was capable of producing only low amounts of bFGF. When these cells were exposed to ALX40-4C, they displayed the same dose-dependent pattern of bFGF reduction, but appeared to be more sensitive than cells that were not starved of ECGS. KSGM lacking ECGS appeared incapable of effectively sustaining KS growth, and these cells demonstrated an increased sensitivity to ALX40-4C. This suggests that the inducers of autocrine growth may be equally important targets for the inhibition of KS proliferation. The KS1 cell line was not assessed in this manner due to it's capability of maintaining proliferation in the absence of medium supplements.

Summary.

KS is a multifocal proliferative disorder that is highly influenced by inflammatory modulators during its development (Ensoli et al. 1989, Miles et al. 1990, Ensoli et al. 1992, Yang et al. 1994, Samaniego et al. 1995). KS appears to arise from the vascular endothelium and progresses as a hyper-proliferative cell type as opposed to a truly transformed neoplasm.

KS hyper-proliferation is suggested to be mediated in part by an autocrine growth mechanism, due to the ability of KS cells to produce cytokines proven to highly stimulatory for their growth (Ensoli et al. 1989, Miles et al. 1990, Ensoli et al. 1992, Yang et al. 1994, Samaniego et al. 1995). The production of these cytokines is influenced by factors such as

inflammatory cytokines, HIV tat protein, and possibly HHV8 infection, although the inability to maintain HHV8 within KS cells *in vitro* has made this difficult to confirm. The partial dependence of KS cell proliferation on an autocrine growth mechanism predicts that interference with these factors or the inducers of these factors may be an effective method of inhibiting KS cell growth.

ALX40-4C is an anti-viral agent that has demonstrated anti-replicative effects on both HIV and human herpesviruses *in vitro* (Sumner-Smith et al. 1995, O'Brien et al. 1996). In addition to it's known mechanism of action with respect to HIV, ALX40-4C has demonstrated activity suggestive of protein-protein interactions.

ALX40-4C demonstrated the capability of selectively inhibiting the proliferation of KS cells in a dose-dependent manner, in the absence of observable cytotoxicity. When culture associated bFGF was assessed in response to ALX40-4C exposure, a dose-dependent decrease in detected bFGF was observed.

These observations lead to a plausible model for the mechanism of ALX40-4C mediated inhibition of KS proliferation (Figure 15). Assessing the different factors that may play a role in inducing and/or maintaining autocrine stimulation of KS, e.g.: inflammatory cytokines, secondary infectious agents (HIV), and HHV8 infection, it is possible that ALX40-4C will inhibit the proliferation of KS by interfering with either the effectors and/or inducers of autocrine stimulation. To address this model further, future investigation should focus on the interactions of ALX40-4C with the potential inducers and effectors of autocrine stimulation in KS. If the model proves correct it may lead to new therapies that will address a specific quality

of KS proliferation, thereby eliminating unpleasant side effects often associated with standard chemotherapy agents.

Figure 15. A model for the inhibition of KS proliferation by ALX40-4C. The proliferation of KS is suggested to be mediated in part by an autocrine growth mechanism. This autocrine growth mechanism may be influenced directly or indirectly by inflammatory cytokines or infectious agents. Based on the suggested biological activity of ALX40-4C, it is possible that this peptide is capable of interfering with the effectors and/or inducers of autocrine growth. The asterisks indicate possible locations where ALX40-4C may be able to interact.

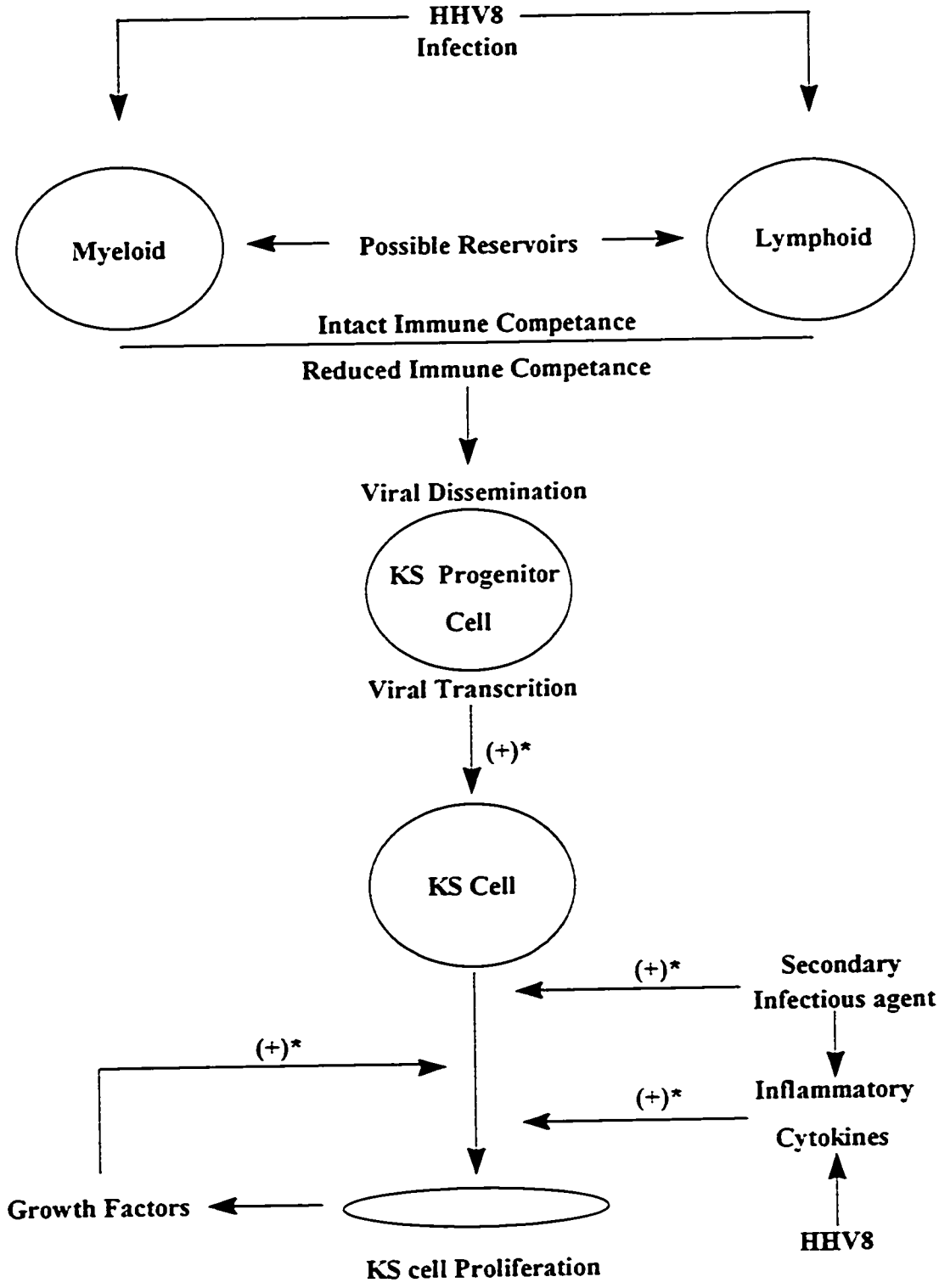

HHV8
Infection
Myeloid
Possible Reservoirs
Lymphoid
Intact Immune Competance
Reduced Immune Competance
Viral Dissemination
KS Progenitor Cell
Viral Transcrition
(+)*
KS Cell
(+)*
Secondary Infectious agent
(+)*
Inflammatory Cytokines
HHV8
Growth Factors
KS cell Proliferation

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IMAGE EVALUATION
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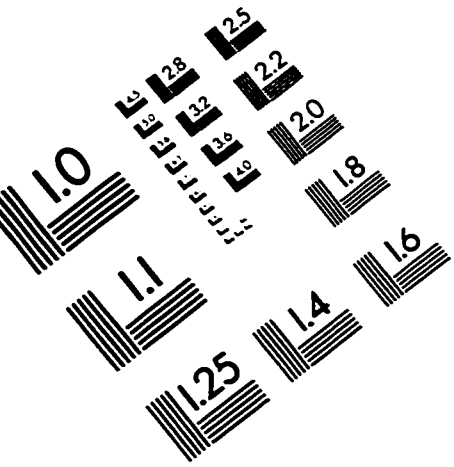

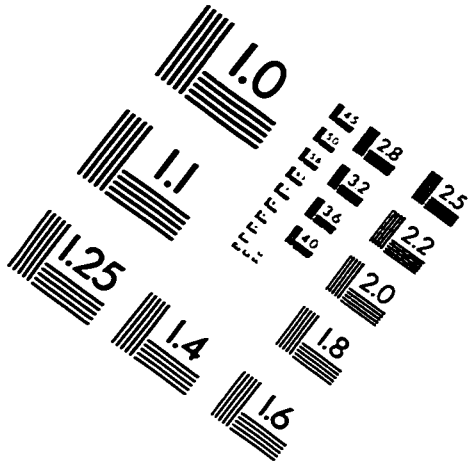

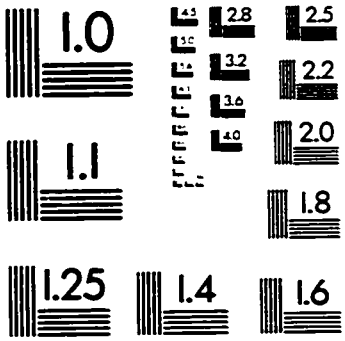

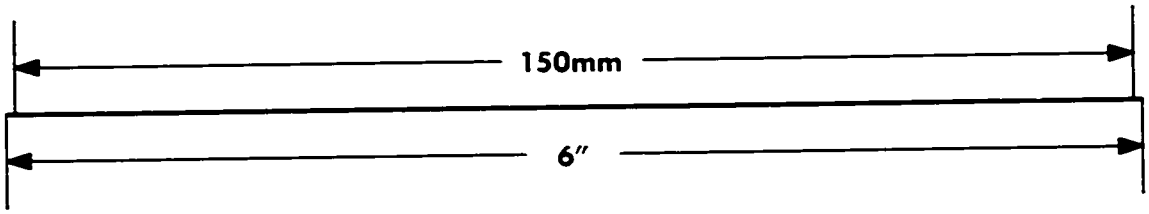

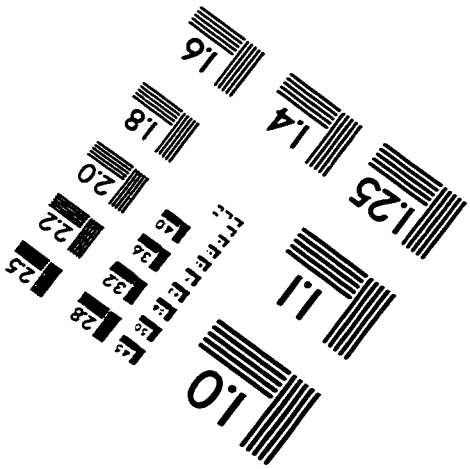

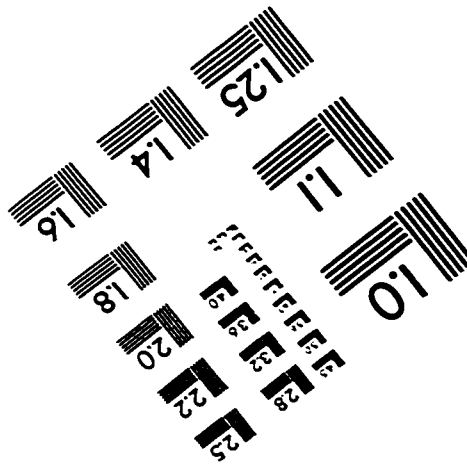

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