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Cytokine-mediated growth promotion of Kaposi's sarcoma and primary effusion lymphoma

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Kaposi's sarcoma (KS) is an angioproliferative disease particularly frequent and aggressive in patients with AIDS but occurring also in post-transplant patients or in immunocompetent individuals of certain geographic areas. At least in its early stages, KS behaves as a reactive hyperplastic process mediated by inflammatory cytokines and angiogenic factors triggered or exacerbated by human herpesvirus-8 (HHV-8) infection. The HIV Tat protein appears to be responsible for the highly aggressive nature of AIDS-KS. Over time, however, KS may evolve into a true sarcoma in association with the expression of oncogenes and/or HHV-8 latency genes endowed with growth and anti-apoptotic properties. HHV-8 infection is also associated with primary effusion lymphoma (PEL), a rare tumor that similarly develops more frequently in the setting of HIV infection. HHV-8 latency genes are likely to contribute to the neoplastic phenotype of PEL cells, whose growth in vivo *may require cytokines and factors from the host, or encoded by the virus*

Key words: angiogenesis / human herpesvirus-8 / inflammatory cytokines / Kaposi's sarcoma / primary effusion lymphoma

Introduction

Kaposi's sarcoma (KS) is a neoplasm of vascular origin arising with multiple independent lesions in the form of purple maculae or plaques that, over time, can progress into a nodular tumor.[1] Several forms of KS are known. Classical KS (CKS) affects elderly men of Mediterranean or Eastern Europe origin; post-transplant KS (PT-KS) arises in allograft recipients treated with cyclosporin; African KS (A-KS) and AIDS-associated KS (AIDS-KS) are aggressive forms involving visceral and/or lymphatic organs and occur in young adults or children from sub-equatorial Africa or in HIV-infected patients, respectively.[1] AIDS-KS represents the most frequent tumor of HIV-infected individuals.[1,2] As discussed below, the Tat protein of HIV may be responsible for the aggressive nature and the high incidence of AIDS-KS.[3–10]

Evidence suggests that, in its early stage, KS is not a true sarcoma but a hyperplastic, reactive process. For example, all forms of KS can start as multiple lesions that appear simultaneously with a symmetrical or dermatome distribution and in the absence of metastasis.[11] In addition, spontaneous regressions of KS lesions can occur in patients with AIDS-KS[11,12] or in PT-KS patients upon the withdrawal of immunosuppressive therapy.[13] Furthermore, AIDS-KS regression can be obtained by treatment with HIV-1 protease inhibitors[14] or upon treatment with IFN-α[15] that strongly inhibits human herpesvirus-8 (HHV-8) infection *in vitro* and *in vivo*.[16,17] KS itself starts as granulation-like tissue, rich in inflammatory cells including lymphocytes and monocyte/macrophages, producing the same inflammatory cytokines (IFN-γ, IL-2, TNF, IL-1, etc.) as do peripheral blood mononuclear cells (PBMC) from KS patients.[18,19] Recent data suggest that this reactive process may be induced or enhanced by infection with HHV-8.[18–20] As discussed below, inflammatory cytokines, in turn, trigger KS lesion formation through the induction of angiogenic factors that mediate KS spindle cell growth, angiogenesis and edema, the prominent features of KS lesions.[3,9,19,21–25] In addition, inflammatory cytokines induce endothelial cells (EC) to acquire the phenotypic features of KS cells[3,19,21,23–26] in-

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cluding the responsiveness to the effects of the HIV-1 Tat protein.[3,4,9,10,19,21,26,27] This viral product is released from acutely infected cells[6,7,28] and induces the growth, migration and invasion of inflammatory cytokine-activated EC[3,4,9,10,21,27,28] and KS spindle cells,[3–7] and thus acts as a progression factor for AIDS-KS.

Other observations, however, suggest the possible malignant nature of late-nodular KS. In fact, evidence of aneuploidy, microsatellite instability, and clonality of KS spindle cells has been found in high-grade KS lesions,[29–31] although microsatellite instability is lacking in CKS[29] and clonality of KS spindle cells has not been confirmed by other studies.[32] This late stage of KS is associated with the deregulated expression of oncogenes or oncosuppressor genes such as c-myc, Bcl-2, or p53[33–35] and with the expression of the HHV-8 latency genes.[36–39] Late KS appears to be independent of growth factors and can be resistant to conventional therapy. In contrast, in its early phases, KS growth is mediated by cytokines and angiogenic factors that can be targeted for therapeutic intervention.

Primary effusion lymphomas (PEL), also termed body-cavity-based lymphomas, are monoclonal lymphomatous effusions of the serous cavities without a clear nodal origin and develop in the absence of an identifiable tumor mass.[40] PEL represent about 3% of all AIDS-associated non-Hodgkin's lymphomas (NHL)[40] and, like KS, arise most frequently in HIV-infected individuals;[40] and their incidence is significantly higher in men than in women.[41] These features, and the observation that PEL can occur in the presence of concomitant or previous KS,[42] suggest that, despite the different etiology and histogenesis, the development of these diseases is associated with a common factor. This is HHV-8 infection which is a specific marker for PEL diagnosis and distinguishes PEL from the other primary lymphomatous effusions.[40–42]

Role of cytokines in KS cell growth

Risk factors and histopathogenesis of KS

All forms of KS share the same histopathologic features such as neoangiogenesis, edema, infiltration of lymphomononuclear cells, presence of activated proliferating EC-forming abnormal blood vessels (slit-like vessels), extravasation of red blood cells, and growth of spindle cells (KS spindle cells, KSC), that are considered to be the tumor elements of KS.[1] As discussed below, spindle-shaped cells expressing markers of KSC are also cultured from the blood of patients with KS or at risk for KS[20,43–45] and they may represent circulating KSC progenitors, possibly explaining the multifocal nature of KS.

Two factors are associated with KS initiation: a disturbance of the immune system leading to activation and infection by the HHV-8. Patients with all forms of KS and individuals at risk of KS, including homosexual men, HIV-1-infected individuals or elderly people of Mediterranean origin show $CD8^+$ T cell and Th1-type activation and increased serum levels of IL-1, IL-6, TNF-α, IFN-γ, soluble CD8, neopterin, and soluble ICAM-1, or show an oligoclonal expansion of $CD8^+$ T cells.[46–51] African subjects are also immunoactivated and show a Th1-type activation probably due to frequent exposure to different infections.[52,53] Despite iatrogenic immunosuppression, in post-transplant patients allogeneic stimulation may result in the emergence of local foci of activated immune cells. In addition, KS can occur in the absence of HIV infection or prior to overt immunosuppression,[54,55] respectively, and A-KS development is not associated with a deficient immunity.[56] As discussed below, immunoactivation and production of inflammatory cytokines also leads to reactivation of HHV-8 infection.[20] Thus immunoactivation is key to KS development, as also indicated by the worsening of KS or KS onset in patients treated with TNFα or IFN-γ.[57,58] However, immunosuppression may play a role in KS progression, for example, by allowing a further increase of HHV-8 load or by indirect effects (see below).[29]

Several lines of evidence suggest that HHV-8 infection is required for KS development. HHV-8 DNA is invariably found in KS lesions[1] and in KSC.[36–38] Furthermore, the incidence of KS is high in populations with a high HHV-8 seroprevalence.[59,60] As compared to healthy subjects, patients with KS or individuals at risk for KS show a higher HHV-8 load and higher antibody titers against latent or lytic virus antigens.[61–63] These determinants are strong risk factors for KS and highly predictive of disease development.[61–63] However, KS is an exceedingly rare disease even in high seroprevalence geographic regions, indicating that HHV-8 infection by itself is not sufficient for KS onset.

Origin of KS spindle cells

Most studies indicate that lesional KSC consist of a heterogeneous cell population dominated by activated vascular and lymphatic EC mixed with

cells of macrophage origin.[1,64,65] Furthermore, some KSC show a phenotype similar to the so-called endothelial macrophages of the lymphatic organs since they co-express markers of macrophages and VE-cadherin, a vascular EC adhesion molecule.[45,66] Circulating KSC have a similar phenotype.[20,43–45,66] These data suggest that they are related cell types and that circulating KSC may be cell progenitors of lesional KSC. As discussed below, normal EC present in KS lesions, lesional KSC, and circulating KSC are all infected by HHV-8.[20,36–39,44]

Evidence indicates that the reactive or hyperplastic KSC of endothelial origin are not transformed, however, they are activated and promote KS-like lesions in nude mice by recruiting and inducing the growth of mouse cells due to the paracrine action of cytokines and angiogenic factors produced by the cells.[5,67–69] This supports the concept that KSC, at least in the early stage, are reactive cells and not tumor cells. However, recent evidence suggests that KSC of endothelial origin are 'trans-differentiated', as they show distinctive functional properties as compared to normal EC (see below).

Inflammatory cytokines and their role in KS initiation

The first histological change of KS is the appearance of an inflammatory cell infiltrate that precedes the spindle cell formation.[1] The factors eliciting this reactive process have not yet been identified. However, as discussed below, HHV-8 infection appears to participate in this early reaction. Immunohistochemical analysis of early KS lesions has shown the presence of T cells, particularly $CD8^+$ T cells, monocyte-macrophages, and dendritic cells ($FXIIIa^+$), whereas B cells are few or absent [Figure 1(a)].[1,19,45,66] Infiltrating cells obtained from KS biopsies (tumor-infiltrating lymphocytes, TIL) and lesional macrophagic spindle cell cultures show the same features of resident cells.[18] These cells, as well as PBMC from patients with KS or at risk of KS, produce Th1-type cytokines including IFN-γ [Figure 1(a)], TNF, IL-1, IL-6, and others[18,19,70–72] that induce the production of angiogenic factors [Figure 1(b)][3,9,19,21–25,73] and activate EC, determining a further recruitment of circulating cells into tissues (Tables 1 and 2).[18,21,74] In contrast, Th2-type cytokines such as IL-4 are absent or produced at low levels by TIL and PBMC from KS patients,[18] although the apoptotic death of Th1 cells from cultured TIL[18] may account for the predominant Th2/Tc2 phenotype of $CD4^+$ or $CD8^+$ T cell clones obtained from KS lesions.[75,76]

Table 1. Effects of inflammatory cytokines in KS initiation

A. Systemic effects of inflammatory cytokines
KSC progenitors proliferation and differentiation. Vessels activation; extravasation of inflammatory cells. Induction of HIV-1 gene expression/replication; production/release of HIV-1 Tat protein. HHV-8 reactivation in circulating cells; virus spreads to tissues.
B. Local (tissue/KS lesion) effects
Recruitment of inflammatory cells; activation and differentiation of monocytic cells into macrophages, endothelial macrophages and dendritic cells. Maintenance of TIL phenotype; TIL survival. Differentiation of E-KSC and M-KSC from precursors (EC, circulating KSC progenitors); KSC proliferation. E-KSC cytokine production and angiogenic activity.
Activation of endothelial cells: spindle morphology, down-regulation of FVIII-RA, expression of activation markers and adhesion molecules, expression and release of angiogenic factors, responsiveness to the effects of the HIV-1 Tat protein, induction of KS-like lesions upon inoculation in nude mice.
Angiogenic effects: induction of KS-like lesions after injection in nude mice (mediated by induction of angiogenic factors).

E-KSC, M-KSC, Endothelial and macrophagic KSC; TIL, tumor-infiltrating lymphocytes; EC, endothelial cells.

The inflammatory cytokines produced in KS lesions have profound growth effects on KSC, since lesional KSC of both endothelial (E-KSC) or macrophage (M-KSC) origin from KS lesions have been established in culture by utilizing the same combination of inflammatory cytokines as found in the lesions (Table 1).[3,18,77] Several of these inflammatory cytokines contribute to induce the long-term growth of hyperplastic E-KSC via a synergistic stimulatory effect on production and release of basic fibroblast growth factor (bFGF)[3,22,25] that, in turn, functions as an autocrine KS cell growth factor.[23,25,67–69] Inflammatory cytokines also promote the growth and differentiation of circulating KSC progenitors from the adherent cell fraction of PBMC from patients with all forms of KS or at risk of KS (Table 1).[20,43]

The same inflammatory cytokines activate normal EC to acquire the phenotypic and functional features of E-KSC, including a typical spindle morphology, the expression of the same markers (down-regulation of FVIII-RA, activation of ELAM-1, ICAM-1, VCAM-1, DR, $\alpha5\beta1$, $\alpha v\beta3$ integrin expression), the angio-

Table 2. Expression and activity of the major cytokines, and angiogenic and chemotactic factors in KS

Factor	Expression[a] *in vivo*	Expression[a] *in vitro*	Possible role in KS pathogenesis
IL-1α, β	+	+	Activation and induction of a spindle morphology in EC. Induction of E-KSC proliferation (mediated by bFGF). Cell recruitment into tissues and cell differentiation.
TNFα, β	+	+	Induction of E-KSC proliferation. Activation and induction of a spindle morphology in EC. Cell recruitment and differentiation. KS progression after systemic inoculation.
IFNγ	+	+	Activation and induction of a spindle morphology in EC. Cell recruitment and differentiation. Induction of Tat responsiveness. KS progression after systemic inoculation. *In vitro* growth of lesional M-KSC.
bFGF	+	+	Potent inducer of E-KSC proliferation and angiogenesis. Synergy with HIV-1 Tat protein and VEGF in induction of angiogenesis and edema.
VEGF	+	+	Synergy with bFGF in inducing angiogenesis and edema.
MCP-1	+	+	Recruitment of circulating cells.
IL-8	+	+	Recruitment of circulating cells.
Rantes	+	n.d.	Recruitment of circulating cells.
MIP1α, MIP1β	+	n.d.	Recruitment of circulating cells.

[a] Expression in primary lesions and in cultured KSC. E-KSC, endothelial KSC; EC, endothelial cells.

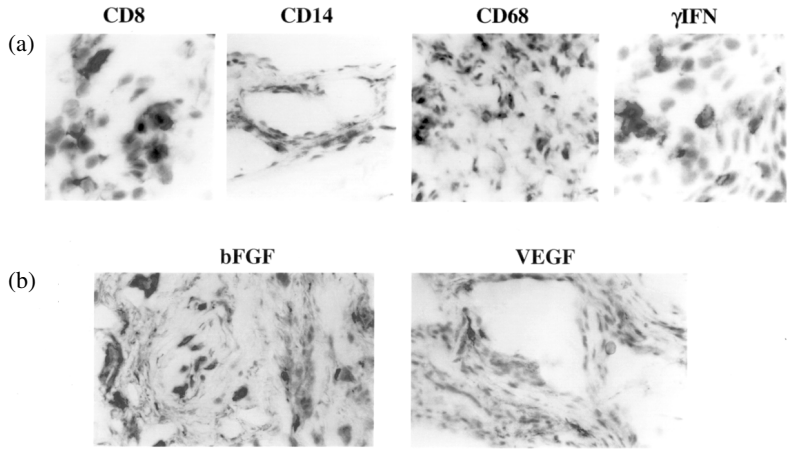

Figure 1. Detection of inflammatory cells, Th1-type cytokines and angiogenic growth factors in KS lesions. (a) Detection of CD8, CD14, or CD68-positive infiltrating cells and γIFN in a KS lesion. Cells expressing γIFN consisted of $CD8^+$, $CD14^+$, and $CD68^+$ mononuclear cells or $CD68^+$ spindle cells (data not shown).[19] (b) High-level expression of bFGF is apparent in vessels and spindle cells from a representative KS lesion; spindle cells also express VEGF.[24] Frozen sections were stained by immunohistochemistry with specific antibodies (APAAP method). (Modified from Fiorelli *et al.*[19] and Samaniego *et al.*[24])

genic phenotype, and the capability of inducing the formation of KS-like lesions in mice (Tables 1 and 2).[3,19,21,23–26] As discussed below, inflammatory cytokines also induce normal EC to become responsive to the adhesive, mitogenic and invasive effects of the extracellular HIV-1 Tat protein,[3,4,9,10,21,27,28] (Tables 1 and 2) as are E-KSC,[3–7] leading to augmented angiogenesis and spindle cell growth in AIDS-KS.[5,19,27] However, a few differences still exist between inflammatory cytokine-activated EC and E-KSC, indicating that E-KSC have acquired a 'trans-differentiated' phenotype although they are not

transformed nor tumorigenic. These include the production of vascular endothelial growth factor (VEGF) and the growth response to RGD peptides.[10, 24, 78, 79] In addition, inflammatory cytokine-activated EC, but not E-KSC, proliferate in response to VEGF, although the level of expression of VEGF receptors is similar in these two cell types.[24, 78, 79]

Inflammatory cytokines that are increased in KS also have profound effects on HHV-8 infection [Table 1, Figure 2(a)], since they maintain HHV-8 DNA or increase the HHV-8 DNA load in cultured PBMC.[20] IFN-γ appears to be key for these effects [Figure 2(b)], although other inflammatory cytokines may contribute to it.[20] Although several mechanisms may be involved, recent studies suggest that the reactivation of latent HHV-8 in these cell types is the major mechanism [Figure 2(c)], similar to the reactivation of human cytomegalovirus observed in PBMC after prolonged allogeneic stimulation.[20]

Reactivation of HHV-8 in circulating cells may play a crucial role for the localization of the virus in tissues (Table 1). In particular, since B cells are rare or absent in KS lesions, circulating monocytes may recruit the virus into tissues and, upon exposure to inflammatory cytokines, they may undergo lytic infection and transmit the virus to neighboring cells, as suggested by the presence in KS lesions of productively infected monocyte-macrophages.[80] Alternatively, upon exposure to inflammatory cytokines, monocytic cells may differentiate into macrophages or spindle-like endothelial macrophages with latent infection as observed in lesional spindle cells.[18, 36] This, and the reactivation of HHV-8 infection in PBMC, may induce systemic and local reactive responses with a further production of inflammatory cytokines and a further recruitment of immune cells into tissues. However, as discussed below, inflammatory cytokines can be detected in early KS lesions and uninvolved tissue even prior to detection of HHV-8 by PCR,[19] suggesting that HHV-8 has an optimal environment in these tissues and may exacerbate these reactive processes but may not initiate them.

Altogether these results indicate that the inflammatory cytokines produced in KS lesions are capable of triggering a cascade of events leading to KSC appearance and growth and lesion development.

Angiogenic and growth factors, and chemokines mediate KSC growth and lesion formation

Several angiogenic molecules, growth factors, and chemokines are produced in KS lesions, many of them in response to inflammatory cytokines (Table 2). These molecules mediate KSC growth or recruit circulating cells that transmigrate through the activated endothelia. Among them bFGF and VEGF appear to be the major mediators.

The angiogenic factor bFGF is released by KSC and inflammatory cytokine-activated EC in the absence of cell death or cell permeability changes[22, 23, 67, 68] and has autocrine and paracrine activities in KS development by stimulating angiogenesis and the proliferation of KSC and EC (Table 2).[22, 23, 25, 69] Due to the production of bFGF, E-KSC[5, 67–69] or inflammatory cytokine-activated EC[19, 21, 23, 24] are highly angiogenic in the chorioallantoic membrane assay and induce KS-like lesions of mouse cell origin in nude mice (Table 2). The expression of bFGF is detected at both the RNA[81] and protein[5] level in primary KS lesions [Figure 2(b)] and KS-like mouse lesions,[21–23] and the inoculation of bFGF itself in nude mice results in the formation of KS-like lesions.[5] Although KSC produce other angiogenic factors (see below), inhibition studies with neutralizing antibodies or antisense oligodeoxynucleotides directed against bFGF have shown that bFGF is absolutely required for the formation of KS-like lesions in mice.[69]

VEGF, another angiogenic factor expressed in KS lesions [Figure 1(b)] and by cultured KSC,[24, 78, 79] synergizes with bFGF in inducing EC growth, angiogenesis[24, 79] and edema, which is another prominent feature of KS (Table 2).[24] VEGF production is induced by inflammatory cytokines in E-KSC but, unlike EC, these cells do not proliferate in response to VEGF, although they express the VEGF receptors KDR/Flk-1 and flt-1 *in vitro* and *in vivo*.[24, 79, 82] VEGF, however, mediates the growth of two transformed KS cell lines established from KS lesions.[83] T cells are induced to produce VEGF in response to HIV infection or, most importantly, to the same inflammatory cytokines that are increased in KS.[24, 73] Of note, activated (i.e. IFN expressing) T cells infiltrate early KS lesions in large numbers.[18, 19]

Inflammatory cytokines produced in KS lesions induce the expression of several chemokines that mediate cell recruitment into tissues, including the monocyte chemotactic protein-1 (MCP-1),[84] IL-8,[84] and Rantes, MIP1α and MIP1β that are produced by tumor-infiltrating lymphocytes (Table 2).[85] IP-10 and Mig are expressed in AIDS-KS lesions by infiltrating inflammatory cells, EC and KSC (B. Ensoli, unpublished data). Some of these chemokines modulate constitutive signaling by the HHV-8-encoded G

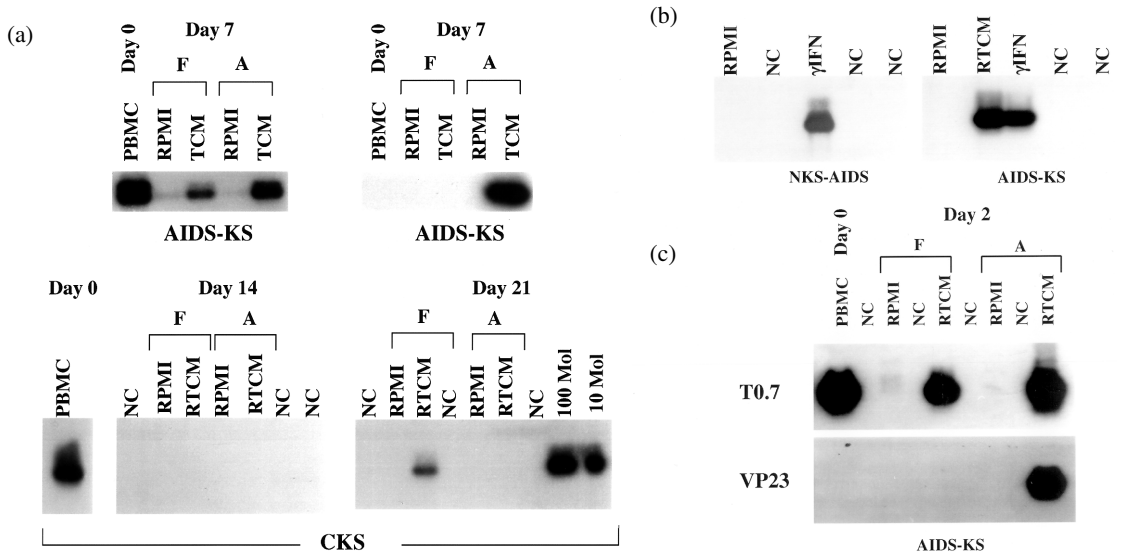

Figure 2. Reactivation of HHV-8 in PBMC by the IC increased in KS.[20] (a) PCR detection of HHV-8 DNA in PBMC (day 0) and in floating [F] or adherent [A] cells from KS patients after short-term (7 days) or long-term (21 days) culture in the presence of IC [RTCM] or in their absence [RPMI]. IC maintained or increased HHV-8 infection to detectable levels in floating or adherent cells from two representative AIDS-KS patients. In a C-KS patient, viral DNA was no longer seen after culture for 14 days but was detected after additional culture for 7 days in the presence of RTCM, suggestive of viral reactivation by long-lasting stimulation by IC. (b) Effect of γIFN on HHV-8 reactivation. HHV-8 DNA was detected in PBMC from two AIDS-KS patients only after short-term culture in the presence of RTCM or γIFN. This result indicates that γIFN alone can reproduce the effects of the IC increased in KS, pointing to a specific role in this cytokine in HHV-8 reactivation. (c) Detection of HHV-8 gene expression in total PBMC at day 0 and in floating [F] or adherent [A] cells cultured with or without IC [RTCM] from a patient with AIDS-KS. The figure shows the result of RT-PCR and hybridization for T0.7, a viral transcript up-regulated upon virus reactivation, or ORF26, a viral gene expressed in productively infected cells. NC, negative control made without adding template DNA; MOL, positive controls made with the indicated numbers of molecules of a control plasmid. PCR products were transferred to nylon membranes and hybridized to a ^{32}[P]-labelled oligonucleotide probe internal to the amplified sequences. (Modified from Monini *et al.*[20])

protein-coupled receptor (vGPCR/vIL-8R) that, in turn, has been suggested to have a role in KS angiogenesis and lesion development (see below).[86,87]

Paracrine actions of HHV-8 lytic genes in KS initiation

HHV-8 encodes for homologues of cytokines and chemokines endowed with paracrine activity. These include vIL-6[88] and three homologues of host chemokines [viral macrophage inflammatory protein (vMIP)-I, -II, -III].[88–90] In addition, the HHV-8 vGPCR may have paracrine actions due to its ability to induce VEGF.[91] None of these viral factors, however, is expressed by latently infected KSC[88,92–95] and, therefore, cannot have a direct transforming action on these cells. Nevertheless, these molecules may play a role in KS upon transient expression during HHV-8 reactivation of infiltrating lymphocytes and monocytes induced by local production of inflammatory cytokines. Although all these viral products have been suggested to be involved in KS pathogenesis due to their ability to induce angiogenesis,[90,91,96,97] this occurs at very high molecule concentration, that is unlikely to be achieved in the few cells undergoing virus reactivation. For example, in KS lesions, v-IL6 is undetectable or expressed at very low levels.[89,93,94]

The HHV-8 MIP homologues show multiple actions including inhibition[89] or promotion[76,90,98] of cell chemotaxis, but they are also potent Th2 cell chemoattractants and inhibitors of monocyte chemotaxis.[76,89,90,98] These effects, however, are in contrast with the immunophenotype of infiltrating cells in KS.[18,19] In transgenic mice, vGPCR can induce KS-like lesions due to the production and release of VEGF in tissues by vGPCR-expressing circulating cells.[99] However, during infection, the time of vGPCR expression can only be transient and in a few lytically infected cells.[95] Recent data have

also shown that vMIP-II acts as a vGPCR inhibitor.[86] Since vMIP-II and vGPCR are both expressed upon HHV-8 reactivation, these data suggest that HHV-8 replication requires a shut-off of vGPCR activity. As large amounts of VEGF are also produced by lesional KSC,[24,78,79] that are negative for vGPCR expression,[95] these data indicate that most of the VEGF production in KS lesion may be sustained by cells that do not express HHV-8-encoded GPCR.

Factors of progression of KS: Bcl-2 and HHV-8 latency genes

Although in its early stages KS behaves as a reactive process, it can evolve into a real tumor and KSC can become monoclonal, likely due to the deregulated expression of oncogenes (Bcl-2, c-myc, c-int, ras) or oncosuppressor genes (p53), or to the long-lasting expression of HHV-8 latency genes (LANA, vCycD, vFLIP, kaposin), that are all expressed by KSC in the late nodular phase of KS. In fact, although HHV-8 terminal repeats have a polyclonal profile in most KS lesions, a few lesions show an oligoclonal or clonal pattern.[100] In addition, the HIV-1 Tat protein acts as a progression factor for AIDS-KS due to its effects on KSC growth and angiogenesis.

Several oncogenes or oncosuppressor genes have been found to be expressed in KS, including Bcl-2,[33,101] ras,[102] int-2,[103] p53[101,104] and c-myc.[34] Bcl-2 is expressed in lesional EC and KSC and its expression increases with lesion stage in all forms of KS and is maximal in nodular lesions, representing a progression marker of KS.[33,101] In addition, regression of KS is observed in patients treated with taxol[105] that is a known inhibitor of Bcl-2 function.[106] Recent data, in fact, indicate that taxol blocks KSC growth and migration, and KS-like lesion formation induced by inoculation of KSC in nude mice, due to induction of apoptosis and down-regulation of Bcl-2 expression.[107]

Heterozygous mutations and overexpression of p53 have been detected in late stage KS lesions but not in early lesions.[101,104] In addition, as discussed below, very recent data point to functional inactivation of p53 by HHV-8 LANA as a mechanism to prevent apoptosis of KSC,[108] a finding indirectly supported by data showing that p53 is functionally inactivated but not mutated in PT-KS lesions.[109] The oncogene c-myc, that is up-regulated in KSC by PDGF-B, is expressed at higher levels in late nodular KS lesions as compared to early lesions, indicating that it may have a role in disease progression.[34]

HHV-8 latency-associated genes encoding for vCycD, vFLIP, LANA and kaposin[110,111] may be involved in KS progression due to their capability of promoting cell growth by direct effects or anti-apoptotic effects. Only few KSC appear to express these genes in early stage KS. However, their expression increases with lesion stage and most KSC in nodular lesions are positive at the RNA or protein level,[36–39] suggesting that the continuous expression of these genes may be required for KS progression.

Expression of vCycD is found in lesional KSC and increases with lesion stage.[37] In addition, vCycD is expressed in peri-lesional EC[37] and also in circulating KSC from KS patients[37].

The anti-apoptotic molecule vFLIP inhibits apoptotic cell death by interfering with the recruitment and activation of the protease FLICE driven by the TNF receptor family members.[112] Transcripts of vFLIP are expressed at very high levels in nodular KS lesions as compared with early lesions, and this correlates with the reduction in apoptosis in KSC in lesions.[38] In addition, overexpression of vFLIP in murine B lymphoma cells allows the growth of aggressive tumors in mice due to the inhibition of FAS-mediated cytotoxic T cell responses.[113] Thus, vFLIP may contribute to both lesion growth and evasion of immune responses against HHV-8-infected KSC.

The HHV-8 kaposin locus encodes for multiple proteins including kaposin A, B, and C and may be involved in KSC transformation and in the progression of KS to the nodular tumor stage, as also suggested by increased expression of Kaposin transcripts in nodular lesions as compared to early lesions.[36,111]

HHV-8 LANA expression is also found in KSC, specifically in association with VEGFR-3.[39] In nodular lesions, the percentage of VEGFR-3 positive cells co-expressing LANA is, again, higher than in early lesions.[39] A prominent function of LANA is to ensure the stable maintenance of the HHV-8 genome in the host cell during latent infection.[114] In addition, LANA may contribute to the survival or transformation of KSC by inactivation and down-regulation of p53 that is associated with anti-apoptotic effects.[108]

Altogether these data support the concept that HHV-8 latency genes may play a role in the progression of KS by providing KS cells with growth and/or anti-apoptotic signals.

HIV-1 Tat protein: a progression factor of AIDS-KS

Several studies indicate that HIV-1 Tat is responsible for the higher incidence and aggressiveness of KS in

HIV-1-infected people. Tat is a transcriptional activator of HIV-1 gene expression produced early after infection and essential for virus replication.[6,115] Tat is released in an active form from HIV-1 acutely infected T cells in the absence of cell death.[6,7,28] Extracellular Tat can induce the growth, migration and invasion of KSC[3–7] and EC[3,4,21,26] However, these effects of Tat on normal EC require a previous exposure of the cells to the same inflammatory cytokines increased in KS.[3,19,21,26,27] IFN-γ appears to play a major role in these processes.[27] Inflammatory cytokines act by inducing in EC the expression of $\alpha5\beta1$ and $\alpha v\beta3$ integrins and of bFGF that, in turn, amplifies the expression of these integrins.[9,10,26,27]

Recent data indicate that the RGD sequence and the basic region of Tat cooperate to induce Tat angiogenic effects by different pathways.[10] The RGD sequence of Tat mediates EC adhesion, migration and invasion by binding to the $\alpha5\beta1$ and $\alpha v\beta3$ integrins. This interaction also provides EC with the adhesion signal required for growth in response to mitogens. In turn, the Tat basic sequence that binds heparin sulfate proteoglycans (HPSG)[28] converts extracellular bFGF, bound to HPSG, into a soluble form by competing for heparin-binding sites.[10] This soluble bFGF mediates Tat-induced vascular cell growth,[10] explaining why bFGF is required for the angiogenic effect of Tat.[5] Consistent with these data, KS lesion formation is induced in a synergistic way by bFGF and Tat in nude mice and is blocked by RGD peptides used as competitors to inhibit the interaction of Tat with $\alpha5\beta1$ and $\alpha v\beta3$ integrins (Figure 3)[5,9] Since these effects resemble those of extracellular matrix proteins, these data suggest that Tat enhances angiogenesis and promotes KS progression by a molecular mimicry of these molecules.

Tat was also reported to stimulate angiogenesis by the binding of its basic sequence to the VEGF receptor KDR/Flk-1.[116] However, several observations suggest that this is unlikely to occur *in vivo*. In fact, Tat has no effects on resting EC[3,4,6,7,19,21,26,27] and alone does not promote angiogenesis in mice.[5,19] Furthermore, competition for VEGF receptors by Tat in KS lesions is unlikely to happen due to the much higher expression of VEGF than Tat.[5,24,79] Finally, KS is found in risk subjects that are not infected by HIV-1, but is more frequent and aggressive in patients with AIDS,[1] indicating a role for Tat as a progression and not an initiating factor.

Tat has also been shown to activate the adhesion of monocyte-macrophages to vessels upon vascular damage and to increase their migration and invasion into tissues by inducing collagenases.[117] Finally, Tat can also activate the expression of cytokines involved in KS pathogenesis, including TNF α and β, IL-6, MCP-1, or TGFβ.[1]

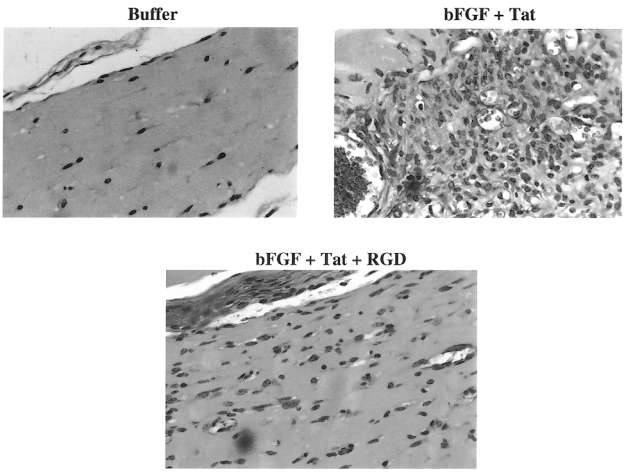

Figure 3. Synergistic angiogenic and KS-promoting effects of bFGF and Tat and inhibition of lesion formation by RGD peptides.[9] Animals inoculated with Tat and bFGF combined (*upper right panel*) develop KS-like lesions characterized by angiogenesis and spindle cell growth that are not induced by control buffer (*upper left panel*) and are inhibited by the simultaneous inoculation of RGD peptides competing for Tat binding to $\alpha5\beta1$ and $\alpha v\beta3$ integrins (*lower panel*). Animals do not develop macroscopic lesions upon inoculation of Tat or bFGF alone at the doses used (data not shown).[9] Shown are hematoxylin-eosin staining of representative tissues from inoculation sites. (Modified from Barillari *et al.*[9])

Role of cytokines in PEL growth

Pathogenesis and cell origin of PEL

The most prominent characteristic of PEL is their growth in serous cavities.[40] PEL cells exhibit a specific homing for the serous membranes to which they adhere, forming multiple foci at sites of membrane thickening, and they can spread along the serous membranes without infiltrative growth.[40] Mesothelial cells activated by the inflammatory cytokines that are increased in AIDS (INF-γ, TNF-α, IL-1β) secrete chemokines, particularly IL-8, and express adhesion molecules (N-CAM, V-CAM)[118] that may play a major role in PEL cell homing and adhesion to mesothelia. In addition, the HIV-1 Tat protein can induce PEL cells to migrate and to adhere to EC,[119] suggesting that Tat may be involved in the migration and homing

of PEL cells to the serous membranes in HIV-infected individuals.

PEL cells have heterogeneous morphology including plurilobated and multinucleated cells. They have an indeterminate phenotype, and lack expression of lineage-associated T or B lymphocyte antigens.[40] However, the monoclonal rearrangement and the pattern of expression of Ig genes identify PEL cells as a clonal population of B cells.[40,41] PEL cells, in addition, are characterized by mutations of the 5′ regulatory region of the Bcl-6 proto-oncogene and lack Bcl-6 expression.[120,121] They express high levels of CD138/syndecan 1,[121] and show evidence of antigen selection.[122] This profile identifies PEL cells as post-germinal center B cells in a pre-terminal differentiation stage. Most PEL lack genetic alterations typical of NHL but they show specific and recurrent chromosomal aberrations.[40,120] In addition, PEL cells are invariably infected by HHV-8 and, in 70% of cases, also EBV.[40] Both viruses are in a latent state and express a limited subset of genes including EBNA1,[123] LANA,[39,124,125] vCycD,[125] vFLIP,[125] kaposin,[111,125] and vIL-6.[93,94,125]

Cytokine-mediated growth of PEL cells

Cell lines established from PEL effusions show the same phenotype and HHV-8 or EBV infection pattern as the *in vivo* lymphoma cells.[40,126] IL-10 and IL-6 are expressed although at high levels by PEL cell lines and released in culture,[127–129] and they are expressed at lower levels also in primary tumors[130] (Table 3). In addition, PEL cells express the IL-6 receptor (IL-6R)[127,128] and IL-10R[129] (Table 3). Altogether these data suggest that IL-6 and IL-10 may be involved in the pathogenesis of PEL by promoting tumor growth. However, recent functional studies have produced conflicting results (Table 3).[127,129] In addition, neither IL-10 nor IL-6 alone stimulates the growth of PEL cells (Table 3),[127–129] suggesting that cooperation of several autocrine growth factors may be required to promote PEL proliferation. Furthermore, unlike anti-IL-6-neutralizing antibodies,[127–129] antibodies against gp130 or IL-6R alone or in combination inhibit PEL proliferation (Table 3).[128] In contrast, the growth of experimental tumors induced in SCID mice by inoculation of PEL cells is efficiently inhibited by the administration to the animals of antibodies to human IL-6, indicating that IL-6 alone may act as an autocrine PEL growth factor *in vivo* (Table 3).[131]

Another factor showing a differential activity for PEL *in vitro* or *in vivo* is VEGF.[132] PEL cell lines produce and release large amounts of VEGF which, however, does not induce their proliferation (Table 3), nor do anti-VEGF-neutralizing antibodies inhibit PEL cell growth *in vitro*.[132] However, such antibodies do prevent the development of PEL-like ascites in irradiated SCID mice inoculated intraperitoneally with PEL cells (Table 3).[132] Thus, these data suggest that VEGF may also act as a PEL autocrine growth factor *in vivo*.

PEL cells produce additional factors with potential growth effects including oncostatin M and scatter factor/hepatocyte growth factor, as well as its receptor c-met.[128,133] However, additional work is required to determine the role of these factors in PEL growth and pathogenesis.

Role of HHV-8-encoded factors in the growth of PEL cells

Like KSC, PEL cells express HHV-8 latency genes including LANA, vCycD, vFLIP, and kaposin both at the RNA and the protein level.[39,111,124,125,134] In addition, PEL tumors express large amounts of vIL-6 with a pattern typical of a latency gene (Table 3),[93,94,124] although in established PEL cell lines vIL-6 RNA is found only in cells undergoing viral lytic infection (B. Ensoli, unpublished data).[125] These data indicate that vIL-6 may play an important role in PEL growth by autocrine and/or paracrine effects.

Recent evidence indicates that vCycD may play a key role in PEL cell growth. In fact, PEL tumors express the p27 cell cycle inhibitor and fail to express the cellular cyclin D1[134] but, in spite of this, PEL cells show a high proliferation index *in vivo* that is associated with high-level expression of vCycD.[134] In contrast, p27 expression and cell proliferation are inversely correlated in HHV-8 negative secondary lymphomatous effusions.[134] Altogether these data suggest that the resistance of vCycD to cdk inhibitors may be a specific mechanism for PEL growth *in vivo*.

Conclusions

In this article we reviewed data suggesting that, at least in its early stages, KS is a reactive hyperplastic disease initiated by inflammatory cytokines that induce EC to acquire the KSC phenotype and to become responsive to the effects of extracellular Tat. Inflammatory cytokines activate vessels and induce chemotactic factors that recruit monocytes and KSC

Table 3. Expression and activity of cytokines in PEL

Factor	Expression[a] *in vivo*[a]	Expression[a] *in vitro*[a]	Relevant effects for PEL growth/pathogenesis
IL-6	+	+	(?) Autocrine growth effects *in vitro*. PEL cell growth is inhibited by blocking IL-6R and/or gp130. Autocrine growth factor *in vivo* (in a mouse model of PEL).
IL-10	+	+	(?) Autocrine growth effects *in vitro*. Lack of growth effects by recombinant IL-10.
vIL-6	+	+	Expressed by most PEL cells *in vivo*. (?) Autocrine growth effects *in vitro*. PEL cell growth inhibited by blocking IL-6R and/or gp130.
VEGF/VPF	n.d.	+	Does not act as a growth factor for PEL cells *in vitro*. Neutralizing antibodies prevent the formation of PEL-like ascites in mice.

[a] Expression in PEL tumors and in cultured PEL cells. n.d., not done; (?) conflicting data IL-6R, IL-6 receptor.

progenitors into tissues, induce these cells to differentiate into macrophages, dendritic cells or KSC with an endothelial macrophage phenotype, and promote EC and KSC proliferation, angiogenesis and edema due to the induction of angiogenic factors.

Inflammatory cytokines reactivate a HHV-8 infection that participates in KS development probably by inducing systemic and local immune responses that, in turn, exacerbate the KS reactive processes. However, inflammatory cytokines can be detected in KS lesions and uninvolved tissue even prior to HHV-8, suggesting that HHV-8-infected cells are recruited into tissues in response to KS reactive processes, rather than initiating KS. Several lines of evidence indicate that HHV-8-encoded factors with paracrine effects are unlikely to play a role in KS initiation, although they may participate in KS progression.

HHV-8 latency genes, host oncogenes, and oncosuppressor genes appear to play a role in KS progression due to their long-lasting expression and their growth or anti-apoptotic properties. The Tat protein of HIV-1 is a progression factor for AIDS-KS, and may be responsible for the higher frequency and aggressiveness of KS in the setting of HIV infection.

PEL pathogenesis is also associated with HHV-8 infection and, as in KS progression, HHV-8 latency genes may be involved in PEL cell transformation toward a neoplastic phenotype. Owing to their clearly neoplastic nature, PEL cells are less dependent on cytokines and growth factors than hyperplastic KSC. However, host and HHV-8-encoded cytokines may play a role in PEL development *in vivo*. Despite the neoplastic phenotype of PEL cells, however, no specific mutations or rearrangements of oncogenes or oncosuppressor genes have so far been associated with PEL development, although recurrent chromosomal changes may suggest the involvement of genetic changes that may play a key role in PEL cell transformation. Additional studies are required to fully understand PEL etiopathogenesis, particularly concerning the possible role of cytokines and viral factors in the transformation of PEL cells from post-germinal center B cells into pre-terminally differentiated neoplastic cells and their role in PEL cell trafficking and homing to the serous membranes.

A complete understanding of the pathogenesis of these HHV-8-associated diseases is required for the development of appropriate therapy for KS and PEL, targeting specific factors including cytokines, growth factors, viral proteins, molecules involved in the homing of circulating KSC or PEL cells, oncogenes, and oncosuppressor gene expression.

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