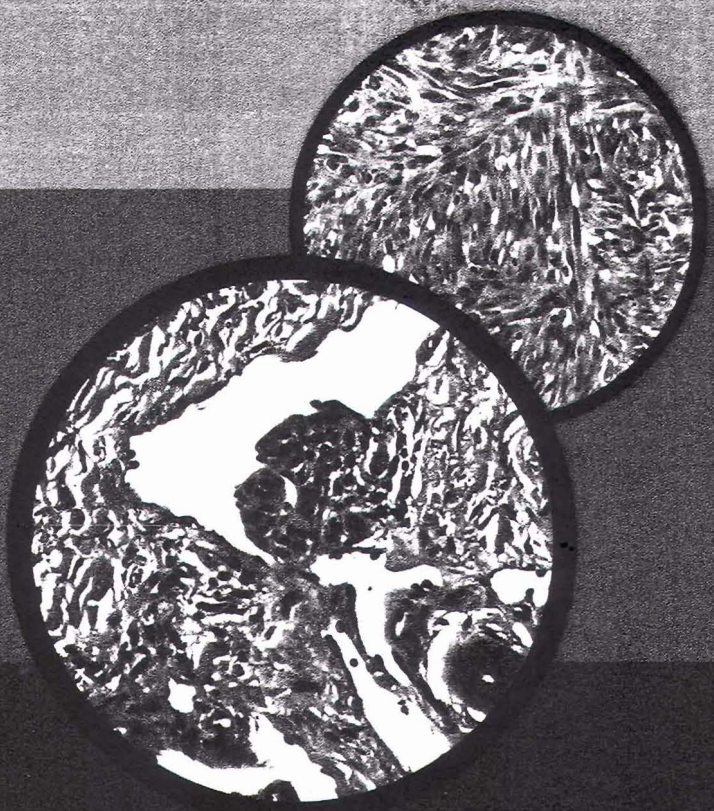

KAPOSI SARCOMA

A MODEL OF ONCOGENESIS

EDITORS: LIRON PANTANOWITZ, JUSTIN STEBBING & BRUCE J. DEZUBE

RESEARCH SIGNPOST

Kaposi Sarcoma: A Model of Oncogenesis

2010

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6. The formation of new blood vessels in Kaposi sarcoma

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Summary. Abnormal and intense new blood vessel formation is a general feature of all clinical-epidemiological forms of Kaposi sarcoma (KS). This chapter summarises key aspects of the scientific literature regarding the genesis of new vessels in KS lesions, in an effort to draw a unifying view of KS initiation and progression. The basic mechanisms leading to blood vessel generation, including angiogenesis and vasculogenesis, are discussed focusing on the growth factors, integrin receptors, and extracellular matrix (ECM)-degrading proteinases which are altered and/or de-regulated in KS. Features of vessels present in early- and late-stage KS lesions are illustrated, paying a particular attention to the role that the KS spindle-shaped cells resembling activated endothelial cells or lymphatic endothelial macrophages play in KS-associated vasculogenesis or angiogenesis. We have also reviewed the pro-angiogenic pathways triggered in KS by oncogenes and oncosuppressor gene products, cytokines and chemokines, Human Immunodeficiency Virus (HIV) Tat protein, and Human Herpesvirus 8 (HHV8). Finally, we have reported about anti-angiogenic molecules which had been or are currently being evaluated for KS treatment. In particular, we have emphasised HIV Protease Inhibitors (PI), a class of antiretroviral drugs effectively inhibiting KS onset or promoting KS regression. In fact, due to their capability of blocking both new blood vessel formation and tumor cell invasion, fundamental steps of tumor progression, PI should be considered as promising anti-cancer tools.

1. Introduction

1.1. Physiologic new blood vessel formation

In the human body, cells are generally located within 200 μm from blood vessels, which is the maximal distance allowing the diffusion of oxygen and nutrients: when tissues

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and organs grow, the 200 μm limit is exceeded, and new blood vessels have to be formed [1]. During human embryonic development, vessels are generated mainly by vasculogenesis, which is accomplished through the mobilization of endothelial cell precursors (ECP) from yolk sack or bone marrow, their homing to sites of new vessel formation, and their differentiation into mature endothelial cells (EC) [1]. Later, new capillaries sprout from existing vessels through a multi-step process termed angiogenesis [1].

Angiogenesis is started when EC degrade and penetrate the blood vessel basement membrane (BM) and, subsequently, the peri-vascular extracellular matrix (ECM): this is followed by EC proliferation and migration toward chemotactic stimuli, leading to EC organization into solid cords [1]. Subsequently, vacuoles form inside endothelial cords, which then evolve into tubes with hollow lumens permitting blood flux [1]. As a consequence of EC shear stress, pericytes are recruited at angiogenic sites, and produce molecules forming the new blood vessel BM [2]. Upon their adhesion onto the reconstituted BM, EC become quiescent, and newly formed capillaries are stabilised [1].

Both vasculogenesis and angiogenesis are regulated by chemical signals [3]. Promoters of new blood vessel formation (the angiogenic factors) include molecules inducing one, few, or all vasculogenic or angiogenic steps [3]. Some molecules act directly on ECP or EC, while others stimulate local stromal or immune cells to release angiogenic factors [3]. Among human angiogenic factors, the vascular endothelial growth factors (VEGF) and the fibroblast growth factors (FGF) are particularly effective at promoting ECP and EC survival, growth, and motility [4,5]. Upon release by producing cells, angiogenic factors remain diffusible or bind to ECM components [4,5]. In most cases, ECM sequesters the angiogenic molecule without compromising its biological activity. For example, ECM-bound, immobilized FGF or VEGF drive migrating EC, guiding the formation of EC cords [4,5]. However, some ECM components, such as thrombospondin (TSP)-1, can prevent FGF or VEGF binding to their receptors [4,5].

Indeed, although it is triggered by soluble factors, new blood vessel formation is modulated by ECM components. In particular, the interactions occurring between EC and the ECM are mediated by the integrins, a family of transmembrane adhesion receptors composed by α and β subunits [6]. Among the integrins, $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 5\beta 1$, $\alpha v\beta 3$ and $\alpha v\beta 5$ are intensely expressed at angiogenic sites, their levels and/or affinity being up-regulated by angiogenic factors [6]. When angiogenesis is started, EC are exposed to both interstitial collagen and non-collagen proteins: the latter are recruited from plasma because of the increase in vascular permeability and form a provisional matrix [7]. EC adhesion to provisional matrix triggers the above mentioned pro-angiogenic integrins, activating signalling pathways which, in turn, support all angiogenic steps [6]. The cross talk of pro-angiogenic integrins and the VEGF or FGF receptors (VEGFR and FGFR) increases the signalling of all these molecules associated with EC migration, survival and growth [6]. In addition to integrin-mediated adhesive interactions, angiogenesis requires ECM remodelling: i.e., degradation of the pre-existing BM and ECM and synthesis of novel BM and ECM [1,2]. When EC invasion is impaired angiogenesis is blocked [8].

EC penetrate the blood vessel BM and perivascular ECM because of the activity of ECM-degrading enzymes. Among them are heparanases, serine proteases (including plasmin and its activators), and cysteine proteases (such as the cathepsins) [8]. However, angiogenesis mostly depends on the matrix-metalloproteinases (MMP), a family of zinc-containing enzymes which digest ECM components including collagens, laminin, fibronectin, and proteoglycans [8]. The human MMP family comprises 23 members which possess distinct but overlapping substrate specificities: all MMP share a basic structural organization comprising a signal peptide that targets them for secretion, a

hemopeptide domain and an N-terminal catalytic domain [8]. MMP activity is regulated at four points gene expression, compartmentalization (i.e., pericellular accumulation of enzyme), pro-enzyme activation, and enzyme inactivation - and it is further controlled by substrate availability and affinity. Specifically, MMP are synthesized as pro-enzymes, and then secreted in a diffusible form, or anchored to the cell membrane (membrane-type MMP, MT-MMP) [8]. Subsequently, soluble MMP pro-forms are activated through the detachment of the hemopexin domain promoted by proteinases including other MMP. For example, MMP-3 processes proMMP-1 into fully active MMP-1 [9].

MMP activity is inhibited principally by the Tissue Inhibitors of MMP (TIMP). Four TIMP have been isolated to date: types-1, -2, and -4 are secreted, diffusible proteins which impair the activity of most soluble MMP by binding them in a 1:1 stoichiometry; by contrast, TIMP-3 is a matrix-associated molecule which inhibits some MT-MMP [10,11]. MMP can also be inhibited by procollagen C-terminal proteinase enhancer, reversion-inducing cystein-rich protein with Kazal motifs, tissue factor pathway inhibitor-2, α2-macroglobulins, and TSP-1 or -2 [10,12,13].

MMP are expressed by quiescent EC at a very low level: when angiogenesis is triggered, EC expression of MMP is up-regulated by growth factors, cytokines, and/or mechanical stress [8]. Major roles in initiating angiogenesis have been attributed to MT1-MMP, MMP-2, and MMP-9. Interestingly, MMP-2 and/or MT1-MMP are present on the EC surface in association with the $\alpha v\beta 3$ integrin [14,15]. In addition, MT1-MMP can co-localize with β1-integrins in EC-EC contacts [16], while MMP-9 interacts with the CD44 cell adhesion molecule [17]. These multi-protein complexes help in focusing MMP proteolytic activity. Besides their ability of degrading the ECM to create space for migrating EC, active MMP: 1) generate pro-migratory ECM fragments; 2) process integrins (such as $\alpha v\beta 3$) in a manner that increases their affinity for the ECM and, thereby, their pro-angiogenic activities; 3) convert latent forms of angiogenic cytokines (e.g. interleukin-8 and transforming growth factor) into an active form; and 4) release ECM- or cell surface-bound inflammatory mediators or angiogenic factors (including FGF and VEGF) into a soluble form [8,18,19]. Moreover, MMP influence EC growth. This is because MMP can break EC-EC adhesions (by digesting intercellular adhesion receptors), and detach pericytes (which produce EC growth inhibitors) from the outer side of blood vessel [20].

1.2. Aberrant new blood vessel formation in KS

In the healthy adult vasculogenesis is extremely rare [21], while angiogenesis occurs almost exclusively in association with tissue repair or female reproductive cycles [3]. This is due to the activity of angiogenesis repressors which, by counteracting angiogenic factors, pro-angiogenic integrins, and ECM-degrading proteases, cause EC apoptosis and/or inhibit EC motility. Negative regulators of new blood vessel formation comprise natural antagonists of angiogenic factors (angiopoietin-2), soluble forms of angiogenic factor receptors (VEGFR1), inhibitors of ECM-degrading enzymes (TIMP), ECM components (TSP-1), or ECM fragments (angiostatin and endostatin) generated by MMP [3,8,22].

In chronic inflammation, metabolic syndromes, or tumors, the balance between negative and positive regulators of angiogenesis is altered in favour of the positive ones, and aberrant new blood vessel formation occurs [3]. This is particularly prominent in KS, a disease characterized by multiple skin or mucosal angioproliferative lesions, which in time evolve into nodular, angiofibrosarcomatous lesions [reviewed in ref. 23]. Indeed, the

Table 1. Features of blood vessels present in late-stage KS lesions.

- Vessel BM is discontinuous: vascular permeability is increased, activated leukocytes and plasma-derived ECM proteins are recruited in KS lesions
- Vessel wall is composed of a mosaic of EC, E-KSC, and PB-KSC which align together in a VE-cadherin-dependent fashion
- Vessels are very unstable, partly because of their high expression of angiopoietin-2
- Vessels are tortuous, occasionally have a dilated diameter, or are compressed by proliferating KSC

Abbreviations are: BM, basement membrane; EC, endothelial cells; ECM, extracellular matrix; E-KSC, endothelial- KS cells; KSC, KS cells; PB-KSC, peripheral blood- KS cells; VE-cadherin, vascular endothelial-cadherin.

formation of new capillaries is very intense at all stages of KS progression and in all KS forms, including classical, post-transplant, African or AIDS-associated KS [24-27]. In the initial stage of KS progression, newly formed capillaries have a regular, continuous wall [24,26]. Soon afterwards, however, the vessel BM becomes discontinuous and locally disrupted: this leads to an increase in vascular permeability that, in turn, causes the recruitment of activated leukocytes and plasma-derived ECM proteins (including fibrin, fibronectin, and vitronectin) in KS lesions [25,27] (**Table 1**). Within a short time, proliferating cells with an elongated ("spindle") shape appear in the lesions, becoming the KS histological hallmark (KS cells, KSC) [23].

KSC are a quite heterogeneous cell population (**Figure 1**). In fact, some of them express macrophage markers such as PAM-1, CD68 and CD14 (macrophagic KSC, M-KSC), while others display markers of activated EC (endothelial KSC, E-KSC) of either blood vessel or lymphatic origin [26,28]. EC markers include factor VIII-related antigen (FVIII-RA), *Ulex europaeus* lectin-1 (UEA), vascular endothelial (VE)-cadherin, platelet endothelial cell adhesion molecule (PECAM)-1 (or CD31), intercellular adhesion molecule (ICAM)-1, D2-40, LYVE-1, VEGFR3 and/or the pro-angiogenic ephrin-1 [26,28,29]. Finally, some KSC express both macrophage and endothelial markers, resembling the so-called endothelial macrophages of lymph node sinuses [26,28].

Irrespective of these phenotypic traits, KSC present in all forms of KS, in either early- or late –stage lesions, are highly proliferating and invasive [30,31]. After the pre-existing ECM of the peri-vascular area has been destroyed, invading KSC secrete a provisional matrix which is similar to that present in early-stage wound healing, being composed by fibronectin, tenascin and collagen-VI [32,33]. Owing to the expression of several pro-angiogenic integrins [26,28], KSC adhere on to the provisional matrix as a support for migrating and proliferating. At the same time, KSC internalize plasma proteins, including fibrinogen [34].

In late-stage KS lesions, KSC become the predominant cell type [23]. Bundles of collagen-I line the spaces between KSC, while BM components including laminin and collagen-IV are distributed at the interface between KSC or EC and the collagen-I bundles [25,35]. At this stage of KS progression, KSC loose EC markers such as FVIII-RA and UEA [36] and, unlike KSC from early stage lesions, they can display features of transformed cells [37-39]. In addition, KSC establish stable VE-cadherin-mediated cell-to-cell contacts with EC or other KSC forming channels which allow blood influx. These channels (the abnormal vessels of late-stage KS), are quite unstable due to the high expression of angiopoietin-2 [33,35,40]. Because of the above mentioned features, KS

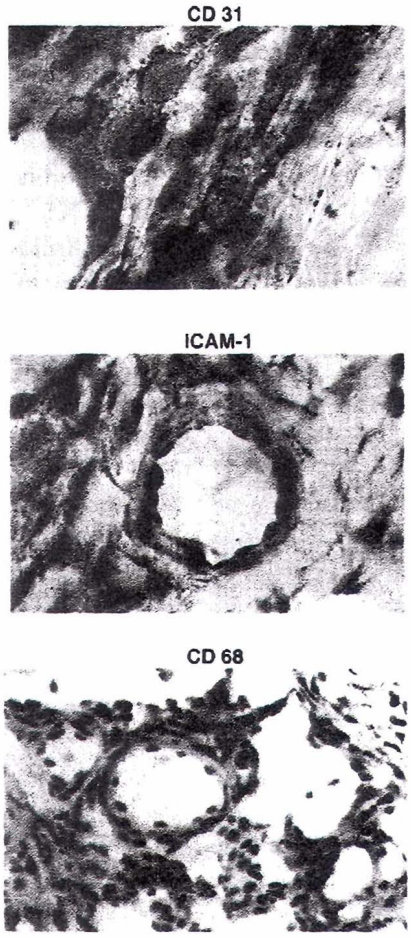

Figure 1. *KS spindle cells (KSC) express both endothelial and macrophage markers.* KSC present in a human KS lesion stain for the endothelial cell marker CD31, the activated endothelial cell marker ICAM-1, and the macrophage marker CD68 (immunohistochemistry, alkaline phosphatase anti-alkaline phosphatase, 100 X magnification). Modified from Ensoli et al. [23], and reproduced in part by kind permission from Elsevier Company.

abnormal blood vessels may favor thrombosis, and/or compromise immune cells homing and binding to EC, extravasation and cytotoxicity. Moreover, these aberrant vessels are tortuous, and sometimes present with a dilated diameter or are compressed by proliferating, densely aggregated KSC [33,35,40]. This impairs blood supply, causes hypoxia and acidosis, and interferes with drug delivery, thus rendering KSC resistant to cytotoxic drugs [3].

Although the intense new blood vessel formation which characterizes KS is generally believed to depend upon neo-angiogenesis [24-27], a particular form of vasculogenesis occurs in KS patients [41,42]. Specifically, adherent elongated, KSC-like cells differentiate from cultured peripheral blood mononuclear cells of patients with KS or individuals at risk for developing KS [41-45]. As for lesional E-KSC, the peripheral

blood KSC (PB-KSC) express both ECP and EC markers (including CD34, UEA, FVIII-RA, PECAM-1, ICAM-1, VEGFR2 and endothelial nitric oxide synthase), or endothelial macrophage markers (VE-cadherin and CD68 or the mannose receptor), while they are negative for the leukocyte markers CD14 and CD45 [41-45]. Most likely, PB-KSC are mobilized from the bone marrow into the blood in response to angiogenic growth factors released by either KSC or activated leukocytes infiltrating KS lesions [41,43,46-54]. This may explain the presence of multiple KS lesions at different body sites. These cells are believed to be KSC progenitors which precede over KS development, and evidence indicates that they can initiate KS lesions when transmitted by otherwise KS free organ donors to transplant recipients [41-45]. Furthermore, as for ECP [55], the spindle-shaped morphology and the expression of endothelial intercellular adhesion molecules [41,42] could allow PB-KSC to align and form, together with lesional EC and/or E-KSC, cord-like structures which will ultimately differentiate into the new, abnormal vessels.

2. Mechanisms for KS-associated new blood vessel formation

The intense, aberrant formation of new capillaries which characterizes KS results from the concurrent action of several factors, many of which are also involved in angiogenesis associated with other tumors and/or microbial products (**Table 2**).

Table 2. Pathways leading to KS-associated abnormal new blood vessel formation.

- Ras oncogene activation: up-regulation of VEGF expression
- PTEN functional loss: PI3K/Akt activation, EC invasion and proliferation
- p53 inactivation: reduction of p53-induced, anti-angiogenic TSP-1; increase in p53-repressed, pro-angiogenic Bcl-2, FGF-2 and MMP-1
- HIF-1α stabilization: up-regulation of VEGF and VEGFR expression
- Increase in inflammatory mediators levels: 1) up-regulation of pro-angiogenic chemokines and angiogenic factors promoting angioproliferative lesion development; 2) increase in the number of circulating PB-KSC; 3) acquirement of the KSC phenotype by EC; 4) KSC proliferation; 5) up-regulation and activation of MMP and other ECM-degrading proteases
- EC and/or KSC infection by HHV8: 1) NF-kB, AP-1, Ets-1 and HIF-1 activation upregulating the expression of angiogenic chemokines, angiogenic factors, and MMP; 2) oncogene deregulation and tumor suppressor functional loss leading to EC/ KSC invasion, survival, uncontrolled growth and, probably, transformation
- HIV-1 Tat release by acutely infected lymphocytes and/or macrophages: stimulation of EC/KSC survival, growth and locomotion through a molecular mimicry of ECM molecules, VEGFR2 triggering and extracellular-bound FGF release into a diffusible form

Abbreviations are: EC, endothelial cells; ECM, extracellular matrix; FGF, fibroblast growth factor; HIF, hypoxia inducible factor; HHV8, human herpesvirus 8; KSC, KS cells; MMP, matrix metalloproteinase; NF-kB, nuclear factor kappa-B; PB-KSC, peripheral blood-KS cells; PI3K, phosphatidylinositol-3 kinase; PTEN, phosphatase and tensin homolog; TSP, thrombospondin; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

2.1. Oncogenes and oncosuppressors

Tumor angiogenesis can be preceded by proto-oncogene mutation into oncogenes, and/or by the functional impairment of tumor suppressor genes [3]. In KS lesions, the Ras oncogene is activated, and this could contribute to the up-regulation of VEGF expression detected in all KS forms [56]. Moreover, the function of the phosphatase and tensin homolog (PTEN) tumor suppressor is often lost in KS [57]: as for other types of tumor, this is followed by the activation of the phosphatidylinositol-3 kinase (PI3K)/Akt pathway, leading to EC invasion and proliferation [58]. Of note, the hypoxia inducible factor (HIF)-1α, which also plays a key role in tumor angiogenesis by activating both VEGF-A and VEGFR2 gene transcription, is expressed in KS lesions, and this directly relates to KS clinical progression [59]. However, at variance with many human tumors [60], the p53 tumor suppressor is not mutated in KS [61]. Nevertheless, the p53 protein is expressed at very low levels in early-stage KS lesions [62,63], and/or it is found in the cytoplasm of KSC, suggesting p53 functional inactivation [64]. Furthermore, although p53 protein levels progressively increase in late-stage KS lesions [65,66], this is associated with the expression of Mdm-2, a potent inhibitor of p53 function [67]. Consistently, the p53-induced anti-angiogenic TSP-1 is reduced or absent in KS lesions [26,68], while the p53-inhibited pro-angiogenic Bcl-2 is highly expressed [63]. Loss of p53 function could also explain the increase in FGF and MMP levels accompanying KS development and progression [69,70].

2.2. Inflammatory cytokines and angiogenic factors

Clinical evidence indicates that all individuals with KS or at risk for developing KS (AIDS-KS patients included) are characterised by a disturbance of the immune system that leads to CD8+ T cell activation [48,52,71-73]. Early lesions from all KS forms are infiltrated by a large number of activated leukocytes which release high levels of inflammatory mediators, including interleukin (IL)-1, IL-6, tumor necrosis factor (TNF), and interferon (IFN)-γ [48,50,52,53]. As for infiltrating leukocytes, EC activated by inflammatory cytokine produce several chemokines (MCP-1, RANTES, IP-10, MIP-1α, and MIP-1β) which attract granulocytes, lymphocytes, monocytes and macrophages [46]. These inflammatory cells, in turn, produce additional cytokines and chemokines, thus amplifying the inflammatory reaction associated with KS initiation. At this time, KSC are also usually detected in lesions. Importantly, inflammatory cytokines either favour the trans-differentiation of peripheral blood mononuclear cells into PB-KSC [41,43], or induce EC to acquire features of the KSC phenotype, including the elongated shape, the expression of ICAM-1, and the disappearance of FVIII-RA [48,51,74,75]. As for cytokine-activated EC, KSC also produce chemokines acting in an autocrine fashion on KSC, which express both the CXCR and CCR chemokine receptors [76-80]. Finally, inflammatory mediators stimulate EC, leukocytes, lesional KSC, and PB-KSC to synthesise angiogenic factors which, in turn, promote the development and progression of KS angioproliferative lesions.

In KS lesions, the most abundant angiogenic factors are FGF-2 and VEGF-A [47,51,54]. These two potent promoters of angiogenesis are produced and released by infiltrating leukocytes, KSC, and inflammatory cytokine-activated EC, and they are present at high levels in sera from KS patients [78,81]. FGF-2 stimulates the motility and growth of both EC and KSC, while VEGF-A enhances FGF-2 effects, and is responsible for vascular permeability and edema formation [47,51,54]. Other angiogenic molecules

expressed in KS lesions are scatter factor/hepatocyte growth factor and platelet-derived growth factor, which both stimulate KSC locomotion and growth [49,53], and insulin-like growth factor, that stabilizes HIF-1α, hence promoting VEGF-A expression [59]. Platelets adhere to vessels damaged upon KSC and EC invasion, and release angiogenic factors including VEGF, platelet-derived endothelial cell growth factor and transforming growth factor β [82]. In such a milieu, recruited granulocytes release platelet activating factor, which, in turn, promotes KSC locomotion [83].

Inflammatory mediators and angiogenic factors synergize in activating the expression and/or function of pro-angiogenic enzymes with a role in KS progression. Among them are cyclooxigenase-2 and endothelial nitric oxide synthase [84,85], both expressed at high levels in lesions from all forms of KS [86,87]. Other enzymes up-regulated by inflammatory mediators and/or angiogenic factors are ECM-degrading proteinases. In particular, activated EC, leukocytes or KSC (from all forms of KS, at either early or late-stages) synthesize and secrete high levels of plasminogen activators (PA) and/or MMP including MMP-1, -2, -3, -7, -9, and -13 [31, 81,88-92] (**Figure 2**). With regard to MMP-2 and MMP-9, high levels of these enzymes are also detected in sera from KS patients [81,88,92]. Both PA and MMP are key, not only for invasion, but also for the proliferation of EC or KSC. In fact, MMP break intercellular adhesions and release ECM-bound FGF-2 and/or VEGF-A in a diffusible form [89-91].

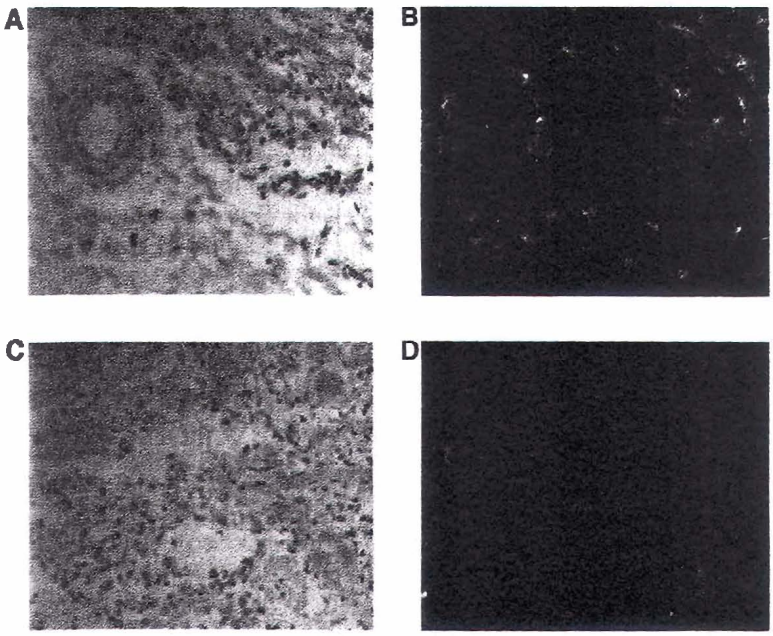

Figure 2. *The matrix metalloproteinase (MMP)-2 is highly expressed in KS lesions.* Thin sections of a human KS lesion had been hybridised with a ^{35}S-radiolabeled antisense MMP-2 RNA probe, as previously described [92]. Bright field and dark field show MMP-2 expression at the periphery (panels A and B) or in the center (panels C and D) of the nodular lesion. Modified from Toschi et al. [92], and reproduced in part by kind permission from the American Society for Cell Biology (ASCB).

2.3. Human herpesvirus 8 (HHV8)

HHV8 is present in a latent form in EC and KSC in all forms of KS [93]. PB-KSC are also infected and, because of their capability of supporting productive viral replication, they represent a reservoir of HHV8 [42-44]. Evidence also indicates that inflammatory cytokines upregulated in KS induce HHV8 reactivation in latently infected cells [43]. This leads to viremia and spreading of the virus to all circulating cell types including monocytes and T cells [43,52]. Reactivation of HHV8 in inflammatory cells infiltrating KS lesions may also be key for the infection of resident EC and KSC, and may lead to the increased viral load that is observed as lesions progress from early to late-nodular stages [43,94,95]. Inflammatory cells also promote the recruitment of HHV8 infected cells into tissues via the activation of the vascular endothelium and induction of chemokines [96,97]. Finally, viral proteins with potential paracrine effects on KS lesion formation are produced during lytic gene expression [97].

HHV8 deeply modifies EC physiology, favoring KS-associated abnormal angiogenesis. In particular, as compared to control EC, HHV8-infected EC, or EC exposed to HHV8 products, synthesize low amounts of the anti-angiogenic TSP-1 [98], and produce high levels of angiopoietin-2, pro-angiogenic chemokines, VEGF, cyclooxigenase-2 and MMP [46,85,99-106]. Consistent with these findings, injection of HHV8-infected EC in mice promotes the development of angioproliferative KS-like lesions [107].

The acquirement of such a strong angiogenic phenotype by EC depends on HHV8 capability of activating the Nuclear Factor-kappa B (NF-kB), Ap-1, Ets-1 and HIF-1α transcription factors [46,99,103,104,106,108-111]. At the same time, HHV8 deregulates the Ras proto-oncogene [108], and/or functionally impairs the PTEN, VHL and p53 tumor suppressors, leading to EC invasion, migration, survival and uncontrolled growth [61,109,112-116]. Noticeably, upon infection by HHV8 or exposure to HHV8 proteins, EC no longer express typical endothelial surface makers, and acquire an elongated shape [99,102,110,111,117]. This phenotype, together with the increase in invasive and angiogenic properties, makes HHV8-infected EC very similar to KSC.

Indeed, the long-lasting functional deregulation of cellular transcription factors, proto-oncogenes and/or oncosuppressors promoted by HHV8 could favor EC or KSC transformation, and KS progression toward a real tumor. In fact, although KSC are considered to represent KS tumor cells, they display features of transformed cells only when they are obtained from late-stage KS lesions [37-39]. This suggests that KS, prior to evolve into a true sarcoma, initiates as a reactive process. Indeed, the finding that in early KS lesions only a small fraction of EC or KSC is infected by HHV8, whereas in late stage lesions most EC and KSC are infected [93,118-120], strongly supports a pathogenetic role for HHV8 after KS initiation. In this regard, it has to be highlighted that the activation of CD8+ T cells triggering KS development can be followed by an impairment of the immune cytotoxic responses [121,122], which may favor the escape of HHV8-infected EC and KSC from immune surveillance.

2.4. HIV-1 Tat protein

The higher incidence and aggressive nature of KS in HIV-infected individuals suggests that HIV-1 could have a role in KS development and progression [123]. However, the finding that EC infection by HIV occurs only in specific anatomical districts and that this is followed by EC death [reviewed in ref. 124], indicated that if HIV

had any role in KS pathogenesis, this should be indirect. Consistently, several *in vitro* and *in vivo* studies had shown that the Tat protein of HIV-1, a trans-activator of the viral gene expression, effectively links HIV-1 infection and the highly aggressive AIDS-KS [reviewed in ref. 125]. In fact, upon its release by acutely infected cells, Tat promotes the motility and growth of activated EC and KSC [125]. These angiogenic, KS-promoting effects are mediated by two Tat regions which recognize receptors expressed on activated EC or KSC surface: the arginine-glycine-aspartic acid sequence, which binds pro-angiogenic integrins, and Tat highly basic residues that interact with VEGFR2. Tat binding to these receptors triggers intracellular signals leading to cell survival and PA/MMP activation [125]. In addition, Tat basic residues compete with ECM-bound FGF-2 for heparin-binding sites, retrieving the sequestered angiogenic factor into a soluble form which, in turn, promotes angiogenesis and KSC proliferation [125]. In fact, Tat synegises with FGF-2 in inducing angioproliferative, KS-like lesions in mice [125].

3. Pathogenetic and anti-angiogenic therapies for KS

Treatment of KS must be determined according to the patient status and to his/ her overall disease. However, at present there is no uniform treatment for KS, nor is there a consensus regarding best treatment. Local therapies, such as surgical removal, laser therapy, cryotherapy, radiation therapy, or topical chemotherapy are generally indicated for patients with early disease, characterised by limited extension and confined to the skin [126,127]. Although these therapies are easy to perform and relatively safe, KS recurrences are frequent. Patients with wide-spread or recurrent KS require extensive treatment including radiotherapy and systemic cytotoxic chemotherapy. As a general rule, the same treatment modalities apply to all KS forms, while response rates and their duration may vary [128-130]. However, the adverse side-effects of cytotoxic chemotherapy are a major concern, particularly in elderly KS patients [128-130].

In recent years, progress in understanding the mechanism underlying KS pathogenesis has fostered a great number of preclinical and clinical studies based on the use of molecules directed against KS pathogenetic targets. The strong association of HHV8 with KS, including individuals at risk of developing KS [93], suggested that drugs directed against this virus could be effective in KS treatment and/or prevention. In one study, the risk of developing KS was decreased in individuals receiving ganciclovir, while foscarnet-treated patients had a delayed KS clinical progression [131]. Several other studies, however, found no correlation between anti-HHV8 therapy and a decreased risk of KS development [132-134].

Since angiogenesis plays such a prominent role in KS development, other clinical trials were designed at targeting pro-angiogenic cytokines and MMP (**Table 3**). Other anti-KS therapeutic approaches employed anti-angiogenic molecules including thalidomide, IFNα, or IL-12. Specifically, phase II clinical studies of oral thalidomide (100-1000 mg/day for 8 weeks) showed a dose-dependent response in 35-40% of treated AIDS-KS patients [135,136]. Thalidomide inhibits FGF-2 and VEGF activity [4], and reduces TNFα production by monocytes, while it increases IL-12 expression [137]. Similarly, AIDS-KS treatment with IFNα (at doses as high as 20 million U/m2 body surface area) led to tumor reduction in up to 38% of patients [138]. This is consistent with results from *in vitro* studies indicating that IFNα decreases both FGF-2 and MMP-9 gene expression [137], inhibits HIV replication and HHV8 reactivation [139-141], and increases NK and monocyte-mediated cytotoxicity against KS-derived targets [138]. More promisingly, IL-12 (100-625 ng/kg, subcutaneously, twice a week) gave a response

Table 3. Anti-angiogenic molecules evaluated for KS treatment.

IFN-α: immunomodulator, antiviral, FGF-2 and MMP-9 inhibitor
IL-12: immunomodulator, IP-10 and Mig inducer
Thalidomide: anti-inflammatory, FGF and VEGF inhibitor
SU5416: VEGFR2 antagonist
IM862: VEGF inhibitor; promoter of NK cell activity
TNP-470: inhibitor of retinoblastoma protein
Imatinib mesylate: inhibitor of c-kit and platelet-derived growth factor receptor
Sorafenib and sunitinib: tyrosine kinase inhibitors
Bevacizumab: monoclonal antibody to VEGF
COL-3: inhibitor of MMP activity, EC and tumor cell invasion
Protease inhibitors: anti-inflammatory; inhibitors of MMP activity, EC and tumor cell invasion.

Abbreviations are: EC, endothelial cells; FGF, fibroblast growth factor; IFN, interferon; IL, interleukin; IP, interferon-inducible protein; MMP, matrix metalloproteinase; NK cell; natural killer cell; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

rate of 71% in AIDS-KS patients [142]. This could depend on IL-12 capability of increasing cytotoxic T lymphocyte and NK cell activity [142], and inducing the production of the anti-angiogenic factors IP-10 and Mig [143,144].

Additional anti-angiogenic compounds which were evaluated in clinical trials in AIDS-KS include TNP-470, a synthetic analogue of fumagillin functionally impairing the retinoblastoma protein [145] and imatinib mesylate, which targets c-kit and platelet-derived growth factor receptor signalling [146]. In particular, when administered at escalating doses (10-70 mg/m2, iv weekly), TNP-470 promoted a partial response in 18% of early-stage AIDS-KS patients, and was well-tolerated even at the highest dose tested [147]. Imatinib mesylate oral treatment (600 mg/day) resulted in partial clinical regression in 50% of KS patients [146]. Interestingly, the finding that only leukemia cells displaying wild-type p53 respond to imatinib [148], suggested that imatinib-resistant KSC have inactive p53. A confirmatory phase II study is in progress. Further anti-angiogenic molecules under evaluation in AIDS-KS patients include the tyrosine kinase inhibitors sorafenib and sunitinib [149], the VEGFR-2 inhibitor SU5416 [150], and bevacizumab, a monoclonal antibody to VEGF [151].

Recently, clinical and epidemiological studies reported a reduced KS incidence and risk of progression in HIV-infected individuals receiving PI-based therapeutic regimens [94,152,153]. By efficiently blocking the production of infectious HIV particles [154], in fact, PI reduce the de-regulation and/or the functional impairment of the immune system, which are key to AIDS-KS initiation and progression, respectively [155]. In addition, PI capability of suppressing HIV replication [154], suggested that the PI inhibitory effect on AIDS-KS possibly depends on a decrease in the levels of HIV-1 Tat protein, a potent promoter of angiogenesis and KS development or progression. However, other work indicated that PI can also inhibit tumor progression in HIV-free experimental models, independently of the immune system [156-160; reviewed in ref. 155]. This raised the possibility that PI could target cellular pathways involved in tumor induction and/or progression. In this regard, the PI indinavir (IDV) or saquinavir (SQV) had been shown to block EC and KSC invasion and MMP activity both *in vitro* and *in vivo*, at

concentrations which are similar to the steady-state or to the peak concentrations present in plasma from treated patients [161]. Due to these actions, IDV or SQV impair angiogenesis, thus inhibiting the development of KS-like lesions, or induce the regression of established KS in animal models [161]. These results are supported by PI capability of affecting the activity and/or expression of several human molecules [157,159,162-173].

When investigating on the anti-angiogenic, anti-KS effects of IDV or SQV, it should be considered that most of the molecules promoting angiogenesis and KS, namely inflammatory cytokines, angiogenic factors, HHV8 proteins or HIV-1 Tat, also activate the NF-kB transcription factor [46,99,108,110,111,159,174-183]. The main mediator of NF-kB activation is the cellular proteasome, a protease complex that regulates intracellular proteins turnover [reviewed in ref. 184]. For example, TNFα or IL-1β stimulate the cellular proteasome to degrade the IkB proteins which, by anchoring NF-kB in the cytoplasm, block it trans-activating functions [179, 184]. As a consequence of IkB proteins degradation, NF-kB translocates into the nucleus, and this is followed by the increase in the expression of NF-kB-targeted genes, including those encoding for MMP [175,176,179,185-192]. In agreement with these findings, inhibitors of the cellular proteasome reduce or block NF-kB activation, MMP expression and EC or tumor cell invasion *in vitro* [179,188,190,191,193], and angiogenesis and tumor growth *in vivo* [194-196]. The anti-angiogenic effect of cellular proteasome inhibitors is also consistent with the fact that, in addition to MMP, NF-kB activates the transcription of other pro-angiogenic molecules, including PA, IL-8, VEGF and VEGFR [186,192,194,197-200]. Strikingly, SQV was shown to inhibit proteasome function, NF-kB activation, and MMP expression in a wide variety of cell types, EC included [162,165-169]. In contrast, experiments on IDV gave diverging results. Specifically, IDV was found to inhibit proteasome function and NF-kB pro-invasive effects in some *in vitro* or *in vivo* experimental settings, comprising models of angiogenesis [156,160,166,170,201,202], but to leave proteasome unaffected in other systems [157,162,165,167,172]. Future work will clarify whether the inhibitory effect IDV and SQV exert on angiogenesis and KS progression occurs through the functional impairment of EC or KSC proteasome.

Based upon PI capability of inhibiting KS-promoting events both *in vitro* and in animal models, we have conducted a phase II trial for the treatment of classical (HIV-negative) KS patients with IDV. The interim analysis indicated that a favourable clinical course is associated with high drug plasma levels, and with a decrease in angiogenesis and tumor invasion surrogate markers including FGF-2 and MMP-2 plasma levels, and circulating EC. As a result, a phase II study is underway for the use of PI in combination with debulking chemotherapy in individuals with advanced, late stage, classical KS.

New blood vessel formation and tumor cell invasion are key to the progression of most human cancers [1,3]. Consistently, blocking cellular invasion and angiogenesis prevents tumor formation, and causes necrosis and regression of established tumors [3,5,8,10,13,84,149-151]. In agreement with these findings, our recent studies indicate that nude mice treatment with anti-invasive, anti-angiogenic concentrations of IDV or SQV potently inhibits the development and/or progression of a variety of prevalent human tumors including lung, breast, colon, and liver carcinoma [155,203]. In this regard, it has to be highlighted that, in addition to inhibiting angiogenesis and tumor cell invasion, PI can also impair tumor cell survival and growth [157,169]. These results, together with the large body of data on PI pharmakokinetics and with the low toxicity of these drugs, as compared to standard anti-cancer chemotherapeutics, strongly encourage the investigation and exploitation of PI as novel anti-tumor tools.

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