Angiogenic and HIV-Inhibitory Functions of KSHV-Encoded Chemokines

Chris Boshoff,* Yoshio Endo, Paul D. Collins, Yasuhiro Takeuchi, Jacqueline D. Reeves, Vicki L. Schweickart, Michael A. Siani, Takuma Sasaki, Timothy J. Williams, Patrick W. Gray, Patrick S. Moore, Yuan Chang, Robin A. Weiss

Kaposi’s sarcoma (KS) is a highly angiogenic multicentric tumor most commonly seen in immunodeficient individuals. Since the acquired immunodeficiency syndrome (AIDS) epidemic, KS has become one of the most common tumors in parts of Africa and is the most common tumor found in HIV-infected individuals (1). Compared to classic KS found in patients from Mediterranean or East European descent, KS in AIDS patients is a more fulminant disease: The angiogenic properties of the HIV-1 Tat protein have been proposed to enhance KS tumor formation (2). Two independent retinoblastoma gene products, Rb and p130, were highly expressed in KS tumors and KS cell lines (3, 4). The Rb protein inactivates the transcription factor E2F (5). Two recent studies demonstrated that carcinoma cells expressing E2F-4 could be selectively killed by an E2F-directed suicide vector (6, 7). This suggests that targeting the E2F pathway may be a potential therapeutic strategy for KS.

Unique among known human herpesviruses, Kaposi’s sarcoma-associated herpesvirus (KSHV or HHV-8) encodes chemokine-like proteins (vMIP-I and vMIP-II). VMP-I was shown to block infection of human immunodeficiency virus-type 1 (HIV-1) on a CD4-positive cell line expressing CCR5 and to a lesser extent on other expressing CCR5, CCR2, or RANTES, but not cellular MIP-1α or RANTES, were highly angiogenic in the chorioallantoic assay, suggesting a possible pathogenic role in Kaposi’s sarcoma.

1. The angiotropism of KS cells is mediated by the expression of the CCR5 chemokine receptor (5). The CCR5 molecule is a G-protein-coupled receptor that is found on the surface of immune cells and is involved in the chemotaxis of CD4+ T cells and monocytes. It is also expressed on the surface of KS cells, suggesting that it may play a role in the angiogenesis of KS.

2. KSHV DNA is present in all KS biopsies, and antibodies to this virus are detectable in all KS diseases. This suggests that KSHV is the likely etiologic agent of KS.

3. The production of KSHV-encoded chemokines, particularly vMIP-I and vMIP-II, may contribute to the angiogenic properties of Kaposi’s sarcoma cells. These chemokines can activate and chemoattract human eosinophils and mononuclear cells, eliciting a localized inflammatory response. This response can lead to the development of KS lesions.

4. The KSHV-encoded chemokines may also play a role in immune evasion strategies of the virus. By recruiting immune cells to KS lesions, KSHV may be able to evade the immune system and continue to replicate within the host.

5. The expression of KSHV-encoded chemokines in KS cells may be a potential target for therapeutic intervention. Small molecule inhibitors that block the activity of these chemokines may be effective in inhibiting KS angiogenesis and furthering the development of targeted therapies for this disease.

6. Additional studies are needed to fully understand the role of KSHV-encoded chemokines in KS pathogenesis and to develop effective therapeutic strategies targeting these molecules.
play a central role in KS tumorigenesis. In KS biopsies, KSHV is present in most of the tumor cells (so-called spindle cells) and in endothelial cells (5). Although it appears that KSHV gene expression in most of these cells is restricted to latent genes (5), a proportion of endothelial and spindle cells in KS lesions harbor hundreds of viral particles, suggesting that lytic viral infection may be necessary to drive KS lesion formation (6) and the vMIPs are expressed in KS lesions (7). KSHV is also etiologically linked to multicentric Castleman’s disease (MCD), a polyclonal lymphoproliferation associated with prominent vasculature (8).

Sequenceing of the KSHV genome has demonstrated colinearity and similarity with the gamma- oncogenic viruses Epstein-Barr virus (EBV) and herpesvirus saimiri (HVS) (9). However, unique among known human herpesviruses, KSHV encodes two genes whose products show sequence similarity to the human CC chemokine family, with highest similarity to macrophage inflammatory protein–1α (MIP-1α) and RANTES: vMIP-I [open reading frame (ORF) K6] exhibits 37.9% amino acid sequence identity and vMIP-II (ORF K4) 41.1% amino acid identity to MIP-1α (10). The amino acid identity between vMIP-I and vMIP-II is 48%, and they are more closely related to each other phylogenetically than to cellular chemokines, suggesting that they have evolved by gene duplication within the virus genome rather than by independent acquisition from the host genome. A third KSHV ORF (K4.1) is also related to the CC chemokine family, but this gene is more distantly related and probably derived independently from another member of the CC chemokine family (9, 11). Both vMIP-I and vMIP-II are expressed in latently infected lymphoma cells, and their expression is induced by phorbol esters (10).

Chemotaxic cytokines (chemokines) and their receptors play a fundamental role in leukocyte migration and activation and in hemopoiesis (12). The finding that the chemokine receptors act as coreceptors for HIV-1 entry into CD4+ cells and that the CC chemokines (MIP-1α, MIP-1β, RANTES, and eotaxin) can suppress some strains of HIV replication in peripheral blood mononuclear cells (PBMCs) and chemokine receptor–transfected cell lines has intensified interest in these proteins (13). vMIP-I can inhibit infection of some primary non–syncytium-inducing (NSI) HIV strains when cotransfected with CCR5, the recently identified coreceptor for NSI HIV-1 strains (10). We generated synthetic proteins (14) of vMIP-I and vMIP-II and assessed their capacity to inhibit HIV infection, induce calcium mobilization, and induce angiogenesis.

We investigated whether vMIP-I and vMIP-II can suppress HIV-1 replication in U87/CD4 cells (a human glioma cell line expressing HIV-1 coreceptors and in primary PBMCs (15). To define which chemokine receptors were most important for inhibition of HIV-1 infection by the vMIPs, we tested U87/CD4 cells stably expressing either CXCR4, CCR3, or CCR5 with the NSI strains SL-2 and SF162 and the dual-tropic HIV-1 strains 89.6 and 2028. Cells were fixed and immunostained for in situ p24 5 days later. p24-positive foci were counted, and average titers are shown. (B) PBMCs were challenged with 89.6 and NSI SL-2 HIV-1 strains in the absence or presence of chemokines vMIP-I, vMIP-II, RANTES, and MCP-1 at the concentrations indicated. Cell supernatants were harvested 9 days later and p24 levels measured. Average p24 concentrations are shown. Bars represent the standard error from triplicate wells. The results are representative of two and three separate experiments for strains 89.6 and SL-2, respectively.

![Image](http://science.sciencemag.org/content/sci/278/5341/1015.full)

**Fig. 1.** Inhibition of HIV replication by vMIP-I and vMIP-II. (A) U87/CD4 cells stably expressing CCR3 were treated with the chemokines vMIP-I, vMIP-II, RANTES, and SDF at the concentrations indicated. Cells were fixed and immunostained for in situ p24 5 days later. p24-positive foci were counted, and average titers are shown. (B) PBMCs were challenged with 89.6 and NSI SL-2 HIV-1 strains in the absence or presence of chemokines vMIP-I, vMIP-II, RANTES, and MCP-1 at the concentrations indicated. Cell supernatants were harvested 9 days later and p24 levels measured. Average p24 concentrations are shown. Bars represent the standard error from triplicate wells. The results are representative of two and three separate experiments for strains 89.6 and SL-2, respectively.
agonist was measured. Data are expressed as the percentage of the desensitizing agent was added 150 s before the response to the second agonist and the subsequent response to the second agonist was measured. Data are expressed as the percentage of the maximal [Ca\(^{2+}\)] induced by vMIP-II (3 nM) or eotaxin (3 nM) alone (filled columns) after treatment of the cells with various concentrations of the desensitizing agent (vMIP-II, 0.3 to 30 nM; eotaxin, 0.3 to 10 nM; RANTES, 3 to 30 nM; and MIP-1\(\alpha\), 3 to 30 nM). Values are the mean ± SEM of three separate experiments with eosinophils purified from different individuals. (Insets) Representative traces of the data from a single experiment showing the dose-response relation between the magnitude of the response to the desensitizing agent and the subsequent response to the second agonist. The desensitization was specific rather than global because none of the desensitizing agents reduced the response of the cells to a subsequent addition of the complement fragment C5a.

Whereas CCR3 is the predominant chemokine receptor through which eotaxin, RANTES, and other CC chemokines activate eosinophils (18), RANTES and MIP-1\(\alpha\) can use CCR1 (21), which is also expressed on eosinophils. To determine the receptor usage by vMIP-II on eosinophils, we performed desensitization studies. vMIP-II exhibited complete cross-desensitization with eotaxin, partial desensitization with RANTES, and no desensitization with MIP-1\(\alpha\) (Fig. 2B), providing functional evidence that vMIP-II binds to CCR3 on eosinophils.

To show that vMIP-II can chemoattract eosinophils, we performed an in vitro chemotaxis assay (22). vMIP-II, but not vMIP-I, was chemotactic for human eosinophils (Fig. 3). This activity of vMIP-II was comparable to that of eotaxin, which correlates with the activity to induce Ca\(^{2+}\) mobilization (Fig. 2A).

Although angiogenesis is central in the pathology of KS and prominent in MCD, the mechanisms by which KSHV induces new blood vessel formation are not known. Angiogenesis is a complex process involving chemotactic migration and proliferation of endothelial cells, followed by lumen formation and functional maturation of the endothelium (23). Often it is associated with the presence of activated inflammatory cells. Apart from their potent biological effects on leukocytes, some members of the chemokine family have been shown to have a direct effect on blood vessel formation. Within the CXC chemokine family, proteins containing the NH\(_2\)-terminal Glu-Leu-Arg (ELR) amino acid sequence motif such as IL-8 are angiogenic factors, whereas non-ELR-containing proteins including IP-10 are angiostatic (24). To our knowledge, there is no information on the angiogenic ability of CC chemokines. We therefore examined the potential of vMIP-I and vMIP-II as mediators of angiogenesis in the chick chorioallantoic membrane (CAM), which is a standard in ovo assay to investigate angiogenic function (25, 26).

Angiogenic responses were observed and photographed 3 days after implantation of a methylcellulose disk containing test samples onto CAM. Most eggs implanted with vMIP-I or vMIP-II showed a clear angiogenic response. The positive controls used, vascular endothelial growth factor (VEGF) and phorbol 12-myristate 13-acetate (TPA), also induced angiogenesis as expected (Fig. 4 and Table 1). In contrast, cellular MIP-1\(\alpha\) and RANTES failed to induce angiogenesis in most eggs, and the few positive responses recorded were not as prominent as those for vMIPs. The potency of vMIPs to induce angiogenesis in chick embryos was demonstrated by clear angiogenic responses in some eggs at doses as low as 0.05 \(\mu\)g, in contrast to poor induction by human CC chemokines at 0.25 \(\mu\)g. Although it is difficult to interpret a comparison of angiogenic activity between chemically synthesized and recombinant proteins, the activity of the vMIPs was not as potent as that of VEGF, but more potent in the CAM assay than that of the ELR-CXC chemokine IL-8 (Table 1). Preliminary results also indicate that eotaxin is not an inducer of angiogenesis in CAM. The finding that KSHV-encoded chemokines can induce new blood vessel formation in the chick CAM is intriguing and could have important implications for the role of KSHV in KS and MCD development.

Our results demonstrate that the vMIPs are biologically active: (i) vMIP-II binds predominantly to CCR3, resulting in potent inhibition of HIV entry by way of this receptor and activation and chemotaxis of eosinophils to eotaxin, and neither vMIP-I nor vMIP-II prevented Ca\(^{2+}\) mobilization in neutrophils induced by IL-8 (data not shown).

Fig. 2. Calcium-mobilization assay on eosinophils and neutrophils. (A) Peak increase of [Ca\(^{2+}\)], concentration in fura-2–loaded human eosinophils and neutrophils in response to vMIP-I and vMIP-II; the CC chemokines eotaxin, RANTES, and MIP-1\(\alpha\); and the CXC chemokines IL-8 and GRO. Data are expressed as the mean ± SEM of three to four separate experiments with cells purified from different individuals. (B) Receptor desensitization of Ca\(^{2+}\) mobilization in human eosinophils between vMIP-II and the CC chemokines eotaxin, RANTES, and MIP-1\(\alpha\). In these experiments the desensitizing agent was added 150 s before the response to the second agonist was measured. Data are expressed as the percentage of the maximal [Ca\(^{2+}\)] induced by vMIP-II (3 nM) or eotaxin (3 nM) alone (filled columns) after treatment of the cells with various concentrations of the desensitizing agent (vMIP-II, 0.3 to 30 nM; eotaxin, 0.3 to 10 nM; RANTES, 3 to 30 nM; and MIP-1\(\alpha\), 3 to 30 nM). Values are the mean ± SEM of three separate experiments with eosinophils purified from different individuals. (Insets) Representative traces of the data from a single experiment showing the dose-response relation between the magnitude of the response to the desensitizing agent and the subsequent response to the second agonist. The desensitization was specific rather than global because none of the desensitizing agents reduced the response of the cells to a subsequent addition of the complement fragment C5a.
Table 1. Angiogenic effect of vMIPs on the chick CAM.

<table>
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<tr>
<th>Sample*</th>
<th>Dose (µg)</th>
<th>Number of embryos</th>
<th>Angiogenic response†</th>
<th>Score‡</th>
<th>Percent maximum score</th>
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<tr>
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<td>+</td>
<td>7.5</td>
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<tr>
<td></td>
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<td>9</td>
<td>+</td>
<td>8.5</td>
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<td>+</td>
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*Angiogenesis induction was performed by a methacrylate disk containing synthesized vMIP-1, vMIP-2, human MIP-1α, and RANTES. VEGF and TPA were used as positive controls. A disk containing vehicle only or 20 µg of BSA was used as a negative control. †Angiogenic responses were judged by three investigators: (+) unanimously positive; (±) unclear or split judgment; (−) unanimously negative. ‡Scores of 1, 0.5, and 0 were counted for (+), (±), and (−) responses, respectively. Percent maximum score is the division of the score by the embryo number. §A CXC ELR-containing chemokine.

REFERENCES AND NOTES

7. Although only the minority of cells in KS lesions at a specific time point undergo lytic replication (K. A. Stasak et al., J. Virol. 71, 715 (1997)), the demonstration that hundreds of virions are produced in such cells indicates that relatively few of HIV-infected KS cells are produced in KS lesions. Our analysis by reverse transcription–polymerase chain reaction showed expression of vMIP-1 and vMIP-II RNA in KS biopsies (30).
15. PBMCS were isolated as described (31). They were stimulated with phytohemagglutinin (PHA, 0.5 µg/ml) and cultured for 2 to 3 days before addition of IL-2 (Boehringer Mannheim, 20 U/ml). IL-2 and SF162 viruses are NSI, macrophage-tropic HIV-1 strains that use CCR5 as a coreceptor (31). Strains 89.6 and 2028 are SI, dual-tropic strains that use CXCR4, CCR5, and CCR3 (31). Strain 89.6 can also use CCR5 (B. J. Doranz et al., Cell 85, 1149 (1996). Virus stocks were produced in PBA–IL-2–stimulated PBMCS. ROD/B is a variant of HIV-2 ROD strain that efficiently infects CD4-negative/H9 cells. For chemokine inhibition, 7.5 µg/ml of viral stock was added and incubated at 37°C for 120 min at 37°C. Virus infection was monitored by way of CCR3 inhibition of HIV infection by way of CCR3 (27). CCR3 is one of the main receptors for HIV-1 entry into microglia, and an antibody to CCR3 can inhibit HIV-1 infection of microglia (28). Patients with KS or high KSHV viral load, or both, may be less likely to develop HIV-related diseases. The potential agonistic activity of vMIP-II for eosinophils contrasts with its antagonistic function shown in other cell systems (27). Although previous studies have shown that HIV Tat is angiogenic (2), this protein does not play a role in the pathogenesis of non-AIDS KS (for example, classic, African endemic, and posttransplant KS). In contrast, KSHV is present at high levels in all epidemiologic types of KS, and the demonstration of angiogenesis induced by these viral-encoded proteins is the first in vivo indication that KHSV-encoded proteins have the potential to directly induce angiogenesis. Although it is unlikely that these viral chemokines are solely responsible for the marked vascularization seen in KHSV-associated tumors, they could contribute with other angiogenic factors involved in KS spindle cell growth or MCD development—for example, VEGF, basic fibroblast growth factor and IL-6 (2, 29).
ing IL-2 and chemokine. Supernatants were harvested on day 9 and assayed for p24 levels. On U87/CD4 cells, virus infectivity was assessed by a focus-forming assay as described [P. D. Ponath et al., J. Exp. Med. 183, 2437 (1996); B. L. Rothgang et al., J. Virol. 69, 2348 (1995)].

17. Neither RANTES, vMIP-I, nor vMIP-II had appreciable effect on SF162 infection of U87/CD4 CCR5 cells, whereas vMIP-I and -II blocked infection of U87/CXCR4 cells in a dose-dependent manner. One hundred FFUs of each virus in 75 μl were added and incubated for 3 hours at 37°C. The cells were then washed three times, and 500 μl of medium containing the appropriate chemokine at the correct concentration was added. After 5 days the cells were fixed for 10 min in cold acetone:mehanol (1:1) and immunostained for in situ p24 as described [I. A. McKeith, P. R. Clapham, R. A. Weiss, Virology 201, 8 (1994)]. Standard errors were estimated from duplicate wells, and the results (Fig. 1) are representative of three separate experiments.


19. Human eosinophils and neutrophils were isolated from the peripheral blood of healthy volunteers as described [I. A. McKeith, J. Exp. Med. 179, 681 (1994)]. Briefly, neutrophils (>95% purity, contaminating cells being a mixture of eosinophils and mononuclear cells) were separated from red blood cells and mononuclear cells by sequential dextran sedimentation and Percoll-plasma density centrifugation. Eosinophils (>98% purity, contaminating cells being mononuclear cells) were isolated from healthy atopic individuals as described above for neutrophils. Neutrophils and mononuclear cells were separated from the eosinophils by the neutrophils with anti-CD16 microbeads as described (32). DNA pools for sensitivity to cleavage by caspase-3. Therefore, we constructed a protein library by translating a murine embryo cDNA library in a caspase-dependent manner in cells stimulated by Fas. Cleaved-gelled gelsolin severs actin filaments in vitro in a Ca2+-independent manner. Expression of the gelsolin cleavage product in multiple cell types caused the cells to round up, detach from the plate, and undergo nuclear fragmentation. Neutrophils isolated from mice lacking gelsolin had delayed onset of both blebbing and DNA fragmentation, following apoptosis induction, compared with wild-type neutrophils. Thus, cleaved gelsolin may be one physiological effector of morphologic change during apoptosis.

Caspase-3-Generated Fragment of Gelsolin: Effect on Morphological Change in Apoptosis

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The caspase-3 (CPP32, apopain, YAMA) family of cysteine proteases has been implicated as key mediators of apoptosis in mammalian cells. Gelsolin was identified as a substrate for caspase-3 by screening the translation products of small complementary DNA (cDNA) clones for sensitivity to cleavage by caspase-3. Gelsolin was cleaved in vivo in a caspase-dependent manner in cells stimulated by Fas. Caspase-cleaved gelsolin severs actin filaments in vitro in a Ca2+-independent manner. Expression of the gelsolin cleavage product in multiple cell types caused the cells to round up, detach from the plate, and undergo nuclear fragmentation. Neutrophils isolated from mice lacking gelsolin had delayed onset of both blebbing and DNA fragmentation, following apoptosis induction, compared with wild-type neutrophils. Thus, cleaved gelsolin may be one physiological effector of morphologic change during apoptosis.

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References

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9. M. J. McCarty, T. Azuma, J. Tang, D. J. Kwiatkowski, Division of Experimental Medicine, Brigham and Women's Hospital, Boston, MA 02115, USA.
14. Human eosinophils (5 × 106 cells/100 μl per well) were placed in 3-μm pore size transwell inserts and placed in cell culture wells containing 400 μl of human eotaxin (500 μg/ml), vMIP-1α or vMIP-2 (500 μg/ml) and various concentrations of buffer (RPMI 1640 + glutamine + 2% fetal calf serum + 10 mM Hepes [pH 7.4]) as described [P. D. Ponath et al., J. Exp. Med. 183, 2437 (1996)]. After incubation at 37°C for 60 min (8% O2, 5% CO2), eosinophils migrating through the transwell were counted on a FACScan flow cytometer (Becton Dickinson).
18. Angiogenic activities of synthetically prepared viral and human chemokines were evaluated by the chick CAM assay as described [T. Okawa et al., Cancer Lett. 59, 57 (1991)]. Fertilized Plymouth Rock × White Leghorn eggs were incubated at 37°C in a humidified atmosphere (relative humidity, ~70%). Test samples were dissolved in sterile distilled water or PBS. Sterilized sample solution was mixed with an equal volume of autoclaved 2% methylcellulose. Controls were prepared with vehicle only (1% methylcellulose solution). The sample solution (20 μl) was dropped on Parafilm and dried up. The methylcellulose disks were stripped off from the Parafilm and placed on a CAM of 10- or 11-day-old chick embryos. After 3 days, the CAMs were observed by means of an Olympus stereo-
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