

Research article

Cultured Kaposi's sarcoma tumor cells exhibit a chemokine receptor repertoire that does not allow infection by HIV-1

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Abstract

Background: HIV-1 is known to play a critical role in the pathogenesis of AIDS-associated Kaposi's sarcoma (KS). However, it remains controversial whether KS cells are target cells for HIV infection. The aim of this study was to investigate the expression of chemokine receptors in KS cell cultures and to determine whether these cells can be infected by HIV-1.

Material and Methods: KS-derived cells and KS-Y1 cells were investigated using RT-PCR for the expression of CD4, CCR3, CCR5, CCR8 and CXCR4 mRNA. HIV infectivity of these cells was determined by p24 antigen and HIV-1 RNA production, as well as by HIV-1 DNA integration.

Results and Discussion: With the exception of CCR8 which is expressed by KS-derived spindle cell cultures but not by KS-Y1 cells, unstimulated KS cells express no significant levels of CD4, CCR3, CCR5 or CXCR4 mRNA. HIV infectivity assays showed that KS cells were unpermissive to HTLVIIIB and JRFL strains. Although the expression of CXCR4 mRNA could be upregulated by interleukin-1 β , stimulation of KS cells by this cytokine did not allow infection by HIV-1.

Conclusions: This shows that KS cells exhibit a chemokine receptor repertoire that does not allow infection by HIV-1. Other cell types making up KS lesions, such as inflammatory cells, are likely to represent the source of HIV-1 products cooperating to promote KS development and progression.

Introduction

Kaposi's sarcoma (KS) is an angioproliferative disease that has aroused considerable interest of late, as it is one

of the major tumors found in patients with the acquired immune deficiency syndrome (AIDS). It is histologically characterized by inflammation, prominent angiogenesis

and proliferating spindle cells which are considered to be the tumor cells of KS. The pathogenesis of KS is not fully understood. The current hypothesis to explain the initiation and progression of the tumor is a multi-step pathway involving cytokine dysregulation and active infection by human herpesvirus 8 (HHV-8) [1,2,3,4]. The extremely high frequency of KS among HIV-infected patients, together with the particular aggressiveness of AIDS-KS suggests that HIV plays a critical role in KS pathogenesis [3]. Several studies point to the HIV-1 Tat protein synergizing with growth factors to promote the growth of AIDS-KS [5,6]. However, whether HIV can directly infect KS cells is still controversial. Delli Bovi et al. did not detect HIV DNA in KS lesions using Southern blot analysis [7] whereas an hybridization study demonstrated the presence of HIV RNA transcripts in AIDS-KS tissues within factor XIIIa dermal dendrocytes [8]. Using polymerase chain reaction (PCR), HIV-1 proviral DNA was usually not found in spindle cells derived from KS lesions of AIDS patients [9,10]. A single study found that KS-derived cells can productively be infected by HIV-1 *in vitro* [11]. This infection was followed by increased expression of interleukin (IL)-1 β and IL-6, which could be relevant to the pathogenesis of AIDS-related KS, knowing the crucial role of cytokines and growth factors in the development of this tumor [1,3,5]. The question of the infectibility of KS cells by HIV-1 raises the question of the ways of attachment and entry of the virus into these cells. Although CD4⁺ spindle-shaped cells have been detected in AIDS-KS tissues [12], it is generally assumed that KS cells do not harbor the CD4 antigen [9,13,14]. However, it is noteworthy that many cell types that do not express CD4, like endothelial cells, are also infectible to various strains of HIV-1 [15].

Cellular entry of HIV is now known to require one of several seven-transmembrane domain G-protein-coupled receptors, principally CXCR4, an α -chemokine receptor, and CCR5 whose natural ligands are β -chemokines [16,17]. CXCR-4, also named fusin, functions as a coreceptor for T-cell-tropic HIV-1 strains. On the other hand, CCR-5 is the most important coreceptor for M-tropic strains which replicate in monocyte-derived macrophages. The role of CCR3 and CCR8, which are other β -chemokine receptors, is more controversial. Interestingly, these chemokine receptors can sometimes support viral entry in the absence of CD4 [15,18]. Whether KS cells express these chemokine receptors and can respond to their natural chemokine ligands is so far unknown. To develop a more detailed understanding of these G-protein-coupled receptors in KS pathogenesis, we examined their expression in KS-derived spindle cell cultures and in an immortalized KS cell culture (KS-Y1). We next determined whether these cells could be infected by HIV-1.

Materials and methods

Reagents

IL-1 β and LPS were obtained from Sigma (Bornem, Belgium). SDF-1 α was purchased from R&D Systems (Abingdon, UK).

Cell cultures

KS-cell cultures were derived from cutaneous KS biopsies from three patients with AIDS (KS-1, -2 and -3). Cell cultures were also obtained from a renal transplant recipient (KS-4) and from a patient with sporadic KS (KS-5). Cultures were established similarly as previously described [14,19]. Briefly, fresh lesions were minced in small pieces and enzymatically digested with collagenase (1 mg/ml) from *Clostridium histolyticum* for 90 min. Cells were grown without additional growth factors in minimum essential medium D-Val (Gibco, Paisley, Scotland) containing 10% inactivated fetal calf serum (FCS) (Gibco), 1% glutamine, 1% non-essential amino acids, penicillin (100 U/ml) and streptomycin (100 μ g/ml). Cells isolated from these cultures were characterized as KS cells morphologically (spindle-shaped morphology) and immunohistochemically (positivity for laminin, vimentin, collagen IV, α -smooth muscle actin, CD13, ICAM-1 and Bel-2 protein, and negativity for cytokeratin, von Willebrand factor, ICAM-2, ELAM-1, VCAM-1, CD4, CD34, CD40, CD45, CD68) in accordance with previously published reports [14,19]. In addition, we and others have shown that these cells constitutively produce a 92-kDa type IV collagenase [20] and can traverse basement membranes [5,21], which suggests they have an invasive phenotype and that they may adequately represent the tumor cells of KS. The experiments presented here were performed on cell cultures between the third and the fifteenth passage.

The KS-Y1 cell line which displays a higher proliferative activity than cutaneous-derived KS cell cultures [10] was provided by Dr. Y. Lunardi-Iskandar (Institute of Human Virology, Baltimore, MA). It was maintained in RPMI (Gibco) containing 10% inactivated FCS (Gibco), 1% glutamine, 1% non-essential amino acids, 1% sodium pyruvate, penicillin (100 U/ml) and streptomycin (100 μ g/ml). No exogenous growth factor was added.

RT-PCR for CD4, CCR3, CCR5, CCR8, CXCR4 and β actin mRNA

Total RNA was isolated by using a commercially available reagent (TripureTM, Roche Diagnostics, Brussels) following manufacturer's instructions. In order to avoid amplification of contaminating genomic DNA, total RNA was treated with 10 units of RQ1 RNase-free DNase (Promega Corporation, Madison, WI) for 30 minutes. DNase was then inactivated by adding 500 μ l of TripureTM, and total RNA was extracted once again. Re-

verse transcription of mRNA was carried out as follows. Eight μl of water containing 500 ng of total RNA were added to 2 μl of oligo dT primer (0.5 $\mu\text{g}/\mu\text{l}$), and incubated at 65°C for 10 min. Samples were chilled on ice, and 10 μl of RT mix containing the following components were added: 1) 4 μl 5 × RT buffer (250 mM Tris HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂); 2) 2 μl deoxynucleotide triphosphate mix (10 mM each); 3) 0.2 μl BSA (1 mg/ml); 4) 0.6 μl (25 U) human placental ribonuclease inhibitor (RNAGuard[®], Pharmacia Biotech, Uppsala, Sweden); 5) 1 μl (200 U) M-MLV reverse transcriptase (Gibco); 6) 0.2 μl H₂O; 7) 2 μl DTT (100 mM). The samples were then incubated at 37°C for 60 min.

The PCR reaction was carried out in a 25 μl final volume containing: 1) 5 μl (3 μl for β actin) cDNA; 2) 1 μl sense and 1 μl antisense primer (0.1 $\mu\text{g}/\mu\text{l}$ each); 3) 0.5 μl deoxynucleotide triphosphate mix (10 mM each); 4) 2.5 μl 10 × PCR buffer (100 mM Tris HCl, pH 8.3, 500 mM KCl, 15 mM MgCl₂); 5) 0.2 μl Taq DNA polymerase (5 U/ μl) (Roche); 6) H₂O up to 25 μl . After an initial denaturation step at 94°C for 4 min, temperature cycling was initiated for each cycle as follows: 94°C for 20 sec, 55°C for 20 sec, 72°C for 45 sec; 35 (30 for β actin) cycles were performed, followed by a final elongation step at 72°C for 8 min. The sequences of the primers were (sense, 5' → 3') CTGGGCAAAAAGGGGATAC, AGTTTGTGCCCCGCTGT, TGGTCCTGCCGCTGCTTG, GCCGTGTATGCCCTAAAGGT, GCCCTCCTGCTGACTATTCC, GGGTCAGAAGGATTCCTATG, and (antisense, 5' → 3'), GTGGGTGT CAGATTGGCAG, TGAAAAACACGCCATGA, TGCTCCCAGTGGATCGG, ATGGCCTTGGTCTTGTGTG, ACTGTGGTCTTGAGGGCCTT, GGTCTCAAACATGATCTGGG, for CD4, CCR3, CCR5, CCR8, CXCR4 and β actin, respectively. The PCR products were run on a 2 % agarose gel, stained by ethidium bromide and then photographed on Polaroid 667-type film. The expected band size, checked against DNA molecular weight markers, was 309, 645, 430, 299, 236 and 237 base pairs for CD4, CCR3, CCR5, CCR8, CXCR4 and β actin, respectively.

Flow cytometric analysis

Cell surface expression of CXCR4 and CD4 receptors was analyzed by flow cytometry. Briefly, 5 × 10⁵ cells were suspended in ice-cold PBS, 0.1% bovine serum albumin. Cells were incubated on ice for 20 min with the primary 44717.111 (anti-CXCR4) antibody (R&D Systems) or SK3 (anti-CD4) antibody (Becton Dickinson, San Jose, CA). Cells were then washed twice with ice-cold PBS, 0.1% bovine serum albumin and FACS analysis was done with a FACScan flow cytometer (Becton Dickinson). Negative controls were provided by replacing the primary antibody with non-immune immunoglobulin fraction.

Ca²⁺ mobilization assay

For measurements of intracellular calcium [Ca²⁺]_i, the cells were washed twice with HBBS without calcium and magnesium (Gibco). After washing, the cells were resuspended at 5 × 10⁶/ml in HBBS and incubated for 30 min at 37°C with 250 μM sulfinpyrazone (Sigma), 100 $\mu\text{g}/\text{ml}$ Pluronic F-127 (Molecular Probes, Eugene, OR) and 7.5 μM of the fluorescent calcium probe Fluo-3AM (Molecular Probes). After incubation, the cells were washed twice with RPMI 1640 (Biowhitakker, Verviers, Belgium) supplemented with 10% FCS and 250 μM sulfinpyrazone. Before analysis, all samples were placed in a 37°C water bath. After a 10 sec period acquisition on a FACScan flow cytometer (Becton Dickinson), SDF-1 α was added from concentration stocks in water.

HIV-1 infection assay

KS cells were seeded at 2 × 10⁴ cells per well in a volume of 1 ml into 24-well microplates. The cells were incubated for 1d to allow attachment and entry into the exponential growth phase and were then treated with IL-1 β (50–500 pg/ml) or LPS (0.5–5 $\mu\text{g}/\text{ml}$). After 2 d incubation at 37°C in 5% CO₂, the cells were inoculated with the HTLVIII-B T cell tropic strain or the JRFL macrophage tropic strain (1000 TCID₅₀/ml). Phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMCs) obtained from a healthy HIV-negative donor and inoculated with both HIV strains were used as positive control. They were cultivated in RPMI 1640 medium containing 15 % FCS and IL-2 and were infected with HTLVIII B or JRFL similarly as KS cells. Non-inoculated KS cells and PBMCs were used as negative controls. After 24 h, virus inoculum was removed, and cells were washed 3 times with Hank's balanced salt solution to remove cell-free virus before replenishing the medium. In order to measure the inoculum background, aliquots of supernatants were removed after the last washing and the addition of fresh medium. The HIV-pulsed cell cultures were maintained for up to 3 weeks. The presence of the p24 antigen was monitored twice a week, using an enzyme immunoassay (Innotest HIV Ag mAb, Innogenetics, Ghent, Belgium). A culture was considered positive when at least 50 pg/ml p24 antigen was detected and when a 4-fold increase in p24 antigen levels on 2 consecutive supernatants was observed.

To further confirm these results, similar experiments were performed for the analysis of the production of HIV-1 RNA. Briefly, KS cells were seeded at 10⁵ cells per well in a volume of 2.5 ml into 6-well plates and treated as described above. The cells were then inoculated with respectively 100000, 200000, and 500000 virions/ml of the HTLVIII-B strain. HTLVIII-B-inoculated PHA-stimulated PBMCs were used as positive controls. Non-inoculated KS cells and PBMCs were used as negative

controls. After 2 h, virus inoculum was removed, and cells were washed 6 times with HBBS to remove cell-free virus before replenishing the medium. Production of HIV RNA in culture supernatants was measured once a week on cell-free supernatant, using the Amplicor HIV1 Monitor test version 1.5 (Roche Diagnostics, Branchburg, NJ).

Cells were also harvested at various time points by scraping and the presence of HIV-1 DNA was measured by semi-quantitative polymerase chain reaction (PCR), as previously described [22]. In order to definitively exclude virus DNA integration, PCR for LTR/gag region, which allows the detection of late reverse transcription product, was performed as previously described [23].

Results

Chemokine receptor expression in KS-derived spindle cells and KS-Y1 cells

The results of the PCR assays are shown in Figure 1. KS cells were examined for the presence of mRNA of CD4 and of the HIV-1 coreceptors CXCR4 and CCR5. The mRNA expression of CCR3 and CCR8 was also investigated. The amplified fragments of CD4 were revealed at very low levels in RNA from KS-derived spindle cells. There was a passage-dependent expression of these receptors, with cells at late passages exhibiting neither CD4 nor CXCR4 detectable levels. No difference could be evidenced between cells obtained from KS cells obtained from AIDS and from non-AIDS patients. With the exception of CCR8, a chemokine receptor which is selectively engaged by HHV-8-encoded vMIP-1 [24], none of the other investigated chemokine receptors was expressed at significant levels in unstimulated KS-derived cells. The KS-Y1 cell line, which may be regarded as a homogenous KS cell culture [10], exhibited barely detectable levels of CD4 mRNA and none of the investigated chemokine receptor mRNA. All PCR performed, except the water controls, were positive for β-actin, showing the presence of amplifiable cDNA.

The cell surface expression of CXCR-4 and CD4 was then evaluated by FACS analysis by using the specific anti-CXCR-4 monoclonal antibody 44717.111 and anti-CD4 monoclonal antibody SK3. This indicated that neither KS-derived spindle cells nor KS-Y1 cells exhibited significant expression of CXCR4 receptors on their surface (Fig. 2). Confirming previous studies, we also found that KS cells do not harbor the CD4 receptor on their surface (data not shown)[14].

To further determine whether KS cells were devoid of functional CXCR4 receptor, our subsequent studies used SDF-1α to assess its ability to induce changes in intracellular levels of Ca²⁺. By contrast to T cells which are

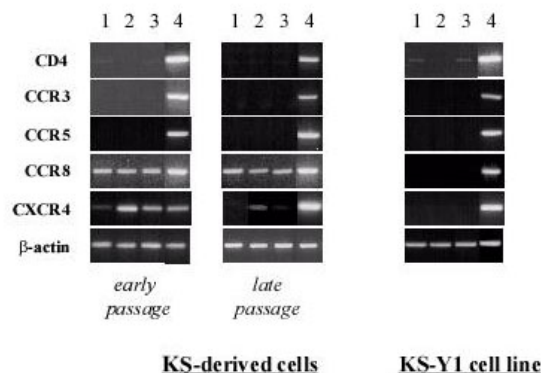


Figure 1
RT-PCR for CD4, CCR3, CCR5, CCR8, CXCR4 and β actin mRNA detection in KS cells. RT-PCR was performed on total RNA extracted from KS-2 cells at early and late passages (passages 4 and 12, respectively) and from KS-Y1 cells, as described in the Materiel and Methods section. Lanes 1, non-stimulated cells; lanes 2 and 3, cells were stimulated for 8 hours with 80 pg/ml IL-1β (lanes 2) or 4 hours with 1 μg/ml LPS (lanes 3); lanes 4, positive controls for RT-PCR, consisting in RT-PCR performed on total RNA extracted from T cells (positive controls for CD4, CCR8, CXCR4 and β actin), LPS-stimulated macrophages (positive control for CCR5) and CHO cells transfected with the CCR3 gene (positive control for CCR3). Similar results were obtained with KS-1, -3, -4 and -5 cells (data not shown).

known to express CXCR4 and to be responsive to its ligand, KS cells exhibited no significant elevation of [Ca²⁺]_i after treatment with SDF-1α (10 -1000 nM) (data not shown).

Since inflammatory cytokines regulate the transcription of several chemokine receptors [25] and are known to play a role in KS pathogenesis through autocrine and paracrine loops, we examined the effects of inflammatory mediators on CCR3, CCR5, CCR8 and CXCR4 mRNA expression in KS cells. Using commercially available ELISA kits (BioSource, Fleurus, Belgium), we found that the treatment of KS-derived cells with IL-1β induced a dose-dependent release of IL-6 and IL-8, indicating effective stimulation of the cells (data not shown). As shown in Fig. 1, IL-1β caused an increase in steady state levels of CXCR4 mRNA in KS-derived spindle cells but not in the KS-Y1 cell line. Maximal increase in CXCR4 levels was reached within 8 h after treatment. The IL-1β-induced expression of CXCR4 mRNA was passage-dependent, with stimulated cells at late passages (> 10) expressing fewer transcripts than cells at early passages. We then investigated whether IL-1β could induce the expression of functional CXCR4 receptor on cell cultures at

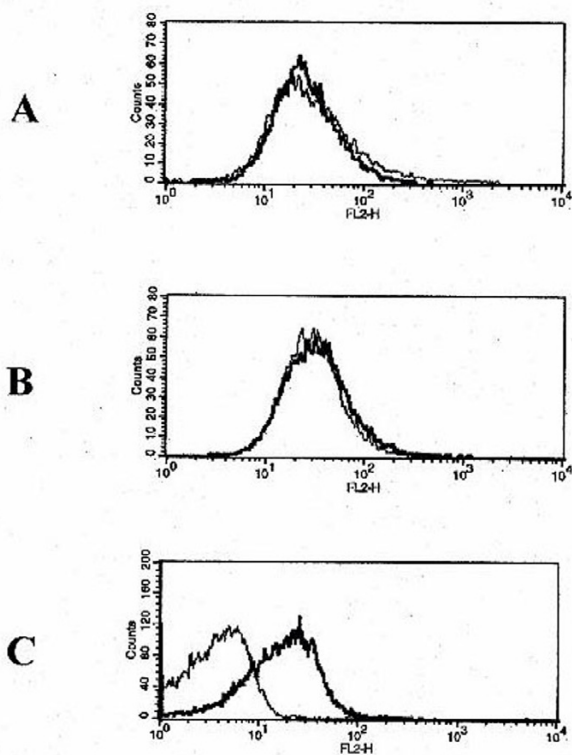


Figure 2
Flow cytometric analysis of CXCR4. (A) KS-2 cells. (B) IL-1 β -stimulated KS-2 cells. (C) T cells. Thin curve, control isotype; thick curve, anti-CXCR4 antibody. Similar results were obtained with KS-1 and -5 cells (data not shown).

passage > 10. Flow cytometry analysis revealed no significant surface expression of CXCR4 after stimulation (Fig. 2), suggesting absence of translation into surface protein expression or expression below flow cytometry sensitivity levels. Similarly, IL-1 β -stimulated KS cells exhibited no significant elevation of [Ca²⁺]_i after treatment with SDF-1 α (10 -1000 nM) (data not shown). No effect of IL-1 β was evidenced on the levels of CD4, CCR3, CCR5 and CCR8 mRNA. LPS achieved similar effects as IL-1 β (Fig. 1).

Sensitivity of KS-derived spindle cells and KS-Y1 cells to HIV-1 infection

We investigated the ability of HTLVIII-B and JRFL strains to infect KS-Y1 cells and KS spindle cells. Three independent infection attempts for each cell type indicated that HIV-1 RNA and p24 antigen production never rose above background values, even after stimulation with IL-1 β or LPS. There was also no HIV-1 proviral DNA integration as assessed by semi-quantitative PCR DNA as well as by PCR for LTR/gag region (Fig. 3). No cytopathic effect could be detected.



Figure 3
PCR for LTR/gag region (detection of late reverse transcription products). PCR were performed on DNA extracted from cell cultures 21 days after infection attempt. Lane 1, positive control for PCR (DNA extracted from PBMC from a HIV-1-infected patient); lane 2, PCR negative control (water); lanes 3 to 6, DNA extracted from PBMC from a HIV-1-negative donor, exposed to 0, 100000, 200000 or 500000 virions respectively; lanes 7 to 10, DNA from KSY-1 cells exposed to 0, 100000, 200000 or 500000 virions respectively; lanes 11 to 14, DNA from KS-2 cells exposed to 0, 100000, 200000 or 500000 virions respectively; lane 15, DNA molecular weight marker.

Discussion

In vitro establishment of KS-derived spindle cell cultures have allowed to show that the aggressive behavior of AIDS-associated KS may be related to HIV-1 products synergizing with growth factors to promote the growth of KS cells [3,5]. However, direct infection of KS cells by HIV-1 remains controversial. In addition to the CD4 antigen, chemokine receptors have emerged as important portals of entry for HIV-1 infection [16,17]. In this report, we found that KS-derived cell cultures and the KS-Y1 cell line express barely detectable levels of CD4 and CXCR4 mRNA. As there was a passage-dependent expression of these receptors, it is suggested that their expression may be related to non-KS cells co-isolated and co-cultured with KS cells during early cell culture passages. CCR8, a β -chemokine receptor whose role as HIV co-receptor remains unclear [26], was expressed by KS-derived spindle cells but not by the KS-Y1 cell line, which may stress the controversy regarding the ontogenic origin of this cell line. By contrast, neither CCR3 nor CCR5 were expressed by unstimulated KS cells. As KS is thought to be an inflammatory cytokine-mediated disease, at least in the early stage of tumor development [1,3], we tested the possibility that these receptors would be expressed under conditions of immune stimulation. One of the most important inflammatory cytokine involved KS pathogenesis is IL-1 β , a pleiotropic cytokine that is synthesized during a broad variety of antigenic stimuli, including viral infections. IL-1 β levels are known to be elevated in homosexual men with HIV-1 infection, which may, at least partly, explain the more aggressive course of KS in these individuals as compared with people with classic KS. Analogously, increased IL-1 β levels might be in-

volved in the reported development and/or progression of KS during opportunistic infections [1,3,27]. Here, we found that IL-1 β induced the expression of significant CXCR4 mRNA levels in KS cells at early passages and induced its expression at low levels in KS cells at late passages. A possible explanation for this passage-dependent induction of CXCR4 mRNA expression is that the effect of IL-1 reflects effects on cell types making up KS lesions and co-isolated with KS cells. For example, IL-1 β has been shown to modulate the transcription of CXCR4 in endothelial cells [25]. Accordingly, the KS-Y1 cell line, which may be regarded as a homogenous KS cell culture [10], did not express CXCR4 after stimulation with IL-1 β . Thus, to avoid misinterpretation with other cell types making up KS lesions [9], we investigated whether IL-1 β could induce the expression of functional CXCR4 receptor on cell cultures at late passages. This indicated that IL-1 β failed to induce CXCR4 on the cell surface at significant levels, suggesting absence of translation into surface protein expression or expression below flow cytometry sensitivity levels. Absence of functional CXCR4 receptor on KS cell surface was further demonstrated by absence of intracellular calcium mobilization with the CXCR4 ligand SDF-1 α . No effect of IL-1 β was evidenced on the levels of CD4, CCR3, CCR5 and CCR8 mRNA.

To test the possibility that HIV-1 could infect KS cells independently of these known HIV-1 receptors and co-receptors, we performed *in vitro* infectivity assays. Neither KS-derived spindle cells nor KS-Y1 cells, could be productively infected by HIV-1 *in vitro*, as evidenced by the absence of production of HIV-1 RNA and p24 antigen as well as the absence of HIV-1 proviral DNA integration. KS-derived cells co-cultured with IL-1 β remained unpermissive to HIV-1. As HIV is known to exert direct cytopathic effects on infected cells, the absence of receptors for viral entry may represent a mechanism that allows KS cells to escape from lysis.

Conclusions

From our data, we conclude that KS cells exhibit a chemokine receptor repertoire that does not allow infection by HIV-1. Other cell types making up KS lesions, such as inflammatory cells, are likely to represent the source of HIV-1 products cooperating to promote KS development and progression.

List of abbreviations used

KS: Kaposi's sarcoma; FCS: fetal calf serum; PHA: phytohemagglutinin; PBMC: peripheral blood mononuclear cells.

Competing Interests

Have you in the past five years received reimbursements, fees, funding, or salary from an organization that may in any way gain or lose financially from the publication of this paper? No

Do you hold any stocks or shares in an organization that may in any way gain or lose financially from the publication of this paper? No

Do you have any other financial competing interests? No

Are there any non-financial competing interests you would like to declare in relation to this paper? No

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