

Purified Tat induces inflammatory response genes in Kaposi's sarcoma cells

Gloria D. Kelly*[¶], Barbara Ensoli[§], Clifford J. Gunthel[‡]
and Margaret K. Offermann*[†]

Objective: Kaposi's sarcoma (KS) is a neoplasm strongly associated with HIV-1 infection and marked by leukocytic infiltration. The infiltrating leukocytes are a possible source of inflammatory cytokines, human herpesvirus 8 (HHV8) and the HIV-1 transactivator protein Tat. This study examines whether Tat directly induces expression of cellular adhesion molecules and cytokines in KS cells and whether this induction differs in kinetics and magnitude from induction by tumour necrosis factor (TNF) α .

Design and method: Changes in gene expression in response to recombinant Tat compared with those to TNF α were evaluated at the messenger (m) RNA and protein level using cells that were cultured from KS lesions.

Results: Tat induced the expression of the adhesion molecules vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) and the cytokines monocyte chemoattractant protein 1 (MCP-1) and interleukin 6 (IL-6). The inductions were observed at both the protein and mRNA levels. The pattern of mRNA induction over time in response to Tat differed from that to TNF α , with higher peak levels that occurred earlier in response to Tat. The expression of these genes is, in part, regulated by the transcription factor NF- κ B. Tat and TNF α activated comparable levels of NF- κ B.

Conclusions: The ability of the HIV-1 Tat to induce the expression of genes with kinetics that are distinct from those seen in TNF α induction suggests that mechanisms in addition to activation of NF- κ B contribute to the observed induction. Tat may contribute to the pathogenesis of AIDS-related KS through induction of cellular genes that are pro-proliferative and proinflammatory and may enhance the recruitment of leukocytes, which are a possible source of further cytokines, Tat and HHV8.

© 1998 Lippincott Williams & Wilkins

AIDS 1998; 12:1753–1761

Keywords: Kaposi's sarcoma, Tat, inflammation, cellular adhesion molecule, cytokine

Introduction

AIDS-associated Kaposi's sarcoma (AIDS-KS) is the predominant neoplasm that occurs in individuals infected with HIV-1. The lesions of KS are multifocal, proliferative vascular lesions of mesenchymal origin that are characterized by abnormal angiogenesis, leukocytic infiltration and proliferation of spindle cells, fibroblasts

and endothelium [1–3]. Experimental evidence suggests that the abnormal proliferation and phenotype of KS cells may result from both changes intrinsic to the KS cells [4,5], including infection with human herpesvirus 8 (HHV8) [6,7], and changes induced by locally elevated cytokines and growth factors such as oncostatin M [8], tumour necrosis factor (TNF) α , interleukin 6 (IL-6) [9] and basic fibroblast growth factor (bFGF) [10].

From the *Winship Cancer Center, [†]Division of Hematology/Oncology and [‡]Division of Infectious Diseases, Department of Medicine, Emory University, Atlanta, Georgia, USA and the [§]Laboratory of Virology, Istituto Superiore di Sanità, Rome, Italy. [¶]G.D. Kelly is a member of the Program in Genetics and Molecular Biology and a member of the Winship Cancer Center.

Sponsorship: This study was supported by NIH grants RO1 CA60345, RO1 CA67382 and P30AR42687 to M. K. Offermann, and NIH grant HG F32 000145–05 to G.D. Kelly.

Requests for reprints to: Dr Margaret K. Offermann, Winship Cancer Center, 1365-B Clifton Rd NE, Atlanta, Georgia 30322, USA.

Date of receipt: 6 March 1997; revised: 2 June 1998; accepted: 12 June 1998.

Although not necessary for the development of KS, infection with HIV-1 is associated with at least a 20 000-fold increase in the incidence of KS among persons with AIDS [11]. HIV-1-infected leukocytes are present in KS lesions [12], and these cells are likely to release the HIV regulatory protein Tat into the extracellular environment where it can be taken up by neighbouring cells [13]. Tat is found to localize within KS lesions [14], and several lines of experimental evidence indicate that Tat may contribute to the development of KS. Skin lesions that resemble KS develop in some Tat transgenic mice [15–17], and Tat stimulates KS cells and cytokine-activated endothelial cells to undergo steps involved in angiogenesis [18]. Tat also synergizes with bFGF in inducing angiogenic KS-like lesions when both are injected subcutaneously into nude mice [14]. Tat is best known as a potent transactivator of HIV-1 gene expression that interacts with an RNA stem-loop structure known as TAR present on all HIV-1 transcripts [19,20] and promotes elongation during transcription [19,21–23]. Tat also can transactivate some other viral promoters [24,25] and some cellular genes such as TNF β , IL-6 and transforming growth factor (TGF) β [26–28] both through interactions with regions that resemble TAR [27] and through distinct mechanisms [29]. Tat also mediates some changes in cellular function through interactions with extracellular receptors. For example, the proliferation of cultured KS cells in response to Tat occurs through an interaction of its RGD domain with integrins [30,31], and Tat promotes angiogenesis, in part, through its binding to and activation of the receptor for vascular endothelial growth factor-A, Flk-1/KDR [32]. Tat also can activate the transcription factor NF- κ B through mechanisms that are not well characterized [33]. Thus, Tat is a complex molecule that has multiple potential mechanisms of action.

Tat was recently reported to induce elevated levels of IL-6, E-selectin, vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) proteins in human umbilical vein endothelial cells [34,35]. These same proteins are expressed at elevated levels in KS lesions [9,36] and are likely to contribute to the disease process. VCAM-1 binds to the counter-receptor very late antigen (VLA) 4 expressed primarily on monocytes and lymphocytes whereas ICAM-1 binds to counter-receptor leukocyte function associated antigen 1 (LFA-1) expressed on a variety of leukocytes [37,38]. Through such interactions, cellular adhesion molecules regulate the numbers and types of leukocyte that accumulate in an area of inflammation. Chemokines and cytokines are also involved in regulating inflammatory cell infiltration. The chemokine monocyte chemoattractant protein 1 (MCP-1) specifically attracts monocytes to areas where it is expressed [37,39] and the cytokine IL-6 activates a number of leukocytes and directly induces KS proliferation [40,41].

In the current studies, we examine the link between Tat, inflammatory cytokines and cellular adhesion molecule expression in cultured KS cells. We demonstrate that Tat induces high levels of ICAM-1, VCAM-1, MCP-1 and IL-6 at both the mRNA and protein levels. Both Tat and TNF α activate comparable levels of the transcription factor NF- κ B, yet Northern blot analysis indicates that Tat induces earlier increases and much higher levels of most of these mRNA forms than does TNF α , suggesting that the Tat-mediated increases in mRNA result in part from a mechanism that is distinct from that utilized by TNF α .

Materials and methods

Cell culture

KS cells were obtained from punch biopsies of clinically involved skin from HIV-1 infected individuals. The biopsies were finely minced and plated onto gelatin-coated plates in RPMI 1640 media (Mediatech, Inc., Herndon, Virginia, USA) supplemented with 10% fetal calf serum (FCS) (Hyclone Laboratories, Inc., Logan, Utah, USA), 2 mM L-glutamine (Gibco-BRL, Life Technologies, Inc., Grand Island, New York, USA), 50 μ g/ml endothelial cell growth supplement (ECGS) (ICN Biomedical, Irving, California, USA), 16 U/ml heparin (ESI Pharmaceuticals, Cherry Hill, New Jersey, USA), and 100 U/ml penicillin-streptomycin mixture (Gibco-BRL, Life Technologies, Inc.). The phenotype of cells cultured from the KS lesions using these conditions has been previously described [5]. Furthermore, some cells cultured under these contain HHV-8 that can be detected by polymerase chain reaction (PCR) [42].

KS modified media contained 10% FCS, L-glutamine, and penicillin-streptomycin as above, but it did not contain ECGS or heparin. This media was used for all experiments involving the incubation of KS cells with the HIV-1 Tat protein. Heparin was omitted because of its ability to bind Tat. ECGS uses heparin as a cofactor and was also omitted.

Cytokines and other reagents

The synthetic double-stranded RNA poly(I:C) was obtained from Pharmacia LKB Biotechnology (Piscataway, New Jersey, USA). Human recombinant TNF α was purchased from Boehringer Mannheim (Indianapolis, Indiana, USA). Lipopolysaccharide (LPS) was purchased from Sigma (St Louis, Missouri, USA). Recombinant human HIV-1 Tat protein (Tat 86) was expressed in *Escherichia coli* and purified to homogeneity as previously described [13,14]. Lyophilized Tat was then resuspended in degassed buffer [phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (BSA)] and used at the indicated concentrations. For

heat inactivation, Tat was incubated at 95°C at 100× concentration in PBS for 5 min and then placed immediately on ice for 5 min. LPS was processed identically. Parallel samples of Tat and LPS were processed identically without the heat inactivation as controls. The Tat or LPS was then added to modified medium and added to cells.

Flow cytometric analysis of CAM expression

Confluent KS cells were detached with versene (0.2 g EDTA•4Na/l in PBS) (Gibco-BRL, Life Technologies, Inc.) and one-third of each sample was processed with each fluorescein isothiocyanate (FITC)-conjugated antibody. The antibodies used were monoclonal mouse anti-human VCAM-FITC (CD106) at 5 mg/ml, mouse anti-human ICAM-FITC (CD54) at 1 mg/ml and a FITC-conjugated non-reactive goat anti-mouse immunoglobulin (Ig) G at 16 mg/ml. All antibodies were obtained from Ancell (Bayport, Minnesota, USA). Samples were incubated with the antibodies for 30 min at 4°C followed by two PBS washes. Cells were resuspended in PBS and fixed with an equal volume of 2% paraformaldehyde in PBS. Relative immunofluorescence was quantified by flow cytometry with log amplification utilizing a fluorescence activated cell sorter (FACS scan IV, Becton Dickinson, Mountain View, California, USA).

Quantification of IL-6, MCP-1 and ICAM-1 by ELISA

Cells were grown to confluence in 96-well plates prior to induction. All experiments were performed using the KS modified media. The conditioned medium from the experiments was removed and assayed by ELISA for secreted IL-6 and MCP-1 using commercially available kits according to the manufacturers' instructions (IL-6 from PerSeptive Diagnostics, Cambridge, Massachusetts, USA and MCP-1 from R&D Systems, Minneapolis, Minnesota, USA). Triplicate assays were performed for the calculation of standard deviation.

Cell surface ICAM-1 expression was assayed on live cells by incubation with mouse anti-human ICAM-1 antibody (84H10) at 1 mg/ml in complete medium for 30 min at 37°C. Wells in which no primary antibody was used were included for background determination. Cells were washed and then incubated for 30 min with peroxidase-conjugated goat-anti-mouse IgG (Biorad, Richmond, California, USA) diluted 1 : 1000 in medium. Cells were then washed three times with PBS to reduce non-specific sticking. Binding of the secondary antibody was assessed by addition of 100 µl 0.1 mg/ml tetramethylbenzidine (Sigma) and 0.3% H₂O₂. The reaction was stopped by the addition of 25 µl 4 mol/l sulphuric acid, and the plates were read on a Biorad model 450 ELISA Reader at 450 nm.

RNA isolation and Northern blot analysis

Cells were rinsed with PBS followed by lysis in 2 ml 4 mol/l guanidium isothiocyanate. Total cellular RNA was obtained by ultracentrifugation of guanidium isothiocyanate on a caesium chloride density gradient [43]. The cellular RNA underwent two ethanol precipitations before being used for Northern blot analysis.

Total cellular RNA was size fractionated using 1% agarose formaldehyde gels containing 1 µg/ml ethidium bromide [44]. The RNA was transferred to nitrocellulose and covalently linked by a Stratilinker UV cross-linker (Stratagene, La Jolla, California, USA). Hybridizations and washes were performed using Stratagene QuikHyb hybridization solution according to the manufacturer's instructions. Approximately 1×10^7 c.p.m./ml labelled probe (specific activity $> 10^8$ c.p.m./µg DNA) was used for each hybridization. Autoradiography was performed using an intensifying screen at -80°C. Quantification of mRNA levels was performed using a Bio-Rad Phosphorimager.

DNA probes

DNA probes labelled with phosphorus-32 were generated using the OLB random primed oligonucleotide labelling kit purchased from Pharmacia LKB Biotechnology. The IL-6 probe was 1.0 kb *EcoRI* fragment of complementary (c) DNA excised from clone pXM309 [45]. The VCAM-1 probe was obtained from a *HindIII-XhoI* fragment of cDNA representing nucleotides 132-1314 [46]. The ICAM-1 probe was generated from an *EcoRI* digestion of human cDNA [47].

Isolation of nuclear extracts and Electrophoretic Mobility Shift Assay (EMSA)

Cells were washed with PBS, detached using Versene, pelleted by centrifugation and resuspended by pipetting gently in Buffer A (10 mmol/l HEPES pH 8.0, 1.5 mmol/l MgCl₂, 10 mmol/l KCl, 200 mmol/l sucrose, 0.05% NP-40 and 0.5 mmol/l DTT containing protease inhibitors at the following concentrations: 1 µg/ml aprotinin, 0.7 µg/ml pepstatin, 0.3 µg/ml antipain, 1 µg/ml leupeptin and 0.5 mmol/l PMSF). The suspensions were incubated for 10 min on ice and then centrifuged. The nuclear pellet was washed once with Buffer A without NP-40. The pellet was resuspended in Buffer B (20 mmol/l HEPES pH 7.4, 25% glycerol, 420 mmol/l NaCl, 1.5 mmol/l MgCl₂, 0.2 mmol/l EDTA containing the same concentrations of protease inhibitors as Buffer A). The cells were incubated for 30 min on ice and vortexed at half speed every 10 min. After the 30 min incubation, the sample was centrifuged at 14 000 r.p.m. for 10 min. The resulting supernatant was saved as the nuclear extract.

EMSAs were performed using a probe representing the tandem NF-κB elements from the VCAM-1 promoter.

The probe was constructed by placing a primer 5'AGGCGGAGGGA-3' and template 5'-CTGCC CTGGGTTTCCCCTTGAAGGGATTTCCCCTCC GCCT-3' (NF- κ B sites underlined) together in annealing buffer (18 mmol/l Tris pH 7.5, 2 mmol/l MgCl₂, 50 mmol/l NaCl) and heated for 5 min at 85°C and allowed to cool to room temperature. After annealing of the primer, the second strand was synthesized using 0.25 mmol/l dATP, dGTP and dTTP, 50 μ Ci [³²P]-dCTP and 9 U Klenow. The non-radioactive competitor was made identically except for the use of non-radioactive dCTP at 0.25 mmol/l. Free nucleotides were removed using a Sephadex G25 spin column. The probe was incubated with the indicated concentration of nuclear extract for 20 min at room temperature in binding buffer [0.08 mol/l KCl, 0.016 mol/l HEPES pH 7.9, 5 mmol/l Tris-Cl pH 7.5, 1 mmol/l EDTA pH 8.0, 1 mmol/l DTT, 1 mmol/l PMSF, 33 μ g/ml poly(dI:dC), 33 μ g/ml salmon sperm DNA and 16% glycerol]. Protein-DNA complexes were resolved from free probe on native 4% Tris-glycine polyacrylamide gels [48].

Results

HIV-1 Tat protein induces expression of multiple inflammatory response genes on cultured KS cells

The cellular adhesion molecules ICAM-1 and VCAM-1 are known to be induced on KS cells by inflammatory mediators such as poly(I:C), IL-1 β and TNF α [5,49]. Flow cytometry was performed to determine if Tat protein could also induce cell adhesion molecule expression on the surface of KS cells. There was no detectable VCAM-1 expression in the control population of KS cells (Fig. 1A). After incubation with Tat for 20 h, the entire KS cell population displayed induced VCAM-1 expression. The induction by Tat was comparable to that of poly(I:C), an agent known to lead to high levels of VCAM-1 expression on KS cells [5]. ICAM-1 expression was also increased in KS cells that were incubated with Tat (Fig. 1B). There were low levels of ICAM-1 expressed in control KS cells, and the entire population of cells responded to Tat with increased expression of ICAM-1 on their surface. The increase in expression was comparable to that observed with cells incubated with poly(I:C).

To address the possibility that the observed inductions were caused by a LPS contaminant within the recombinant Tat preparation, both Tat and LPS were heated for 15 min at 95°C prior to the addition to KS medium. This treatment eliminated the ability of the heat-labile Tat to induce protein expression but did not affect the ability of LPS to induce ICAM-1 expression (Fig. 2A). The heat sensitivity of Tat and the lack of detectable

LPS by limulus assay indicate that Tat-mediated inductions were not caused by a LPS contaminant.

Having determined that the protein expression of two different inflammatory response genes was induced by Tat, we next examined the ability of Tat to induce MCP-1 and IL-6 proteins in cultured KS cells. The cells were incubated with various concentrations of the recombinant HIV-1 Tat protein, and the levels of MCP-1 and IL-6 secreted into the conditioned medium were assayed by ELISA. The levels of MCP-1 found in non-treated control cells were low (Fig. 2B). In contrast, cells incubated with Tat protein for 20 h showed a significant increase in MCP-1. The increase in MCP-1 levels was detectable using 0.1 μ g/ml Tat, with a greater induction being observed using 0.5 μ g/ml Tat. Tat at 0.01 μ g/ml did not induce MCP-1 protein expression. The induction seen with Tat at the higher concentration (0.5 μ g/ml) was comparable to the known inducer poly(I:C) and even greater than that seen with TNF α . The ability of Tat to induce MCP-1 was eliminated when it was heated prior to addition to the medium. Although the 0.5 μ g/ml concentration of Tat appeared to lead to a greater induction of protein expression, Tat was used at the concentration 0.1 μ g/ml for subsequent experiments since this concentration displayed a clear response.

The effect of Tat on IL-6 protein expression by KS cells was then examined. Cells incubated with the Tat protein (0.1 μ g/ml) for 20 h displayed clear induction of IL-6, with even higher levels resulting from incubation with 0.5 μ g/ml (Fig. 2C). The induction of IL-6 protein expression by Tat was less than that with poly(I:C) and comparable to the induction seen with TNF α . Heat treatment of the Tat eliminated the induction of IL-6 secretion.

HIV-1 Tat leads to an increase in mRNA levels for ICAM-1, VCAM-1, MCP-1 and IL-6

To determine the time course and magnitude of induction by Tat at the molecular level, Northern blot hybridizations were performed on total RNA isolated from KS cells incubated with Tat (0.1 μ g/ml) or with the positive inducer TNF α (10 ng/ml) for various times. The pattern of message induction in response to the two inducers differed in the time required for peak levels to occur. This was most dramatically illustrated by examining IL-6 mRNA levels (Fig. 3A). By 2 h, Tat induced high IL-6 mRNA levels that remained elevated at 4 and 18 h. A decrease in IL-6 mRNA was observed at 8 h, suggesting a biphasic pattern of induction by Tat. This pattern contrasts sharply with IL-6 mRNA levels seen in response to TNF α , which did not begin to increase significantly until 18 h. Differences in time course in response to Tat and TNF α were observed with other mRNAs as well.

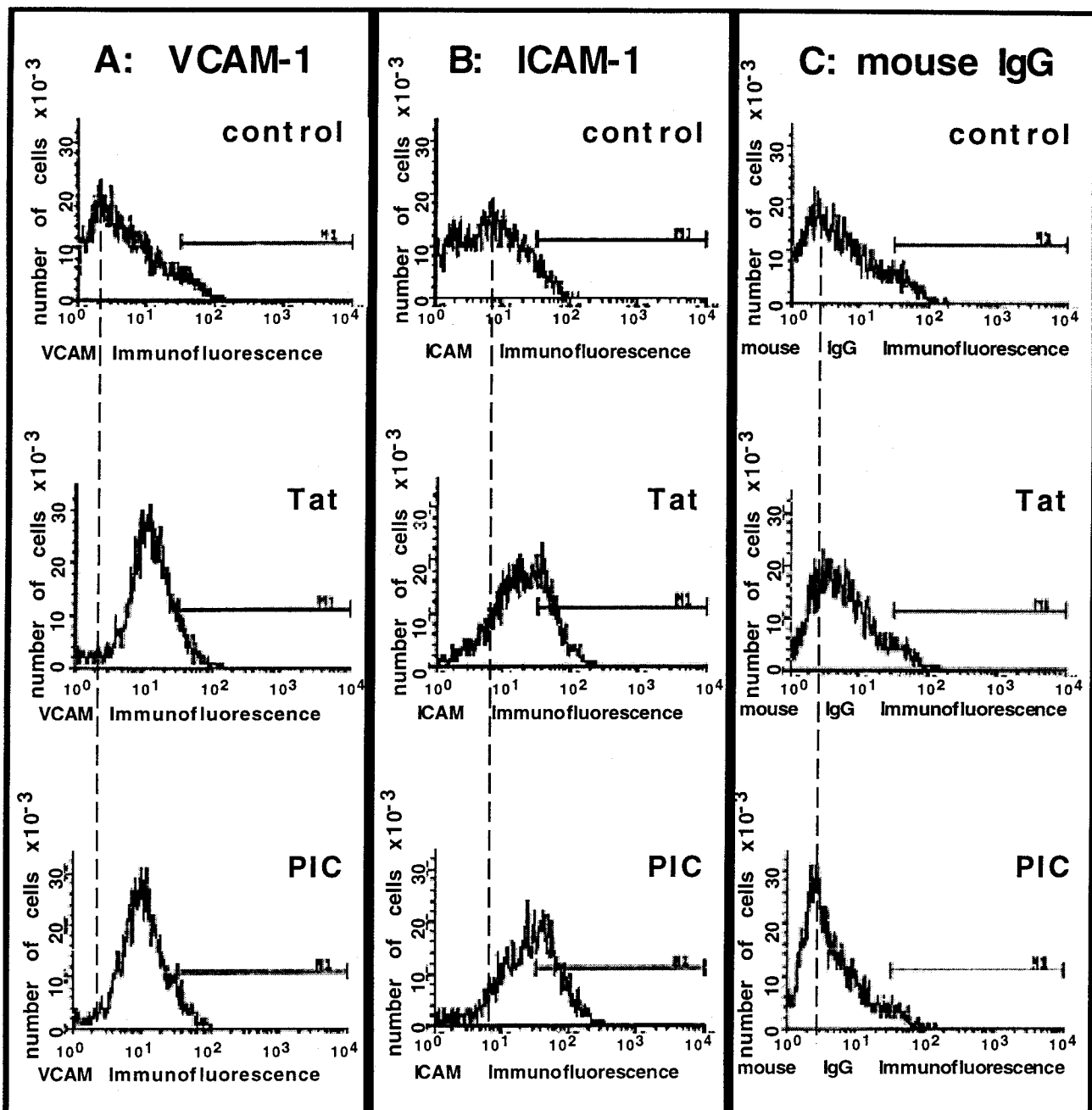


Fig. 1. Exogenously added HIV-1 Tat protein induces cell adhesion molecule expression in Kaposi's sarcoma (KS) cells. Surface cell adhesion molecule expression was determined on KS cells that were incubated in modified media (control) or modified media containing recombinant Tat (0.1 $\mu\text{g/ml}$) or poly(I:C) (100 $\mu\text{g/ml}$) (PIC) for 20 h. Amount of cell surface vascular cell adhesion molecule 1 (VCAM-1) (A) or intercellular adhesion molecule 1 (ICAM-1) (B) was determined by directly labelling with fluorescein isothiocyanate (FITC)-conjugated antibodies and quantified by fluorescence activated cell sorter (FACS) analysis. The background fluorescence that resulted from incubation with a FITC-conjugated anti-mouse immunoglobulin G antibody (non-reactive to human cells) is shown in (C).

VCAM-1 mRNA was induced to peak levels by 2 h with Tat, whereas induction by $\text{TNF}\alpha$ occurred more gradually (Fig. 3B). ICAM-1 mRNA was also induced rapidly and to high levels in response to Tat, whereas ICAM-1 was poorly induced by $\text{TNF}\alpha$ (Fig. 3C).

Although IL-6, VCAM-1 and ICAM-1 mRNAs were induced more effectively by Tat than by $\text{TNF}\alpha$, MCP-1 mRNA was as responsive, if not more so, to incubation with $\text{TNF}\alpha$ as to Tat. Although some variability in the amount of β -actin mRNA was detected,

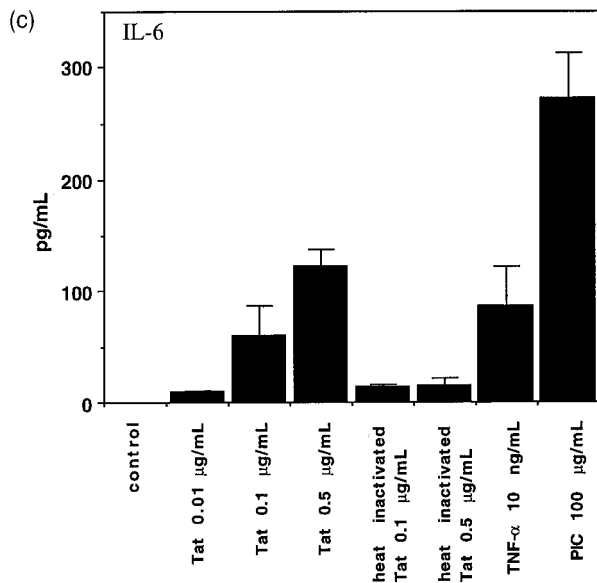
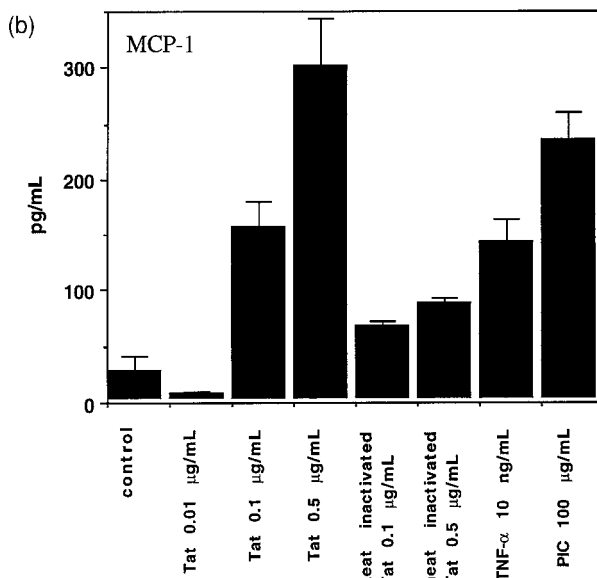
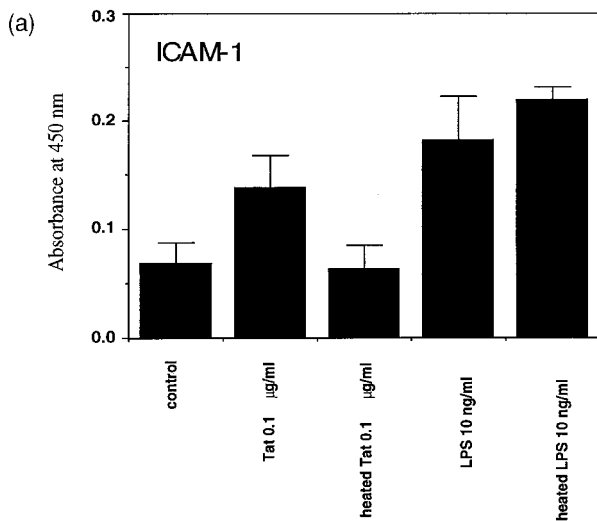


Fig. 2. Induction of inflammatory response genes in AIDS-associated Kaposi's sarcoma (KS) by Tat is heat-sensitive. Identical numbers of confluent KS cells were untreated (control) or incubated with the indicated agents for 20 h and assayed for intercellular adhesion molecule 1 (ICAM-1) expression (a) by ELISA using monoclonal antibodies directed against ICAM-1 as described in the Methods. Conditioned media were assayed for monocyte chemoattractant protein (MCP) 1 (b) or interleukin (IL) 6 (c) by sandwich ELISA, as described in the Methods. Data are shown as mean ± standard deviation of measurements from triplicate wells.

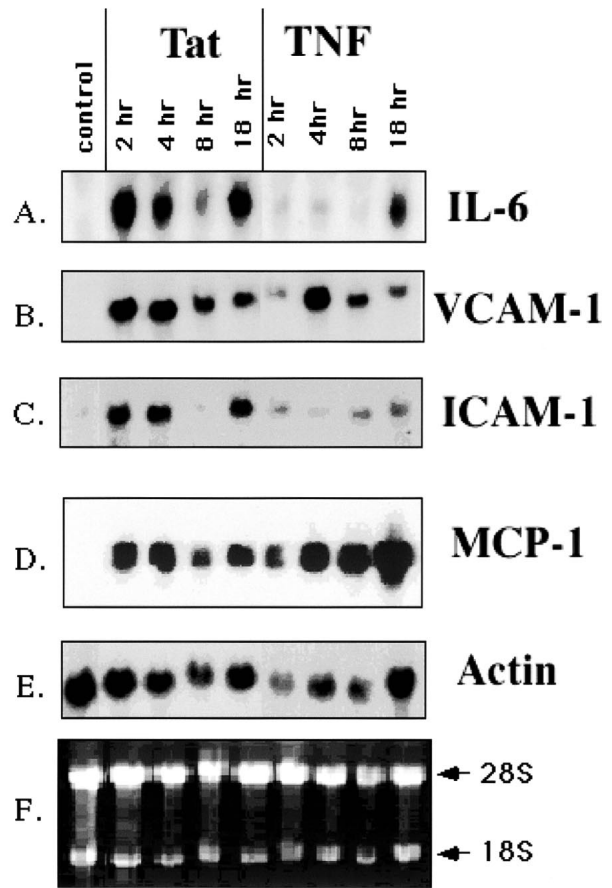


Fig. 3. Northern blot analysis of AIDS-associated Kaposi's sarcoma (KS) mRNA expression over time in response to HIV-1 Tat and tumour necrosis factor (TNF) α. Confluent KS cells were incubated with Tat (0.1 µg/ml) or TNFα (10 ng/ml) for the indicated times. Each lane represents 10 µg total cellular RNA that was size fractionated, transferred to nitrocellulose and serially probed as described in the Methods. The ethidium bromide staining of the nitrocellulose that was serially probed is shown in (F) to document amounts of RNA in each lane.

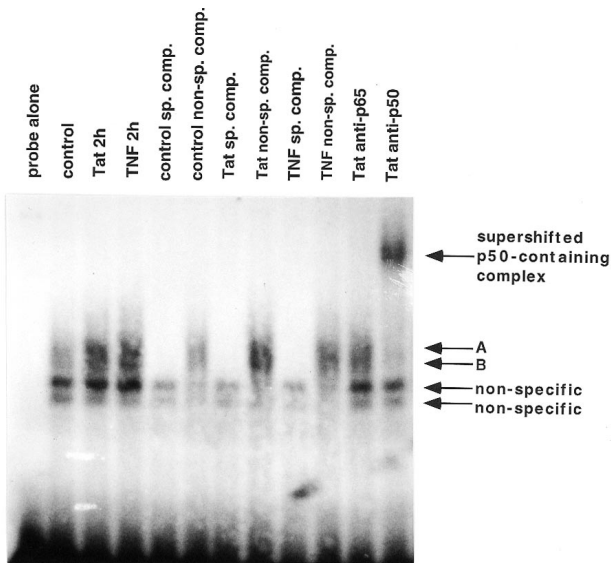


Fig 4. The effect of HIV-1 Tat and tumour necrosis factor (TNF) on NF- κ B binding activity in Kaposi's sarcoma (KS) cells. Nuclear extracts were prepared from confluent KS cells that were untreated or incubated with Tat (0.1 μ g/ml) or TNF α (10 ng/ml) for 2 h. The extracts (10 μ g) were incubated with a 32 P-labelled vascular cell adhesion molecule 1 (VCAM-1) NF- κ B oligonucleotide probe and fractionated on a non-denaturing acrylamide gel as described in the Methods. Complexes denoted as 'A' and 'B' represent NF- κ B complexes specifically competed away by a 100-fold excess of cold NF- κ B probe but not by an unrelated cold oligonucleotide probe. Supershift analysis was performed by the inclusion of antibody (1 μ g) to p50 or p65 subunits during incubation.

standardization to actin using a phosphoimager for quantification confirmed that for all mRNAs examined, except MCP-1, induction occurred earlier and with higher peak levels in response to incubation with Tat than in response to incubation with TNF α .

Tat and TNF α induce NF- κ B binding to the VCAM-1 promoter

The transcription factor NF- κ B is, in part, involved in the transcriptional regulation of all four genes under study. To determine whether differences in NF- κ B activation might account for differences in mRNA induction observed in response to Tat and TNF α , nuclear extracts from cells treated with Tat or TNF α for 2 h were analysed for NF- κ B activation by gel shift analysis. There was a basal level of binding of two NF- κ B complexes of different molecular weights (Fig. 4, bands A and B). Both Tat and TNF α induced comparable increases in the binding of these NF- κ B complexes. The binding of complexes A and B was prevented competitively by an excess (100-fold) of cold specific competitor but not by an unrelated sequence. Supershift analysis indicated that these complexes were composed of p50-containing and a little, if any, p65-containing NF- κ B subunits.

Discussion

We provide evidence that the HIV-1 Tat protein enhances both mRNA and protein expression for IL-6, MCP-1, ICAM-1 and VCAM-1 in cultured KS cells. Although Tat has been shown to induce IL-6, ICAM-1 and VCAM-1 expression in other cell types [26,29], we provide the first demonstration that Tat induces expression of these genes in KS cells. Furthermore, this is the first report that examines changes in ICAM-1, VCAM-1 and MCP-1 mRNA levels in response to Tat. We demonstrate that the induction of IL-6, ICAM-1 and VCAM-1, but not MCP-1, by Tat occurs much more rapidly than induction by TNF α , an inducer whose action is in large part dependent on the activation of NF- κ B. The concentration of Tat that led to increased gene expression in our experiments was within the same range (nanomolar) seen to lead to activation of HIV-1 LTR gene expression and the expression of several cytokines through direct interaction with TAR or TAR-like domains. In contrast, other Tat activities that result from interactions with cell surface proteins such as integrins or the VEGF receptor Flk-1/KDR generally occur at lower (picomolar) concentrations of Tat [31,32]. These data are suggestive of a mechanism that requires higher concentrations of Tat for uptake and nuclear localization rather than interaction with a cell surface receptor.

We demonstrate that both Tat and TNF α activate NF- κ B in KS cells and that the genes induced by Tat all contain NF- κ B sites in their promoters. Tat has been shown to activate NF- κ B in several other cell types, including HeLa cells and monocytic and lymphocytic cell lines [33], and activation by Tat was shown to be blocked with inhibitors of the proteasome complex and anti-oxidants [33], suggesting that activation of NF- κ B by Tat shares some common events with other activation pathways for NF- κ B. NF- κ B activation alone is not sufficient for the induction of the genes reported here since NF- κ B was activated by phorbol ester in cultured KS cells but did not induce expression of the inflammatory response genes (data not shown). For all of the genes examined except MCP-1, induction by Tat occurred earlier and with higher peak levels than seen in induction by TNF α . There were no detectable differences in the NF- κ B that was activated by Tat and TNF α to explain these differences. For Tat-mediated induction of the cellular genes for TNF β and IL-6, transactivation involves putative TAR-like regions in the 5' UTR sequences of their mRNAs [26,27,50]. In addition, Tat complexes with CCAAT Enhancer binding protein β (C/EBP β), a major mediator of IL-6 promoter function, and enhances its binding to the C/EBP site in the IL-6 promoter. Although VCAM-1 and ICAM-1 mRNA were also shown to be induced more rapidly and to higher levels by Tat than by TNF α , neither of these genes are known to contain

C/EBP elements or TAR-like regions. Coimmunoprecipitation studies in other cells demonstrate that Tat can interact directly with several components of the transcription machinery including TFIID, Sp1 and NF- κ B [51,52]. Whether such interactions are involved in the early induction of these genes by Tat remains to be determined.

As we have previously reported, some, if not all, of the cultured AIDS-KS cell lines used in these studies contain HHV8 [42]. Reports from other laboratories have shown an absence of HHV8 genomic sequences in many AIDS-KS spindle cell cultures [53,54]. Differences in isolation technique and culture conditions may contribute to these differences in HHV8 content. Whether the presence of HHV8 has any effect on the responses to Tat is under investigation.

KS develops more commonly in HIV-infected individuals, but it also occurs in individuals that are not HIV-1 infected, suggesting that although HIV gene products may increase the risk for the development of KS, they are not an absolute requirement. Despite the lack of a signal sequence, extracellular Tat can be released from cells and taken up by neighbouring cells [13]. Our results identify several genes in KS cells that are induced by Tat and whose expression might contribute to the development of AIDS-KS. The HIV-infected leukocytes that are characteristically found in AIDS-KS lesions could provide Tat, which could increase expression of IL-6, a cytokine that activates leukocytes and induces the proliferation of KS cells. Induction of the chemokine MCP-1 and of cellular adhesion molecules on the surface of KS cells could promote further leukocytic infiltration. These infiltrated leukocytes could provide additional Tat and cytokines to KS cells as well as providing leukocytes infected with HHV8. Therefore, although Tat is not required for the development of KS, the ability of Tat to induce directly proinflammatory and proliferative genes in KS could contribute to the pathogenic process.

References

1. Kaposi M: **Idiopathisches multiples Pigmentsarkom der Haut.** *Arch Dermatol Syphil* 1872, **4**:742.
2. Mitsuyasu RT: **Clinical aspects of AIDS-related Kaposi's sarcoma.** *Curr Opin Oncol* 1993, **5**:835-844.
3. Templeton A. **Pathology.** In *Kaposi's Sarcoma: Pathophysiology and Clinical Management.* Edited by Ziegler J and Dorfman R. New York: Marcel Dekker. 1988: 23-70.
4. Salahuddin SZ, Nakamura S, Biberfeld P, et al.: **Angiogenic properties of Kaposi's sarcoma-derived cells after long-term culture in vitro.** *Science* 1988, **242**:430-433.
5. Yang J, Xu Y, Zhu C, Hagan K, Lawley T, Offermann MK: **Regulation of adhesion molecule expression in Kaposi's sarcoma cells.** *J Immunol* 1994, **152**:361-373.
6. Staskus KA, Zhong W, Gebhard K, et al.: **Kaposi's sarcoma-associated herpesvirus gene expression in endothelial (spindle) tumor cells.** *J Virol* 1997, **71**:715-719.
7. Boshoff C, Schulz TF, Kennedy MM, et al.: **Kaposi's sarcoma-associated herpesvirus infects endothelial and spindle cells.** *Nature Med* 1995, **1**:1274-1278.
8. Cai J, Gill PS, Masood R et al.: **Oncostatin-M is an autocrine growth factor in Kaposi's sarcoma.** *Am J Pathol* 1994, **145**:74-79.
9. Oxholm A, Oxholm P, Permin H, Bendtzen K: **Epidermal tumour necrosis factor alpha and interleukin 6-like activities in AIDS-related Kaposi's sarcoma: an immunohistological study.** *Apmis* 1989, **97**:533-538.
10. Li JJ, Huang YQ, Moscatelli D, Nicolaides A, Zhang WC, Friedman-Kien AE: **Expression of fibroblast growth factors and their receptors in acquired immunodeficiency syndrome-associated Kaposi sarcoma tissue and derived cells.** *Cancer* 1993, **72**:2253-2259.
11. Beral V, Peterman TA, Berkelman RL, Jaffe HW: **Kaposi's sarcoma among persons with AIDS: a sexually transmitted infection?** *Lancet* 1990, **335**:123-128.
12. Mahoney SE, Duvic M, Nickoloff BJ, et al.: **Human immunodeficiency virus (HIV) transcripts identified in HIV-related psoriasis and Kaposi's sarcoma lesions.** *J Clin Invest* 1991, **88**:174-185.
13. Ensoli B, Buonaguro L, Barillari G, et al.: **Release, uptake, and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral reactivation.** *J Virol* 1993, **67**:277-287.
14. Ensoli B, Gendelman R, Markham P, et al.: **Synergy between basic fibroblast growth factor and HIV-1 Tat protein in induction of Kaposi's sarcoma.** *Nature* 1994, **371**:674-680.
15. Vogel J, Hinrichs S, Reynolds R, Luciw P, Jay G: **The HIV tat gene induces dermal lesions resembling Kaposi's sarcoma in transgenic mice.** *Nature* 1988, **335**:606-611.
16. Corallini A, Altavilla G, Pozzi L, et al.: **Systemic expression of HIV-1 tat gene in transgenic mice induces endothelial proliferation and tumors of different histotypes.** *Cancer Res* 1993, **53**:5569-5575.
17. Frazier AL, Garcia JV: **Retrovirus-mediated transfer and long-term expression of HIV type 1 tat gene in murine hematopoietic tissues.** *AIDS Res Hum Retrovir* 1994, **10**:1517-1519.
18. Albini A, Barillari G, Benelli R, Gallo RC, Ensoli B: **Angiogenic properties of human immunodeficiency virus type 1 Tat protein.** *Proc Natl Acad Sci USA* 1995, **92**:4838-4842.
19. Garcia-Martinez LF, Mavankal G, Peters P, Wu-Baer F, Gaynor RB: **Tat functions to stimulate the elongation properties of transcription complexes paused by the duplicated TAR RNA element of human immunodeficiency virus 2.** *J Mol Biol* 1995, **254**:350-363.
20. Dingwall C, Ernberg I, Gait MJ, et al.: **HIV-1 tat protein stimulates transcription by binding to a U-rich bulge in the stem of the TAR RNA structure.** *EMBO J* 1990, **9**:4145-4153.
21. Selby MJ, Bain ES, Luciw PA, Peterlin BM: **Structure, sequence, and position of the stem-loop in tar determine transcriptional elongation by tat through the HIV-1 long terminal repeat.** *Genes Devel* 1989, **3**:547-558.
22. Bengal E, Aloni Y: **Transcriptional elongation by purified RNA polymerase II is blocked at the trans-activation-responsive region of human immunodeficiency virus type 1 in vitro.** *J Virol* 1991, **65**:4910-4918.
23. Laspi MF, Wendel P, Mathews MB: **HIV-1 Tat overcomes inefficient transcriptional elongation in vitro.** *J Mol Biol* 1993, **232**:732-746.
24. Chowdhury M, Taylor JP, Tada H, et al.: **Regulation of the human neurotropic virus promoter by JCV-T antigen and HIV-1 tat protein.** *Oncogene* 1990, **5**:1737-1742.
25. Kim YS, Risser R: **TAR-independent transactivation of the murine cytomegalovirus major immediate-early promoter by the Tat protein.** *J Virol* 1993, **67**:239-248.
26. Buonaguro L, Barillari G, Chang, HK, et al.: **Effects of the human immunodeficiency virus type 1 Tat protein on the expression of inflammatory cytokines.** *J Virol* 1992, **66**:7159-7167.
27. Buonaguro L, Buonaguro FM, Giraldo G, Ensoli B: **The human immunodeficiency virus type 1 Tat protein transactivates tumor necrosis factor beta gene expression through a TAR-like structure.** *J Virol* 1994, **68**:2677-2682.
28. Cupp C, Taylor JP, Khalili K, Amini S: **Evidence for stimulation of the transforming growth factor beta 1 promoter by HIV-1 Tat in cells derived from CNS.** *Oncogene* 1993, **8**:2231-2236.

29. Scala G, Ruocco MR, Ambrosino C, et al.: **The expression of the interleukin 6 gene is induced by the human immunodeficiency virus 1 TAT protein.** *J Exp Med* 1994, **179**:961–971.
30. Ensoli B, Barillari G, Salahuddin SZ, Gallo RC, Wong-Staal F: **Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients.** *Nature* 1990, **345**:84–86.
31. Barillari G, Gendelman R, Gallo RC, Ensoli B: **The Tat protein of human immunodeficiency virus type 1, a growth factor for AIDS Kaposi sarcoma and cytokine-activated vascular cells, induces adhesion of the same cell types by using integrin receptors recognizing the RGD amino acid sequence.** *Proc Natl Acad Sci USA* 1993, **90**:7941–7945.
32. Albini A, Soldi R, Giunciuglio D, et al.: **The angiogenesis induced by HIV-1 tat protein is mediated by the Flk-1/KDR receptor on vascular endothelial cells.** *Nature Med* 1996, **2**:1371–1375.
33. Demarchi F, Di Fagagna F, Falaschi A, Giacca M: **Activation of transcription factor NF- κ B by the Tat protein of human immunodeficiency virus type 1.** *J Virol* 1996, **70**:4427–4437.
34. Dhawan S, Puri R, Kumar A, Duplan H, Masson J-M, Aggarwal B: **Human immunodeficiency virus-1-Tat protein induces the cell surface expression of endothelial leukocyte adhesion molecule-1, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 in human endothelial cells.** *Blood* 1997, **90**:1535–1544.
35. Hofman FM, Wright AD, Dohadwala MM, Wong-Staal F, Walker SM: **Exogenous tat protein activates human endothelial cells.** *Blood* 1993, **82**:2774–2780.
36. Huang YQ, Friedman-Kien AE, Li JJ, Nickoloff BJ: **Cultured Kaposi's sarcoma cell lines express factor XIIIa, CD14, and VCAM-1, but not factor VIII or ELAM-1.** *Arch Dermatol* 1993, **129**:1291–1296.
37. Butcher E: **Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity.** *Cell* 1992, **67**:1033–1036.
38. Springer TA: **Adhesion receptors of the immune system.** *Nature* 1990, **346**:425–434.
39. Peri G, Milanese C, Matteucci C, et al.: **A new monoclonal antibody (5D3-F7) which recognizes human monocyte-chemotactic protein-1 but not related chemokines. Development of a sandwich ELISA and in situ detection of producing cells.** *J Immunol Meth* 1994, **174**:249–257.
40. Miles SA, Rezai AR, Salazar GJF, et al.: **AIDS Kaposi sarcoma-derived cells produce and respond to interleukin 6.** *Proc Natl Acad Sci USA* 1990, **87**:4068–4072.
41. Yang J, Hagan M, Offermann M: **Induction of IL-6 gene expression in Kaposi's sarcoma cells.** *J Immunol* 1994, **152**:943–955.
42. Offermann M, Lin Y-C, Mar E-C, Shaw R, Yang J, Medford R: **Antioxidant-sensitive regulation of inflammatory-response genes in Kaposi's sarcoma cells.** *J Acquir Immune Defic Syndr* 1996, **13**:1–11.
43. Chirgwin J, Przybyla R, MacDonald R, Rutter W: **Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease.** *Biochemistry* 1979, **18**:5294–6000.
44. Selden RF: **Analysis of RNA by northern hybridization.** In *Current Protocols in Molecular Biology*. Edited by Ausubel FM, Brent R, Kingston RE et al.: New York: Wiley, 1987: 4.9.1–4.9.8.
45. Sutherland GR, Baker E, Callen DF, et al.: **Interleukin 4 is at 5q31 and interleukin 6 is at 7p15.** *Hum Genet* 1988, **79**:335–337.
46. Osborn L, Hession C, Tizard R, et al.: **Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes.** *Cell* 1989, **59**:1203–1211.
47. Tomassini JE, Graham D, DeWitt CM, Lineberger DW, Rodkey JA, Colonno RJ: **cDNA cloning reveals that the major group rhinovirus receptor on HeLa cells is intercellular adhesion molecule 1.** *Proc Natl Acad Sci USA* 1989, **86**:4907–4911.
48. Chodosh L. **DNA-protein interactions.** In *Current Protocols in Molecular Biology*. Edited by Ausubel FM, Brent R, Kingston RE et al.: New York: Wiley, 1987:12.2.1–12.2.6.
49. Fiorelli V, Gendelman R, Samaniego F, Markham PD, Ensoli B: **Cytokines from activated T cells induce normal endothelial cells to acquire the phenotypic and functional features of AIDS-Kaposi's sarcoma spindle cells.** *J Clin Invest* 1995, **95**:1723–1734.
50. Ambrosino C, Ruocco M, Chen X, et al.: **HIV-1 Tat induces the expression of the interleukin-6 (IL6) gene by binding to the IL6 leader RNA and by interacting with CAAT enhancer-binding protein β (NF-IL6) transcription factors.** *J Biol Chem* 1997, **272**:14883–14892.
51. Jeang KT, Chun R, Lin NH, Gatignol A, Glabe CG, Fan H: **In vitro and in vivo binding of human immunodeficiency virus type 1 Tat protein and Sp1 transcription factor.** *J Virol* 1993, **67**:6224–6233.
52. Kashanchi F, Piras G, Radonovich MF, et al.: **Direct interaction of human TFIIID with the HIV-1 transactivator tat.** *Nature* 1994, **367**:295–299.
53. Ambroziak JA, Blackburn DJ, Herndier BG, et al.: **Herpes-like sequences in HIV-infected and uninfected Kaposi's sarcoma patients.** *Science* 1995, **268**:582–583.
54. Lebbe C, de Cremoux P, Rybojad M, Costa da Cunha C, Morel P, Calvo F: **Kaposi's sarcoma and new herpesvirus.** *Lancet* 1995, **345**:6.