HIV-1-Tat Protein Activates Phosphatidylinositol 3-Kinase/
AKT-dependent Survival Pathways in Kaposi’s Sarcoma Cells*

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In this study we found that Tat protected vincristine-treated Kaposi’s sarcoma cells from apoptosis and from down-regulation of several anti-apoptotic genes such as AKT-1, AKT-2, BCL2, BCL-XL, and insulin-like growth factor I and induced the de novo expression of the interleukin-3 gene. Moreover, we found that Tat enhanced phosphorylation of AKT and BAD proteins. The inhibition of phosphatidylinositol 3-kinase with two unrelated pharmacological inhibitors, wortmannin and LY294002, abrogated both the anti-apoptotic effect and the phosphorylation of AKT induced by Tat. After treatment with Tat, the AKT enzymatic activity showed a biphasic increase: an early activation (15 min), independent from protein synthesis; and a delayed activation (24 h), which was significantly decreased upon blockage of protein synthesis. Experiments with a function blocking antivascular endothelial cell growth factor receptor-2 antibody suggested that both the early and delayed AKT activation and the protection from apoptosis were triggered by the interaction of Tat with vascular endothelial cell growth factor receptor-2. Moreover, experiments with function-blocking antibodies directed against insulin-like growth factor I/insulin-like growth factor I receptor or interleukin-3 indicated their involvement in the delayed activation of AKT and their contribution to the anti-apoptotic effect of Tat on vincristine-treated Kaposi’s sarcoma cells.

Kaposi’s sarcoma (KS)*1 is a hemangiosarcoma containing spindle-shaped cells, fibroblasts, inflammatory cells, vascular endothelial cells, and smooth muscle cells characterized by the presence of an intense neoangiogenesis (1–3). KS is frequently associated with immune-depressed conditions, including HIV-1 infection and long term post-transplantation therapy (4). All forms of KS are associated with infection by human herpesvirus 8 (HHV8) (5, 6). AIDS-associated KS is particularly aggressive, and it is one of the principal neoplasms in regions of Africa affected by both high endemic HHV8 and epidemic HIV infection (7).

Several studies (8–10) suggest a primary role for the protein encoded by the tat gene of HIV-1 in the induction and development of Kaposi’s sarcoma in patients affected by AIDS. HIV-1 tat gene transgenic mice have been shown to develop Kaposi’s sarcoma-like skin lesions (1, 11–13). Furthermore, Tat has been shown to activate the vascular endothelial growth factor (VEGF) receptor 2/KDR (VEGFR2) on endothelial (14) and KS cells (15, 16) and to stimulate angiogenesis (17). It has been also shown that Tat, VEGF, and basic fibroblast growth factor synergized in the induction of KS (18).

The growth and diffusion of KS have been ascribed not only to a dysregulation of cellular proliferation but also to a resistance against apoptotic signals derived from the activation of endogenous or exogenous execution death programs. Tat has been shown to prevent apoptosis of different cell lines of lymphoid, epithelial, and neuronal origin (19). However, Tat does not have an univocal role in modulating apoptosis in lymphocytes as it decreases apoptosis of HIV-1-infected T-cells in peripheral blood and induces apoptosis of uninfected T-cells (20) through a Fas-dependent mechanism (21). Recently, we found that Tat is a survival factor for KS and endothelial cells (22), suggesting a putative role in tumor development in a microenvironment characterized by immune system impairment.

The aim of this study was to investigate the molecular mechanisms through which HIV-1-Tat protein inhibits vincristine-induced apoptosis of KS cells. The analysis of the expression of genes involved in modulation of apoptosis prompted us to focus on the role of phosphatidylinositol 3-kinase (PI3K)-dependent activation of AKT.

EXPERIMENTAL PROCEDURES

Reagents—Purified Tat and rabbit anti-Tat IgG were obtained from Intracell (London, UK). Polyclonal goat antibody against human AKT and phosphorylated AKT (P-AKT), polyclonal rabbit antibodies against human BAD and phosphorylated BAD (P-BAD), and rabbit polyclonal neutralizing anti-VEGFR2 antibody were purchased by Santa Cruz Biotechnology (Santa Cruz, CA). Anti-AKT1/PKBα/β-P domain antibody cross-linked to protein G-agarose, BAD-agarose, and active AKT1 body cross-linked to protein G-agarose, BAD-agarose, and active AKT1 were from Upstate Biotechnology, Inc. (Lake Placid, NY). Recombinant human insulin-like growth factor I (IGF-I), monoclonal anti-human IGF-I, and anti-human IGF-I receptor antibodies (IGF-IR) were purchased from R & D Systems (Minneapolis, MN). Human recombinant interleukin-3 (IL-3) was obtained from Sandoz Pharmaceutical (Basel, Switzerland). Polyclonal rabbit anti-IL-3 antibody was from Genzyme (Cambridge, MA). Anti-PiH12 (Chemicon International), anti-desmin D33, von Willebrand factor F8/86, anti-SM α-actin, and anti-vimentin

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1 The abbreviations used are: KS, Kaposi’s sarcoma; HIV-1, human immunodeficiency virus type 1; IGF-I, insulin-like growth factor I; IGF-IR, IGF-I receptor; IL-3, interleukin-3; PI3K, phosphatidylinositol 3-kinase; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling; HHV8, human herpesvirus 8; VEGF, vascular endothelial growth factor; MOPS, 4-morpholinopropanesulfonic acid.
antibodies (Dako, Glostrup, Denmark) and myeloperoxidase, monoclonal anti-VEGFR1, and anti-VEGFR2 antibodies (Chemicon International Inc., Temecula, CA) were used for the characterization of KS cells. Mouse monoclonal anti-β-actin and vincristine were obtained from Sigma.

**Cell Cultures**—A primary culture of Kaposi’s sarcoma cells (KS cells) was obtained from a cutaneous lesion of a patient bearing renal allograft under immunosuppressive therapy and compared with the well-characterized spontaneously immortalized iatrogenic KS cell line (designated KS-9252) (23). Endothelial cells from human umbilical veins obtained and propagated as described previously were used as control in the characterization of KS cells (24).

The KS cells were cultured with RPMI 1640 (Invitrogen) containing 10% fetal calf serum (HyClone Laboratories, Logan, UT) and 2 mM glutamine (Invitrogen).

**Assessment of Apoptosis**—Cell viability and apoptosis were assessed using several different methods.

**DNA Fragmentation**—Apoptosis was accompanied by fragmentation of DNA (25–26). To determine the occurrence of DNA fragmentation, total DNA was extracted from unstimulated and stimulated KS cells. The culture medium was removed and centrifuged at 3,000 g for 5 min to collect detached cells. Adherent cells were lysed with a hypotonic lysis buffer (10 mM Tris-HCl, pH 8, containing 10 mM EDTA and 0.5% Triton X-100) and then pooled with pellets made of detached cells. RNA and proteins were digested using 0.1 mg/ml RNase at 37 °C for 1 h, followed by protease K treatment for 2 h at 50 °C. DNA was homogenized in the TRI Reagent (Sigma) for 10 min at room temperature. After centrifugation, the aqueous phase was gently removed, and the DNA pellet was resuspended with 100% ethanol and centrifuged at 6,000 × g for 5 min at 4 °C. The ethanol was then removed, and the DNA pellet was washed twice with a solution containing 0.1 M sodium citrate in 10% ethanol for 3 min at room temperature under agitation and was centrifuged at 6,000 × g for 5 min at 4 °C. Next, the DNA pellet was suspended in 75% ethanol, shaken for 15 min at room temperature, and centrifuged at 6,000 × g for 5 min at 4 °C. Again, the ethanol was removed, and the DNA pellet was briefly air-dried at room temperature. The DNA pellet was dissolved in 8 mM NaOH by slowly passing through a pipette and centrifuged at 10,800 × g for 10 min at 4 °C. The supernatant was collected and resolved in a 2% agarose gel stained with 0.5 μg/ml ethidium bromide.

**XTT Reduction**—This assay was based on the capability of mitochondrial dehydrogenases of viable cells to cleave the tetrazolium ring of the sodium salt of XTT (Sigma), generating orange crystals that are soluble in water (27). Briefly, KS cells were cultured in 96-well flat-bottomed microtiter plates (Falcon Labware, Oxnard, CA) at a concentration of 5 × 10^4 cells/well for 24 h. The cells were then washed twice with PBS and incubated at 37 °C with 250 μM XTT in medium without phenol red in the presence of 0.25 μg/ml vincristine alone or in combination with different stimuli. The absorption values were measured at 620 nm in a microplate reader (Bio-Rad Laboratories, Hercules, CA). The percentage of apoptotic cells was determined using the CellTiter96 AQueous One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer’s instructions.

**TUNEL Assay**—KS cells were subjected to terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay analysis (ApopTag Oncor, Gaithersburg, MD). Briefly, KS cells were plated on tissue culture flasks with 0.25 μg/ml vincristine and different stimuli for 24 h. KS cells in 24-well plates were suspended in PBS, fixed in 1% paraformaldehyde in PBS, pH 7.4, for 15 min at 4 °C, washed twice in PBS, and then post-fixed in precooled ethanol/acetone 2:1 for 5 min at −20 °C. Samples were then washed twice with PBS, and the cells were treated with terminal deoxynucleotidyltransferase enzyme and incubated in a humidified chamber at 37 °C for 1 h. The cells were then washed three times in PBS, treated with warmed anti-digoxigenin conjugate with fluorescein, and incubated in a humidified chamber for 30 min at room temperature, avoiding exposure to light. After washing four times in PBS, samples were mounted in medium containing 1 μg/ml propidium iodide, and the cells were analyzed by fluorescence microscopy.

**Western Blot Analysis**—For Western blot analysis of Akt, P-AKT, BAD, and P-BAD expression by KS cells, cells were lysed at 4 °C for 1 h in a lysis buffer (50 mM Tris-HCl, pH 8.3, containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 100 units/ml aprotinin). After centrifugation of the lysates at 15,000 × g, protein contents of the supernatants were measured by the Bradford method. Aliquots containing 30–50 μg of protein per lane were subjected to 10% SDS-PAGE under reducing conditions and electroblotted onto nitrocellulose membrane filters. The blots were blocked with 5% nonfat milk in 20 mM Tris-HCl, pH 7.5, 500 mM NaCl plus 0.1% Tween (TBS-T). The membranes were subsequently incubated overnight at 4 °C with polyclonal goat antibody against human Akt and P-AKT or with a polyclonal rabbit antibody against human BAD and P-BAD (Santa Cruz Biotechnology) at a concentration of 500 ng/ml. After extensive washing with TBS-T, the blots were incubated for 1 h at room temperature with peroxidase-conjugated protein A (200 ng/ml; Amersham Biosciences), washed with TBS-T, developed with ECL detection reagents (Amersham Biosciences) for 1 min, and exposed to X-Omat film (Eastman Kodak Co.).

**AKT Kinase Assay**—To assay for AKT kinase activity, cells were serum-starved, submitted to different experimental conditions, washed twice in cold PBS, and lysed in ice with 900 μl of lysis buffer containing 1% Triton X-100, 10% glycerol, 157 mM NaCl, 20 mM Tris-HCl, pH 7.5, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM Na3P04, and 1 mM Na2VO3. Equal amounts of lysates (300 μg) were precleared by centrifugation and preabsorbed with protein A-protein G (1:1) agarose slurry. Immunoprecipitation was carried out for 18 h using anti-AKT1/PRKBo-PH domain antibody cross-linked to protein G-agarose (Upstate Biotechnology, Inc.). Immunoprecipitates were washed three times with lysis buffer, once with water, and once with the AKT kinase buffer (20 mM HEPES, pH 7.4, 10 mM MgCl2, 10 mM MnCl2). Kinase assays were performed for 30 min at 25 °C under continuous agitation in kinase buffer containing 0.05 mg/ml histone H2B (Roche Molecular Biochemicals), 5 μM ATP, 1 mM dithiothreitol, and 10 μCi of [γ-32P]ATP (Amersham Biosciences). Samples were analyzed in a 15% SDS-polyacrylamide gel, transferred to nitrocellulose membranes (Bio-Rad), and exposed. Resulting autoradiograms were quantified. Data for the kinase activity were expressed as fold induction with respect to the activity exhibited by unstimulated serum-starved cells. In parallel, to assess the level of expression of AKT, the same amounts of immunoprecipitates were submitted by Western blot, using polyclonal goat antibody against human AKT (1 μg/ml) (Upstate Biotechnology, Inc.). Bands were developed by ECL detection reagents (Amersham Biosciences) using peroxidase-conjugated protein A (200 ng/ml; Amersham Biosciences).

AKT activity was also measured using recombinant BAD as substrate for serine 136 phosphorylation (28–30). Briefly, lysates of unstimulated or stimulated KS cells in DIM lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EGTA; 1 mM phenylmethylsulfonyl fluoride; 1 μg/ml each aprotinin, leupeptin, pepstatin; 1 mM sodium orthovanadate; and 1% Triton X-100

**Table I**

**Characterization of KS cell primary culture**

| Markers | HUVECs | KS cells | KS imm |
|---------|--------|----------|--------|
| Vimentin | +/+    | +/+      | ++     |
| PH1H2   | ++/+   | ++       | +/+    |
| PVIII   | ++     | -/+      | +      |
| Desmin  | -      | -        | +      |
| SMα-actin | -    | -        | +      |
| Myeloperoxidase | - | -        | +      |
| VEGFR1  | ++     | +/+      | +      |
| VEGFR2  | +      | +        | +      |

*PH1H2 monoclonal antibody detects the CD146 of an endothelial cell marker (32).*

**Fig. 1. Comparison of the anti-apoptotic effect of Tat on primary KS and KS-imm cell cultures.** The percentage of apoptotic cells subjected to the TUNEL assay after 24 h of incubation with vehicle alone or in the presence of 0.25 μg/ml vincristine or 0.25 μg/ml vincristine plus 10 ng/ml Tat is shown. Primary KS cells, **black column;** KS-imm cells, **open column.** Data are expressed as mean ± 1 S.D. from three different experiments.

**Tat Inhibits KS Cell Apoptosis**

25196

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25196
for 10 min) were prepared. Cell lysates (1 mg) diluted to roughly 1 µg/ml total cell protein were added to 10 µl of BAD-agarose (Upstate Biotechnology) and 50 µl of magnesium/ATP mixture (20 mM MOPS, pH 7.2; 25 mM β-glycerolphosphate; 5 mM EGTA; 1 mM Na3VO4; 1 mM dithiothreitol; 75 mM MgCl2; 500 mM ATP). The reaction mixture was shaken for 10 min at 30 °C, and the agarose beads were collected by centrifugation. Fifty ng/µl active Akt1 (Upstate Biotechnology, Inc.) was used as positive control. The beads were washed three times with ice-cold PBS, resuspended in Laemmli sample buffer, boiled for 5 min, and then collected by a pulse in a microcentrifuge (29–31). Samples were subjected to SDS-PAGE, and proteins were transferred to nitrocellulose. The nitrocellulose membranes were blocked in freshly prepared TBS with 0.5% Tween 20 for 15 min. Specific reactions were revealed with the ECL Western blotting detection reagent (Amersham Biosciences). The nitrocellulose was washed four times in TBS containing 0.5% Tween 20 for 15 min and incubated with the secondary antibody (horse-radish peroxidase-conjugated goat anti-rabbit IgG, a 1:10,000 dilution in 10% BSA/TBS with 0.5% Tween 20 overnight at 4 °C).

The nitrocellulose was washed four times in TBS containing 0.5% Tween 20 for 15 min and incubated with the secondary antibody (horse-radish peroxidase-conjugated goat anti-rabbit IgG, a 1:10,000 dilution in 10% BSA/TBS with 0.5% Tween 20 overnight at 4 °C).

RESULTS

Immunohistochemistry using antibodies directed against factor VIII, P1H12 (an endothelial cell marker), vimentin, CD145 detected by the anti-P1H12 monoclonal antibody (32). Moreover, both cell lines were positive for VEGFR2 but not for VEGFR1.

As reported previously (22), HIV-1-Tat acted as a survival factor for KS cells and prevented vincristine-induced apoptosis of KS-imm. Similar results were obtained in KS cell primary culture exposed to vincristine (0.25 µg/ml) in the presence of Tat (10 ng/ml) (Fig. 1).

Gene array technology was used to investigate the effect of vincristine and Tat on the expression of genes that modulate apoptosis by KS cells (Table II and Fig. 2). Fig. 2A shows gene
denatured salmon sperm DNA (Amersham Biosciences) to block non-specific hybridization. The filters were then hybridized overnight at 68 °C with denatured biotinylated cDNA probes in 5 ml of hybridization solution in hybridization bottles rotated at 5 rpm. The filters were then extensively washed at low and high stringency conditions in hybridization bottles at a rotation speed of 15 rpm. After membrane incubation with alkaline phosphatase-conjugated streptavidin (AP-streptavidin 1:5,000), gene expression was detected by chemiluminescence signal using the alkaline phosphatase substrate, CDP-Star, and exposed to x-ray film.

Each GEArray membrane was spotted with a negative control of pUC18 DNA in position G1 and G2 as well as two positive control genes, β-actin and GAPDH, in positions G3, G4, E8, F8, G5, G6, and G7. The G axis served as internal hybridization control.

For 10 min) were prepared. Cell lysates (1 mg) diluted to roughly 1 µg/ml total cell protein were added to 10 µl of BAD-agarose (Upstate Biotechnology) and 50 µl of magnesium/ATP mixture (20 mM MOPS, pH 7.2; 25 mM β-glycerolphosphate; 5 mM EGTA; 1 mM Na3VO4; 1 mM dithiothreitol; 75 mM MgCl2; 500 mM ATP). The reaction mixture was shaken for 10 min at 30 °C, and the agarose beads were collected by centrifugation. Fifty ng/µl active Akt1 (Upstate Biotechnology, Inc.) was used as positive control. The beads were washed three times with ice-cold PBS, resuspended in Laemmli sample buffer, boiled for 5 min, and then collected by a pulse in a microcentrifuge (29–31). Samples were subjected to SDS-PAGE, and proteins were transferred to nitrocellulose. The nitrocellulose membranes were blocked in freshly prepared TBS with 0.5% Tween 20 for 15 min. Specific reactions were revealed with the ECL Western blotting detection reagent (Amersham Biosciences). The nitrocellulose was washed four times in TBS containing 0.5% Tween 20 for 15 min and incubated with the secondary antibody (horse-radish peroxidase-conjugated goat anti-rabbit IgG, a 1:10,000 dilution in 10% BSA/TBS with 0.5% Tween 20 overnight at 4 °C).

The nitrocellulose was washed four times in TBS containing 0.5% Tween 20 for 15 min and incubated with the secondary antibody (horse-radish peroxidase-conjugated goat anti-rabbit IgG, a 1:10,000 dilution in 10% BSA/TBS with 0.5% Tween 20 for 1 h and then incubated with anti-phospho-BAD (P-BAD) diluted 1:1000 in 10% BSA/TBS with 0.5% Tween 20 overnight at 4 °C).

The nitrocellulose was washed four times in TBS containing 0.5% Tween 20 for 15 min and incubated with the secondary antibody (horse-radish peroxidase-conjugated goat anti-rabbit IgG, a 1:10,000 dilution in 10% BSA/TBS with 0.5% Tween 20 for 1 h at room temperature. The nitrocellulose was washed four times in TBS with 0.5% Tween 20 for 15 min. Specific reactions were revealed with the ECL Western blotting detection reagent (Amersham Biosciences). Gene Array—The human BCL-2 family and regulatory network GE-Array Kit (Biomol Feinchemikalien GmbH, Hamburg, Germany) was used to characterize the gene expression profiles associated with different experimental conditions. The kit included duplicate spots of 23 human family genes and other genes in-
expression in unstimulated cells. Hybridization of RNA extracted from KS cells treated with vincristine was associated with a loss of expression of some anti-apoptotic genes such as AKT-1 and AKT-2, BCL2, BCL-X, MCL-1 and IGF-I, and with enhanced expression of pro-apoptotic genes such as BIK (Fig. 2C). After addition of Tat to vincristine-treated KS cells, we observed the reappearance of expression of the anti-apoptotic genes AKT-1 and AKT-2, BCL-2, BCL-X, MCL-1 and IGF-I and the de novo expression of IL-3 gene (Fig. 2D). Stimulation of KS cells with Tat alone, in addition to the genes mentioned above, induced the expression of BAX, BCL-6, and BAG-1 (Fig. 2B). No significant variation in the expression of the housekeeping genes (β-actin and GAPDH) was observed in the different experimental conditions. The bacterial plasmid pUC18 was always negative.

The observation that Tat abrogated the effect of vincristine on AKT gene expression prompted us to test whether AKT was involved in the anti-apoptotic effect of Tat. It has been shown that the anti-apoptotic effect of AKT depends on PI3K activation (33–35). Thus, we evaluated the effect of two unrelated PI3K pharmacological inhibitors, wortmannin and LY294002 (36). As shown in Fig. 3 vincristine-induced apoptosis of KS cells was inhibited by Tat. Pretreatment with wortmannin (0.1 μM) and LY294002 (10 μM) completely abrogated the anti-apoptotic effect of Tat. Fig. 4 shows that vincristine slightly decreased the expression of AKT protein consistently with the down-regulation of AKT gene expression observed by gene array analysis. However, vincristine mainly impaired the ability of AKT to undergo phosphorylation (P-AKT). Treatment with Tat not only restored the expression of AKT protein but also its phosphorylation. When PI3K was inhibited by wortmannin (0.1 μM) and LY294002 (10 μM), Tat-induced phosphorylation of AKT was significantly prevented. These compounds alone had no significant effect on AKT kinase activity in serum-starved cells but inhibited AKT activity induced by Tat (Fig. 5A). Phosphorylation of BAD at serine 136 is one of the potential mechanisms through which AKT inhibits apoptosis (28, 29). We therefore studied whether phosphorylation of BAD paralleled that of AKT. As shown in Fig. 5B, vincristine induced a concomitant reduction of P-AKT and P-BAD. Tat treatment of KS cells incubated with vincristine restored both AKT and BAD phosphorylation. No significant changes in BAD protein expression were observed under the different experimental conditions.

To evaluate whether Tat directly induced AKT enzymatic activity, AKT kinase activity was measured after AKT immunoprecipitation from KS cell lysates at different times of incubation. In parallel, phosphorylation of recombinant BAD used as substrate for AKT (28, 29, 31) was evaluated on the same KS cell lysates. As shown in Fig. 6, two waves of AKT kinase activity and BAD phosphorylation were observed after incubation of KS cells with Tat. Tat induced an early increase in AKT activity that peaked at 15 min and decreased after 60 min. This early AKT activation was not inhibited by cycloheximide, an inhibitor of protein synthesis (data not shown). In addition, Tat induced a delayed AKT activation that was detectable after 24 h; this activation was significantly decreased by cyclohexi-
mide, indicating that a Tat-induced synthesis of secondary mediators may contribute to this AKT activation. Previous studies (22) demonstrated that Tat stimulates KS cells through VEGFR-2. As shown in Fig. 7A, pretreatment of KS cells with a function blocking anti-VEGFR-2 antibody significantly reduced the survival effect of Tat on vincristine-treated KS cells. However, when the anti-VEGFR-2 antibody was added 1 h after Tat stimulation, the inhibitory effect on Tat-induced survival was less effective. These results suggest that the effects of the anti-VEGFR-2 blocking antibody were mainly due to interference of Tat interactions with VEGFR-2 rather than any indirect effects such as Tat-induced synthesis of VEGF. As shown in Fig. 8, the anti-VEGFR-2-blocking antibody also inhibited both the early and late enhancement of BAD phosphorylation induced by Tat.

Because gene array analysis indicated that Tat was able to trigger IGF-I gene expression, we investigated whether IGF-I may contribute to the anti-apoptotic effect of Tat in KS cells. IGF-I is known to induce resistance to apoptosis in several cell types via the activation of AKT (37). We observed that the delayed activation of AKT-dependent BAD phosphorylation (Fig. 9) and the survival effect of Tat (Fig. 7B) were significantly reduced after treatment of KS cells with a combination of anti-IGF-I and anti-IGF-IR antibodies. These results suggested that the synthesis of IGF-I triggered by Tat contributed to the delayed AKT activation and to the resistance to apoptosis. Indeed, IGF-I significantly prevented vincristine-induced KS cell death. Moreover, Tat was shown to induce the expression of the gene coding for IL-3, a cytokine known to activate AKT (28). An anti-IL-3 function-blocking antibody also reduced the survival effect of Tat on vincristine-treated cells (Fig. 7B) and the delayed activation of AKT-dependent BAD phosphorylation (Fig. 9). The inhibitory effect on delayed Tat-induced AKT-dependent BAD phosphorylation was particularly evident when cells were treated with anti-IGF-I/anti-VEGFR-2. As shown in Fig. 7A, pretreatment of KS cells with a function blocking anti-VEGFR-2 antibody significantly reduced AKT kinase activity. Kinase reactions and Western blot analyses were performed on anti-AKT immunoprecipitates obtained from KS cell lysates, as described under “Experimental Procedures.” Serum-starved KS cells, unstimulated or stimulated with 10 ng/ml Tat (for 15 min), were preincubated for 30 min with or without 0.1 μM wortmannin or 0.1 μM LY294002. The autoradiogram corresponds to a representative experiment repeated twice. "P-Labeled products (52P-H2B) as well as specific bands detected by the anti-AKT antibody are indicated.

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IGF-IR and anti-IL-3 blocking antibodies. In contrast, no effect was observed on the early activation of AKT induced by Tat (data not shown).

**DISCUSSION**

KS tumorigenesis seems to depend not only on the dysregulation of cellular proliferation but also on resistance to apoptotic signals. Indeed, KS cells are resistant to Fas-mediated apoptosis despite the expression of Fas on their surface (38). Moreover, KS cells derived from AIDS patients are often resistant to chemotherapy drugs (39). We demonstrated recently (22) that Tat can increase survival of vincristine-treated KS cells. This increased survival mainly depended on an anti-apoptotic effect of Tat that was detectable at concentrations of Tat well below those observed in AIDS patients (40). The mechanisms by which Tat induces resistance to apoptosis remain largely unknown. However, because KS cells express KDR/VEGFR-2 and Tat is able to bind to and activate endothelial (14) and KS (15, 16) cells expressing the KDR/VEGFR-2 receptor, KDR/VEGFR-2 activation may transduce the anti-apoptotic signal of Tat.

We therefore investigated the molecular mechanisms involved in the anti-apoptotic effect of Tat on vincristine-treated KS cells.

Gene expression analysis by gene array technology demonstrated that vincristine treatment induced a down-regulation of several anti-apoptotic genes, including AKT-1 and AKT-2, BCL2, BCL-XL, and IGF-I and enhanced the expression of...
pro-apoptotic genes such as BIK. Tat treatment abrogated the effect of vincristine on the anti-apoptotic genes AKT-1 and AKT-2, BCL-2, BCL-XL, and IGF-I and induced the de novo expression of IL-3 in vincristine-treated KS cells. These results are consistent with an enhanced transcription of several genes induced by Tat, which includes not only anti-apoptotic genes but also pro-apoptotic genes such as BAX and BAG-1. However, the final effect appears to be protection from apoptosis.

It is known that AKT regulates cell survival and apoptosis at a post-mitochondrial level (35). On the basis of the observation that vincristine down-regulated and that Tat restored AKT gene expression, we investigated whether AKT mediated the anti-apoptotic effect of Tat on KS cells.

The PI3K/AKT is one of the central pathways involved in survival signaling (34). Several receptors, including those for VEGF (41), IGF-I (37), and IL-3 (28), transmit survival signals through these pathways. PI3K activation catalyzes the transfer of a phosphate group from ATP to the D3 position of phosphatidylinositol, thus generating 3'-phosphatidylinositol phosphates (42). These latter compounds serve as binding sites for proteins that possess pleckstrin homology domains such as AKT. The binding of AKT to 3'-phosphatidylinositol phosphate results in its translocation from cytosol to plasma membrane and phosphorylation of threonine 308 and serine 473 residues. Phosphorylation of threonine 308 and membrane localization depend on the activation of a phosphatidylinositol-dependent kinase-1, which also contains a pleckstrin homology domain (43).

Inhibition of PI3K with two unrelated pharmacological inhibitors, wortmannin and LY294002, abrogated AKT phosphorylation and the protective effect of Tat on vincristine-induced apoptosis of KS cells, suggesting that the PI3K/AKT pathway is involved in Tat-mediated anti-apoptotic effect.

Several studies (33–35) have shown that AKT is the major effector of PI3K survival signaling. However, the mechanism by which AKT suppresses death is incompletely known. Phosphorylation of BAD at serine 136 is one of the potential mechanisms involved in AKT-dependent survival (28). Non-phosphorylated BAD binds to BCL-XL in the mitochondrial membrane thereby preventing BCL-XL from promoting cell survival. When phosphorylated, BAD is released from BCL-XL, which can then prevent apoptosis by inhibiting the release of cytochrome c and is sequestered in the cytoplasm by 14-3-3 survival factors. In the present study we observed that Tat...
Tat Inhibits KS Cell Apoptosis

Induces concomitant phosphorylation of BAD and AKT suggesting the involvement of this pathway in Tat-induced KS cells survival. Indeed, the inhibitors of PI3K/AKT pathway also abrogated BAD phosphorylation (34). The AKT-dependent regulation of cell metabolism may also contribute to the anti-apoptotic effect on KS cells (44, 45).

AKT-mediated cell survival may also depend on its phosphorylation and inactivation and recruitment of Forkhead transcription factor FKHR1 (46–48). In the absence of phosphorylation, FKHR1 migrates to the nucleus leading to the transcription and the surface expression of FasL that in turn mediates a cell death cascade (46). However, the involvement of this pathway in the anti-apoptotic effect of Tat is unlikely, as we have demonstrated previously (22) that Tat did not affect Fas/FasL expression by KS cells.

HIV-1-Tat protein has been shown to down-regulate cAMP-response element-binding protein transcription factor expression in PC12 neuronal cells through PI3K/AKT/cyclic nucleotide phosphodiesterase pathway (31). Moreover, the PI3K/AKT pathway was found to be activated by Tat in T-lymphoblastoid Jurkat cells (49).

In the present study we demonstrated that Tat induced two waves of AKT activity in KS cells. The early activation of AKT was independent of protein synthesis, suggesting a direct activation of PI3K/AKT pathway by Tat. This early AKT activation was inhibited by anti-VEGFR-2 antibodies more efficiently when added before Tat stimulation than when added 1 h after. These results are consistent with the recent observation (16) that the stimulation of KS cells by Tat mainly occurs through the VEGFR-2. The delayed AKT activation detectable at 24 h was significantly decreased by cycloheximide, an inhibitor of protein synthesis, suggesting that a Tat-induced synthesis of secondary mediators may contribute to AKT activation. One could speculate that Tat could stimulate the synthesis of VEGF, which in turn mediates the anti-apoptotic effect of Tat. However, because anti-VEGFR2 blocking antibodies only partially inhibited the survival effect of Tat when added 1 h after Tat stimulation, it is reasonable to assume that beside VEGF other mediators could be involved in mediating the Tat effect.

Because gene array analysis indicated that Tat was able to trigger IGF-I and IL-3 gene expression, we investigated whether these cytokines may contribute to the anti-apoptotic effect of Tat in KS cells. IGF-I is known to induce resistance to apoptosis in several cell types via the activation of AKT (37). IL-3 also mediates an anti-apoptotic effect on lymphocytes by the PI3K/AKT pathway (30). We found that IGF-1 and, to a less extent, IL-3 inhibit apoptosis of KS cells induced by vincristine. Moreover, the treatment of KS cells with anti-IGF-1 receptor and anti-IL-3 blocking antibodies significantly reduced the anti-apoptotic effect of Tat and the delayed activation of AKT. These results suggested that the synthesis of IGF-I and IL-3 triggered by Tat might contribute to the resistance to apoptosis induced by Tat.

It has been described that Tat has opposite effects on apoptosis not only on different cell types but also on the same cell type under different conditions. Indeed, Tat induces apoptosis of uninfected T-cells (20) through a Fas-dependent mechanism (21), whereas in peripheral blood HIV-1-infected T-cells prevent apoptosis (20). Moreover, Tat was shown to prevent apoptosis of different tumor cell lines of lymphoid, epithelial, and neuronal origin (19, 22), suggesting its putative role in tumoral development in a microenvironment characterized by immune system impairment. We observed that Tat not only enhances the transcription of anti-apoptotic but also of some pro-apoptotic genes such as BAX and BAG-1. Moreover, Tat is known to induce production of several cytokines and growth factors that may influence the sensitivity to apoptosis. Therefore, the different effects of apoptosis elicited by Tat may depend not only on a differential activation of pro- and anti-apoptotic genes but also on cytokines and growth factors released in the cell.

FIG. 8. Effect of anti-VEGFR-2 blocking antibody on Tat-mediated activation of AKT detected as phosphorylation of recombinant BAD. AKT-induced phosphorylation of BAD was measured in KS cell lysates at different times of incubation with 10 ng/ml Tat using recombinant BAD as substrate (see “Experimental Procedures”). A representative experiment of phosphorylation of recombinant BAD (3.3 µg) by KS cell lysates (1 mg of protein) was obtained after 15 and 30 min and 24 h of incubation with Tat. Where indicated, 10 µg/ml anti-VEGFR-2 antibody was added 10 min before stimulation with Tat. The densitometric analysis was performed, and data are expressed as fold induction over the unstimulated control. Data are representative of three independent experiments.

FIG. 9. Effect of anti-IGF-I/anti-IGF-IR and anti-IL-3 blocking antibodies on Tat-mediated activation of AKT detected as phosphorylation of recombinant BAD. AKT-induced phosphorylation of BAD was measured in KS cell lysates after 24 h of incubation in the following conditions: row 1, unstimulated control; row 2, 0.25 µg/ml vincristine; row 3, 0.25 µg/ml vincristine plus 10 ng/ml Tat; row 4, vincristine plus 10 ng/ml IGF-I; row 5, vincristine plus 10 ng/ml Tat plus 10 µg/ml anti-IGF-I and 10 µg/ml anti-IGF-IR antibodies; row 6, vincristine plus 10 ng/ml IGF-I plus 10 µg/ml anti-IGF-I and 10 µg/ml anti-IGF-IR antibodies; row 7, vincristine plus 10 ng/ml Tat plus 10 µg/ml anti-IL-3 blocking antibodies; row 8, vincristine plus IL-3 (20 µg/ml); row 9, vincristine plus 20 ng/ml IL-3 plus 10 µg/ml anti-IL-3 blocking antibodies; row 10, vincristine plus 10 ng/ml Tat plus 10 µg/ml anti-IGF-I and 10 µg/ml anti-IGF-IR antibodies plus 10 µg/ml anti-IL-3 blocking antibody. The densitometric analysis was performed, and the data, expressed as fold induction over unstimulated control, are representative of three independent experiments.
microenvironment. In this context, stimuli that activate the AKT-dependent pathway may contribute to the anti-apoptotic effect of Tat.

Recently, it has been shown (50) that the HHV8 G protein-coupled receptor also promotes endothelial cell survival through an AKT-dependent pathway. Several lines of evidence indicate a role of HHV8 in the pathogenesis of KS. Endothelial cells in early KS lesions are infected with HHV8, and HHV8 has been shown to be able to infect endothelial cells in vitro, inducing a spindle-shape morphology and increasing the proliferative lifespan in all the cells in culture, despite the indication that only a limited portion appeared to be infected (51, 52). This suggests that paracrine effects induced by HHV8 infection are able to phonotypically modify neighboring cells (53). Therefore, in AIDS patients the HIV-1-Tat protein may contribute to HHV8 infection with the activation of the PI3K/AKT pathway in sensitive cells concurring to the pathogenesis of KS.

In conclusion, the results of the present study indicate that the HIV-1-Tat protein acts as a survival factor for KS via a PI3K/AKT pathway. This pathway is mainly triggered by the stimulation of KS cells through VEGFR-2, which leads to the transcription of several anti-apoptotic genes including IGF-I and IL-3. These cytokines may then contribute with Tat to the activation of PI3K/AKT-mediated survival signals.

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HIV-1-Tat Protein Activates Phosphatidylinositol 3-Kinase/AKT-dependent Survival Pathways in Kaposi’s Sarcoma Cells

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