

The Tat Protein of Human Immunodeficiency Virus Type-1 Promotes Vascular Cell Growth and Locomotion by Engaging the $\alpha 5\beta 1$ and $\alpha \nu\beta 3$ Integrins and by Mobilizing Sequestered Basic Fibroblast Growth Factor

By Giovanni Barillari, Cecilia Sgadari, Valeria Fiorelli, Felipe Samaniego, Sandra Colombini, Vittorio Manzari, Andrea Modesti, Bala C. Nair, Aurelio Cafaro, Michael Stürzl, and Barbara Ensoli

The Tat protein of human immunodeficiency virus type-1 (HIV-1) has been shown to be released during acute infection of T cells by HIV-1 and to promote angiogenesis and Kaposi's sarcoma (KS) development in infected individuals. In this study, we investigated the molecular mechanisms responsible for the angiogenic effects of Tat. The results shown herein indicate that two different Tat domains cooperate to induce these effects by different pathways. The arginine-glycine-aspartic acid (RGD) sequence present at the carboxy-terminal of Tat mediates vascular cell migration and invasion by binding to the $\alpha 5\beta 1$ and $\alpha \nu\beta 3$ integrins. This interaction

DURING ACUTE INFECTION of T cells by human immunodeficiency virus type-1 (HIV-1), Tat, a transactivator of viral gene expression,¹ is released extracellularly.²⁻⁴ In this form, Tat exerts activities that have linked the protein to the pathogenesis of Kaposi's sarcoma (KS), a disease of vascular origin that is very common and aggressive in HIV-1-infected individuals (acquired immunodeficiency syndrome-KS [AIDS-KS]) but mild and indolent in the absence of HIV infection.⁵ In particular, Tat promotes the locomotion and growth of spindle cells of endothelial origin derived from AIDS-KS lesions (KS cells) and of normal endothelial cells,^{2,3,6-10} which are considered to be the precursors of KS cells.¹¹ However, endothelial cells become responsive to the effects of Tat only after activation with inflammatory cytokines (IC), such as interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), and interferon- γ (IFN- γ).⁶⁻¹⁰ These IC are the same found to be increased in the lesions and blood of KS patients.¹²⁻¹⁴

The requirement of other factors for Tat angiogenic effects is also observed in vivo, because inoculation of Tat protein alone in nude mice has little or no effect.¹⁵ In contrast, when Tat is injected with IC or with suboptimal (non-lesion-forming) amounts of basic fibroblast growth factor (bFGF), it promotes the development of angioproliferative KS-like lesions in the inoculated animals.^{9,15} It is of interest that bFGF production is enhanced in KS cells or induced in endothelial cells by the same IC that are required to promote endothelial cell responsiveness to Tat.¹⁶⁻¹⁸

Tat has also been shown to bind F1K-1/KDR, one of the receptors for the vascular endothelial growth factor (VEGF),¹⁹ suggesting an additional mechanism for Tat to exert angiogenic activity. Both bFGF and VEGF, two potent angiogenic factors,^{20,21} are highly expressed in AIDS-KS lesions,^{15,22-25} where they synergize in promoting neoangiogenesis and edema.^{18,25}

Tat can also promote KS and endothelial cell adhesion through the binding of its arginine-glycine-aspartic acid (RGD) region to the $\alpha 5\beta 1$ and $\alpha \nu\beta 3$ integrins.²⁶ These receptors, which bind the RGD sequence of extracellular matrix (ECM) proteins, such as fibronectin (FN) and vitronectin (VN),²⁷ are constitutively expressed by KS cells both in vitro and in primary lesions,^{15,26} and their levels are increased in normal endothelial

also provides endothelial cells with the adhesion signal they require to grow in response to mitogens. At the same time, the Tat basic sequence retrieves into a soluble form extracellular basic fibroblast growth factor (bFGF) bound to heparan sulfate proteoglycans by competing for heparin-binding sites. This soluble bFGF mediates Tat-induced vascular cell growth. These effects resemble those of extracellular matrix proteins, suggesting that Tat enhances angiogenesis and promotes KS progression by a molecular mimicry of these molecules.

© 1999 by The American Society of Hematology.

cells by the same IC that induce bFGF expression and cellular responsiveness to Tat.^{10,26} Other data indicated that the basic region of Tat binds $\alpha \nu\beta 5$, an integrin that recognizes similar sequences in VN.²⁸

Altogether, these results indicated that Tat has properties similar to both angiogenic factors and ECM proteins and that it requires the cooperation of inflammatory or angiogenic cytokines to exert its effects. However, they did not explain the mechanism(s) by which Tat can promote angiogenesis and KS progression.

We report here that the angiogenic effects of Tat are mediated by two domains of the protein. Specifically, the RGD region of Tat induces the migration and invasion of KS and endothelial cells by binding to the $\alpha 5\beta 1$ and $\alpha \nu\beta 3$ integrins. Additionally, the Tat basic sequence, because of its affinity for heparin, releases preformed extracellular-bound bFGF into a soluble form that mediates Tat-promoted vascular cell growth.

From the Laboratory of Virology, Istituto Superiore di Sanità, Rome, Italy; the Department of Experimental Medicine, University "Tor Vergata," Rome, Italy; the Department of Allergy and Clinical Immunology, University "La Sapienza," Rome, Italy; the Institute of Human Virology, University of Maryland at Baltimore, Baltimore, MD; Advanced BioScience Laboratories Inc, Kensington, MD; the GSF-National Research Center for Environment and Health, Bavarian Nordic Research Institute AS, Martinsried, Germany; and the Technical University of Munich, Institute of Virology, Munich, Germany.

Submitted January 13, 1999; accepted March 24, 1999.

Supported by grants from the Associazione Italiana per la Ricerca sul Cancro (AIRC), the Italian Ministry of Health (IX AIDS Project), the Deutsche Forschungsgemeinschaft (DFG, SFB464), and the Bundesministerium für Bildung und Forschung (BMBF, BioFuture Program).

Address reprint requests to Barbara Ensoli, MD, PhD, Director of Research, Laboratory of Virology, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy; e-mail: ensoli@virus1.net.iss.it.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1999 by The American Society of Hematology.

0006-4971/99/9402-0019\$3.00/0

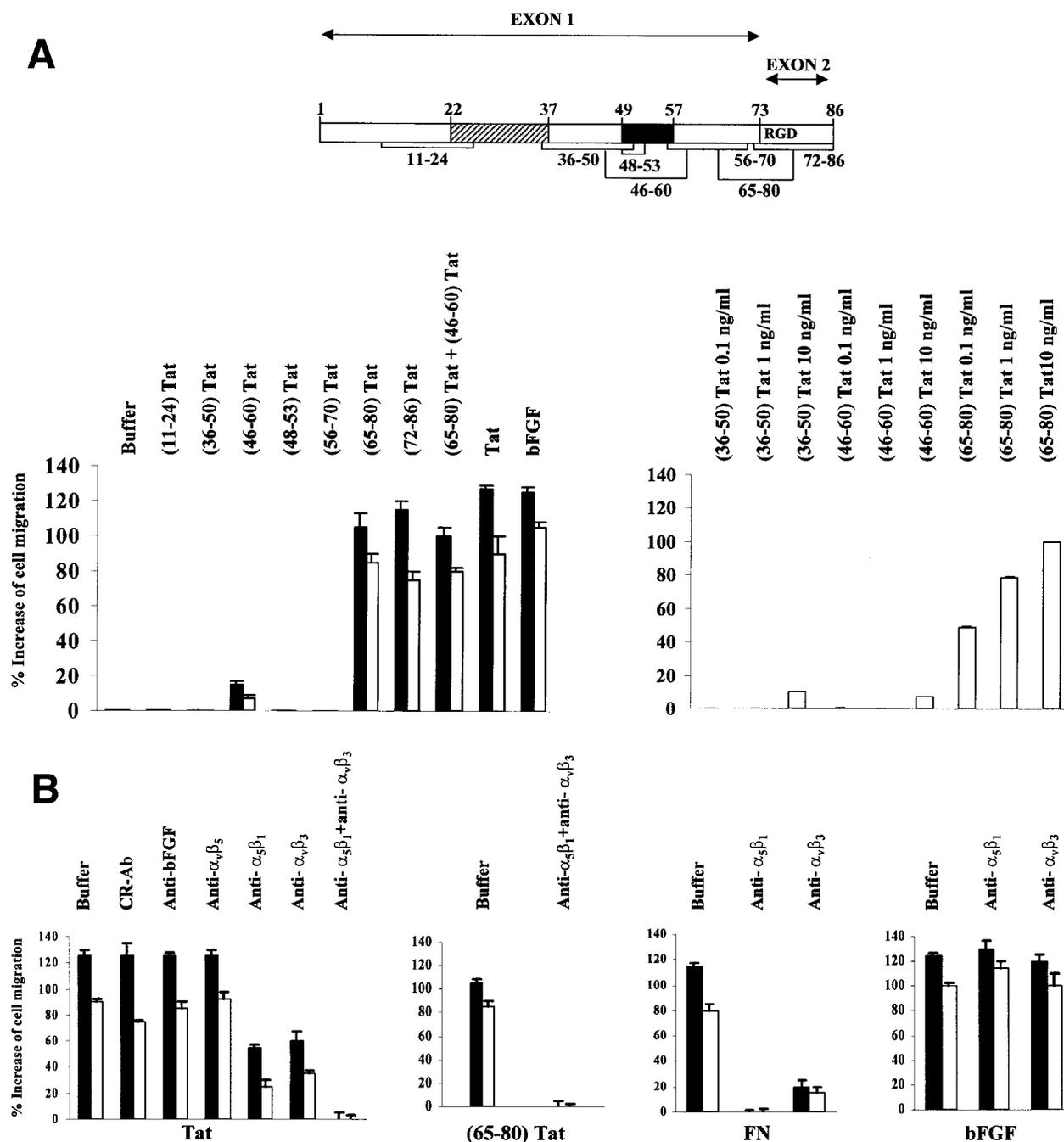


Fig 1. Tat-promoted vascular cell locomotion and collagenase IV activation are mediated by the binding of Tat-RGD region to $\alpha_5\beta_1$ and $\alpha_v\beta_3$. (A) shows the results of the migration assays with KS cells (■) and IC-HUVE cells (□). bFGF (20 ng/mL) and Tat (20 ng/mL) were used as the positive controls, whereas the peptide resuspension buffer (PBS-0.1% BSA) was the negative control. In the left panel, Tat peptides were used at concentrations equimolar to Tat. In the right panel, Tat peptides were serially diluted to test whether their effect was dose-dependent. The location of the peptides in Tat amino acid sequence is shown on the top of the figure. (B) shows KS (■) and IC-HUVE cell (□) migration to Tat (20 ng/mL), [65-80] Tat (4 ng/mL), FN (30 μ g/mL), or bFGF (20 ng/mL) after preincubation of the cells with MoAbs (2 μ g/mL each) directed against the α and β chains of $\alpha_5\beta_1$ (anti- $\alpha_5\beta_1$), $\alpha_v\beta_3$ (anti- $\alpha_v\beta_3$), or $\alpha_v\beta_5$ (anti- $\alpha_v\beta_5$). Immunohistochemical analyses indicated that KS and HUVE cells express all these integrin chains²⁶ (data not shown). Antibodies directed against CD34 or factor VIII-related antigen (antigens that are expressed by KS and IC-HUVE cells, respectively)^{30,31} (data not shown) were used as controls (CR-Ab). Polyclonal antibodies neutralizing the activity of bFGF (anti-bFGF)²² were used to determine the role of this cytokine in Tat-induced cell migration. The antibody dilution buffer (PBS-0.1% BSA) was the negative control. For (A) and (B), results (from 4 experiments, each in duplicate) refer to the number of migrated cells/field (average of 5 fields/filter) and are expressed as the percentage increase of cell migration over the number of cells migrated toward buffer (0% increase), which was 20 (\pm 2) cells/field for KS cells and 15 (\pm 1) cells/field for IC-HUVE cells.

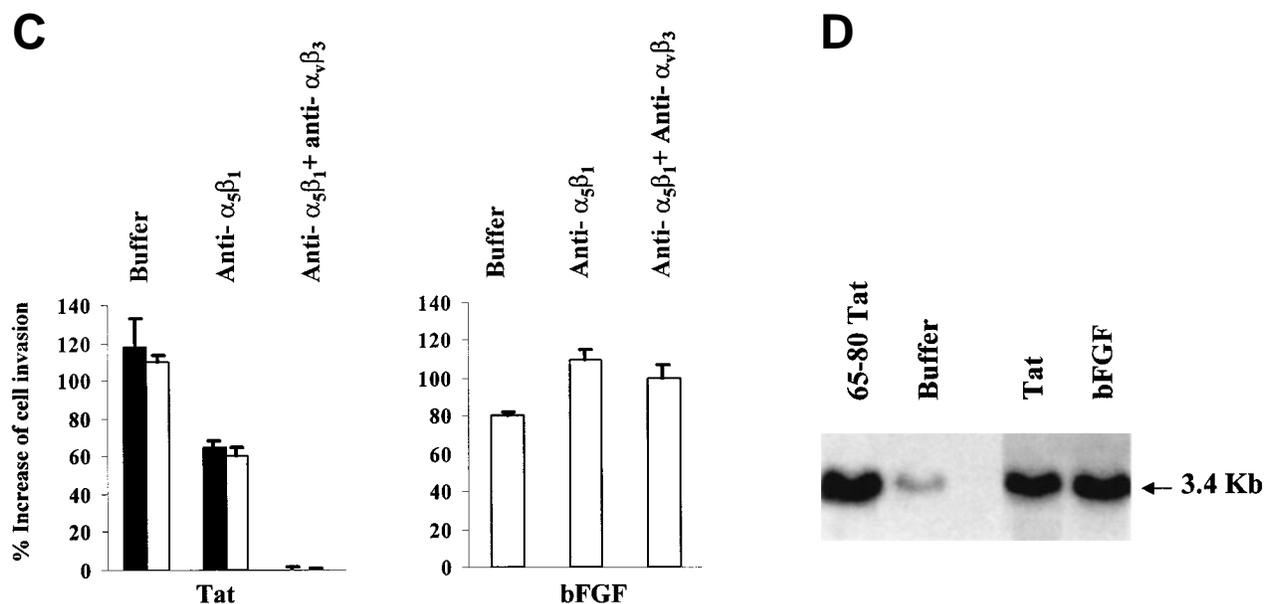


Fig 1 (Cont'd). (C) shows KS (■) and IC-HUVE (□) cell invasion to Tat or bFGF (20 ng/mL/each) after preincubation of the cells with anti- $\alpha_5\beta_1$ and/or anti- $\alpha_v\beta_3$ MoAbs (2 μ g/mL) or with buffer (PBS-0.1% BSA), as described in Materials and Methods. The results shown are from 3 experiments, each in duplicate, and they are relative to the number of invaded cells per field (average of 5 fields/filter). Data are expressed as the percentage increase of cell invasion toward Tat or bFGF over the number of invaded cells in the presence of buffer (0% increase), which was 10 (\pm 2) cells/field for KS cells and 9 (\pm 1) cells/field for IC-HUVE cells. (D) shows the Northern blot analysis of collagenase IV 72-kD gene expression in HUVE cells incubated with Tat (10 ng/mL), [65-80] Tat (2 ng/mL), bFGF (1 μ g/mL, positive control), or dilution buffer (PBS-0.1% BSA, negative control). The amount of RNA loaded in the gels was always monitored by ethidium bromide staining before Northern blotting. Repeated experiments (4 times) gave similar or identical results. The Tat concentration used is the most active in inducing collagenase IV 72-kD mRNA expression.¹⁵ At all the concentrations tested, Tat and [65-80] Tat were equally potent (data not shown). Preincubation of HUVE cells with [72-86] Tat reproduced the results obtained with [65-80] Tat (data not shown).

MATERIALS AND METHODS

Reagents. Recombinant HIV-1 Tat protein (from the IIIB isolate) was obtained and handled as previously described.^{2-4,15} The peptides [11-24] Tat, [36-50] Tat, [46-60] Tat, [56-70] Tat, [65-80] Tat, and [72-86] Tat were purchased from American Biotechnologies Inc (Cambridge, MA). The peptide [48-53] Tat, the FN cyclic peptide GRGDSP, and the mutated cyclic peptide GKGESP were purchased from Research Genetics (Huntsville, AL). Human recombinant IL-1 α and IL-1 β , IL-2, IL-6, oncostatin M, TNF- α and TNF- β , IFN- γ , bFGF, and FN (from human plasma) were purchased from Boehringer Mannheim (Indianapolis, IN). VN (from human plasma), heparin (sodium salt, from porcine intestinal mucosa), human collagen IV, and bovine serum albumin (BSA) fraction V were from Sigma (St Louis, MO). Human recombinant acidic FGF (aFGF) or endothelial cell growth supplement (ECGS), an extract from bovine hypothalamus containing aFGF,²⁹ were purchased from Collaborative Research Inc (Bedford, MA). Human recombinant VEGF, anti-bFGF rabbit polyclonal antibodies, and bFGF enzyme-linked immunosorbent assay (ELISA) kit were from R&D Systems (Minneapolis, MN). The affinity-purified monoclonal antibodies (MoAbs) directed against CDw49e (α_5 chain of one of the FN receptors), CD29 (β_1 chain of one of the FN receptors), CD51 (α_v chain of one of the VN receptors), CD61 (β_3 chain of one of the VN receptors; for review, see Hynes²⁷), and the MoAb QBEND/10 directed against CD34, a marker expressed by both KS and endothelial cells,³⁰ were purchased from Amac Inc (Westbrook, ME). MoAbs raised against the whole $\alpha_5\beta_1$ or $\alpha_v\beta_3$ integrins were purchased from Chemicon (Temecula, CA). MoAbs raised against $\alpha_v\beta_5$ were a gift from Dr E. Ruoslahti (La Jolla Cancer Research Foundation, La Jolla, CA) or were purchased from Telios, Inc (La Jolla, CA). The MoAb directed against factor VIII-related antigen³¹ was from Dakopatt (Carpinteria, CA). The sequence, synthesis, and purification of antisense bFGF or sense bFGF

phosphorothioate oligodeoxynucleotides (24 mers) directed against the splice donor-acceptor site 1 of bFGF RNA have been previously described.²⁴ Cell culture media and media supplements were purchased from GIBCO-BRL Life Technologies, Inc (Gaithersburg, MD).

Cell cultures. Different strains of KS cells (AIDS-KS3, KS4, KS6, KS7, and KS8; passage 6-12) were established and cultured as described previously.³² Three different strains of endothelial cell derived from human umbilical vein (HUVE cells; passage 4-10) were activated with combined IC (IC-HUVE cells), as reported elsewhere.^{6-10,17}

Migration, invasion, and growth assays. The migration assays were performed in the Boyden chamber, as previously described.⁸ Polycarbonate filters (12- μ m pore; Nucleoprobe Inc, Cabin John, MD) were coated with type IV collagen or recombinant Tat protein. The invasion assays were performed as the migration assays, with the difference that the filters were coated first with collagen IV and then with matrigel (Collaborative Research), a reconstituted basement membrane derived from a tumor cell line,³³ to prevent the migration of noninvasive cells.⁸ Growth assays were performed by both the cell counting and the thymidine incorporation methods, as described previously,^{2,3,6,7} with cells seeded onto plates precoated with 1.5% gelatin or with recombinant HIV-1 Tat protein.

In the blocking experiments with antibodies or competitor peptides, cells were seeded onto culture plates (for the growth assays) or resuspended by trypsinization (for the migration and invasion assays) and then preincubated on rotation in RPMI-0.01% BSA containing the competitor peptides or antibodies at the indicated concentrations for either 2 hours at 4°C or for 30 minutes at room temperature. Growth, migration, and invasion assays were then performed as described above.

RNA analysis. HUVE cells were incubated for 12 to 14 hours with Tat, [65-80] Tat, bFGF, or their dilution buffer (phosphate-buffered

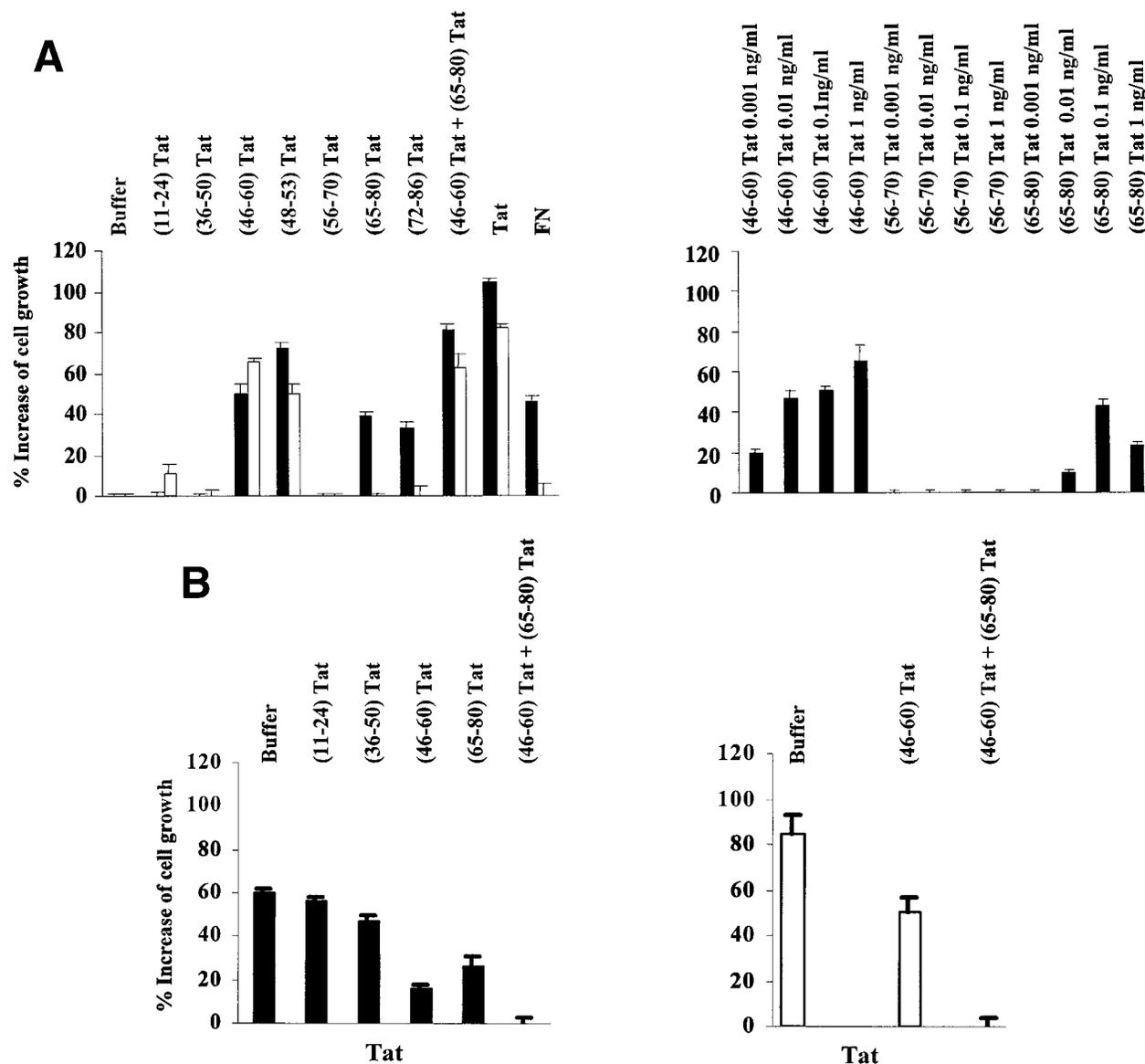


Fig 2. Both the basic and RGD region mediate Tat-promoted vascular cell growth. (A) shows the proliferative response of KS (■) and IC-HUVE (□) cells to Tat peptides, Tat (1 ng/mL), or FN (30 ng/mL). In the left panel, Tat peptides were used at concentrations equimolar to Tat. In the right panel, Tat peptides were serially diluted to determine the dose-dependency of their effect. (B) shows peptide competition experiments of KS (left panel, ■) and IC-HUVE (right panel, □) cell growth. Cells were preincubated with an excess of Tat peptides (5 µg/mL each) and then stimulated to growth with Tat (1 ng/mL), as described above. Preincubation of the cells with buffer was the negative control. For (A) and (B), experiments were performed by cell counting. Data (from 3 experiments, each in duplicate) are expressed as the percentage increase of cell growth over the number of cells grown in the absence of mitogens (basal cell growth). This was 1×10^4 cells/well for KS cells and 1.2×10^4 cells/well for HUVE cells and was given a 0% increase value. Results were also reproduced by the ^3H -thymidine uptake method (data not shown).

saline [PBS]-0.1% BSA). Total RNA was then extracted from the cells and subjected to electrophoresis (10 µg for each lane) and Northern blot analysis. A ^{32}P -labeled oligodeoxynucleotide corresponding to the sequence +59 to +99 of collagenase IV cDNA (encoding the 72-kD form), which detects a 3.4-kb transcript, was used as a probe, as previously described.¹⁵ The amount of RNA loaded in each lane was the same as detected by ethidium bromide staining of the gels.

Measurement of extracellular soluble bFGF retrieved from cell- or ECM-associated heparan sulfate proteoglycans (HSPG). KS cells were incubated for 2 days with conditioned media from activated T cells or with combined IC, which increase bFGF production and release,¹⁶ or were cultured in the presence of exogenous bFGF. Cells were lifted nonenzymatically with a dissociation buffer (PBS-based chelating

solution; GIBCO-BRL), washed with PBS, and resuspended in RPMI. The plates were also rinsed with PBS. Cells or plates were then treated for 20 minutes with the control buffer (PBS-0.1% BSA), Tat, equimolar concentrations of Tat peptides, or heparin. A limited trypsin digest of cells or plates was used to retrieve the total bFGF bound to cells or ECM.¹⁶ Supernatants were centrifuged and tested for bFGF content by ELISA. To avoid the loss of bFGF, all samples were handled in plastic ware precoated with PBS-0.1% BSA.

RESULTS

Binding of Tat-RGD region to the $\alpha 5\beta 1$ and $\alpha \nu\beta 3$ integrins mediates vascular cell migration, invasion, and collagenase IV

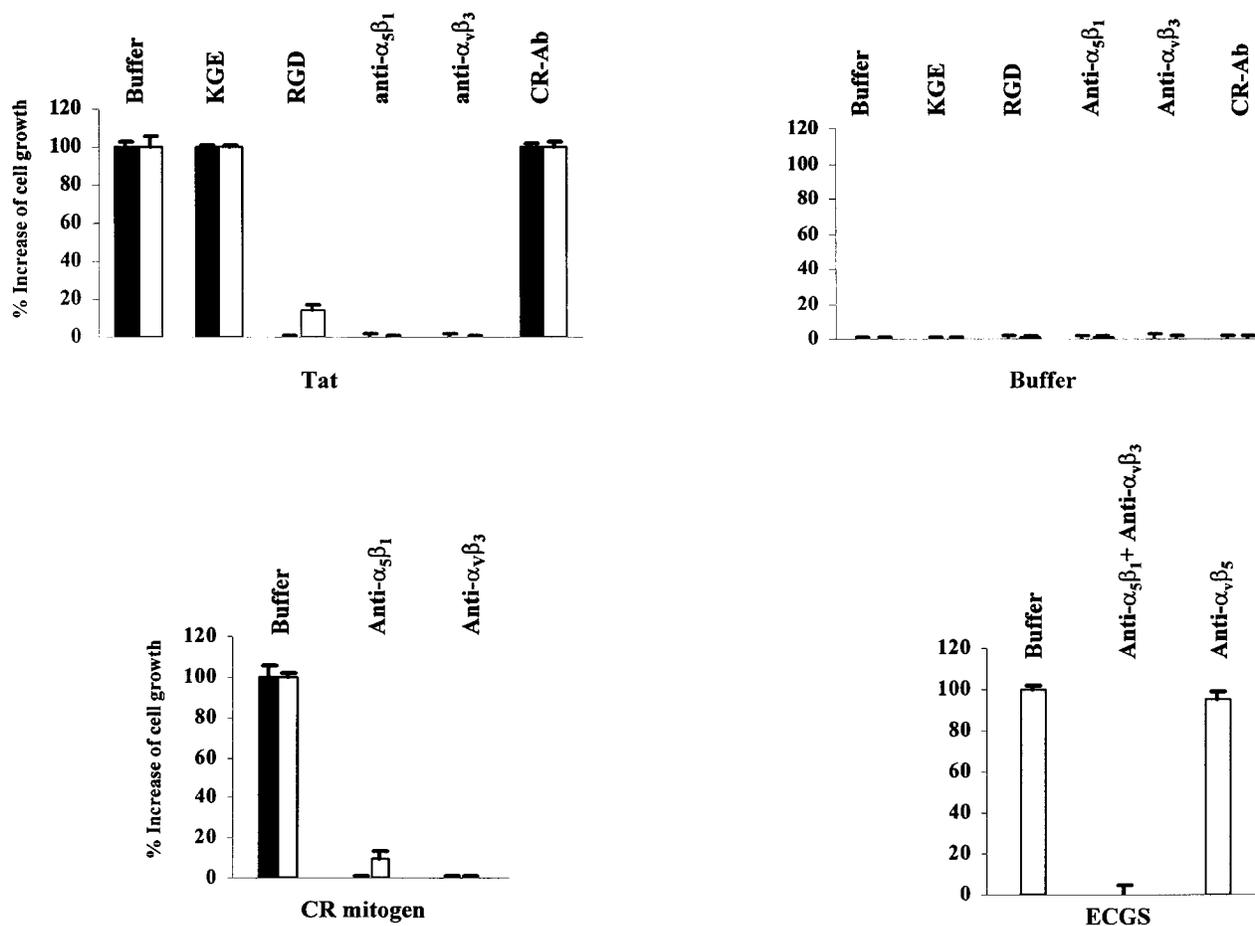


Fig 3. Tat RGD region provides vascular cells with the adhesion signal required by the cells to respond to mitogens. KS (■) and IC-HUVE (□) cells were preincubated with RGD or mutated KGE peptides (1 μg /each) or with anti- $\alpha_5\beta_1$ and/or anti- $\alpha_v\beta_3$ antibodies (4 $\mu\text{g}/\text{mL}$) and were then stimulated with Tat (1 ng/mL; upper left panel). No effects of the competitor peptides or antibodies were observed on cells grown in the absence of stimuli (upper right panel). Anti-CD34 or anti- $\alpha_v\beta_5$ MoAbs (4 $\mu\text{g}/\text{mL}$) were used as control antibodies (CR-Ab) for KS cells. Anti-factor VIII-related antigen MoAbs (4 $\mu\text{g}/\text{mL}$) were used as control antibodies (CR-Ab) for HUVE cells. TNF- α (10 ng/mL) or ECGS (45 $\mu\text{g}/\text{mL}$) were used as control mitogens (CR mitogen) for KS and IC-HUVE cells, respectively (lower left panel). The preincubation of the cells with antibody or peptide dilution buffer was the negative control. In the upper panels and in the lower left panel, KS and IC-HUVE cells were seeded onto gelatin-coated plates. In the lower right panel, IC-HUVE cells were seeded onto plates coated with Tat (5 $\mu\text{g}/\text{mL}$), and then they were stimulated to grow with ECGS (45 $\mu\text{g}/\text{mL}$) in the presence or absence of anti- $\alpha_5\beta_1$, - $\alpha_v\beta_3$, or - $\alpha_v\beta_5$ antibodies (4 $\mu\text{g}/\text{mL}$). Results (from 3 experiments, each in duplicate) are expressed as the percentage increase of cell growth relative to the growth of cells preincubated with buffer and stimulated with Tat, TNF- α , or ECGS (assumed as 100% of cell growth increase).

activation induced by Tat. To elucidate the mechanism(s) of Tat activity, initial studies were focused on identifying the domains required for the effect of Tat on KS and endothelial cell migration and invasion. This was performed by using overlapping Tat peptides. These mapping studies indicated that only the peptides containing the RGD region, namely [65-80] Tat and [72-86] Tat, can induce the migration of KS and IC-activated HUVE cells that was observed at concentrations equimolar to Tat (Fig 1A, left panel). The migration induced by Tat-RGD region was dose-dependent (Fig 1A, right panel).

Therefore, blocking experiments were performed with antibodies directed against RGD-binding integrins expressed by KS and IC-HUVE cells, such as $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$.²⁶ As shown in Fig 1B, KS and IC-HUVE cell migration to Tat or to the [65-80] Tat RGD peptide was inhibited by either anti- α_5 and anti- β_1 antibodies or anti- α_v and anti- β_3 antibodies but not by anti- $\alpha_v\beta_5$ antibodies. In addition, inhibition was complete when

both anti- $\alpha_5\beta_1$ and anti- $\alpha_v\beta_3$ antibodies were added together to the cells. To the contrary, antibodies directed against other antigens (CD34 and factor VIII-related antigen) expressed by KS and IC-HUVE cells, respectively^{26,30,31} (data not shown), had no effects on Tat-promoted cell migration.

Additional experiments performed with MoAbs directed against the whole $\alpha_5\beta_1$ or $\alpha_v\beta_3$ yielded similar results. Specifically, 70%, 75%, and 90% inhibition of Tat-induced IC-HUVE cell migration was observed with anti- $\alpha_5\beta_1$, anti- $\alpha_v\beta_3$, or combined anti- $\alpha_5\beta_1$ and anti- $\alpha_v\beta_3$ antibodies, respectively. Moreover, the anti- $\alpha_5\beta_1$ or anti- $\alpha_v\beta_3$ antibodies inhibited FN-induced, but not bFGF-induced, cell migration (Fig 1B), indicating that the antibodies were specific³⁴ and nontoxic to the cells. Lastly, migration to Tat was not affected by anti-bFGF antibodies (Fig 1B), indicating that the chemotactic effect of Tat is not due to the bFGF that is produced by the cells.

Experiments were then performed to investigate the role of

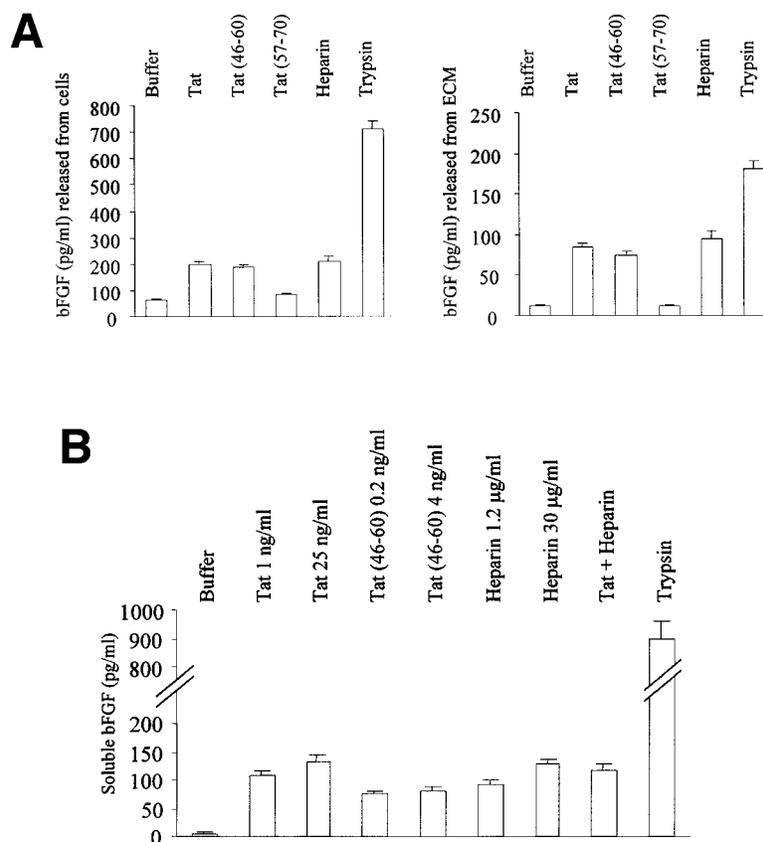


Fig 4. Tat basic region retrieves sequestered bFGF into a soluble form that mediates Tat-promoted vascular cell growth. (A) shows the mean of bFGF levels (in picograms per milliliter) retrieved from cells (left panel) or ECM (right panel). KS cells were lifted with a cell dissociation buffer, and both the suspended cells or the plates containing the ECM produced by the cells were incubated with Tat (25 ng/mL), [46-60] Tat (4 ng/mL), [56-70] Tat (4 ng/mL), heparin (30 μg/mL), or dilution buffer. A limited trypsin digest of suspended cells or plates was used to retrieve the total bFGF bound to cells or ECM.¹⁶ bFGF was then measured in the supernatants by ELISA. (B) shows the mean values of bFGF (in picograms per milliliter) maintained in a soluble form by Tat or Tat basic peptide. KS cells were cultured in the presence of exogenous bFGF (1 ng/mL) and then incubated for 20 minutes in control buffer (PBS-0.1% BSA), Tat (1 or 25 ng/mL), [46-60] Tat (0.2 or 4 ng/mL), heparin (30 μg/mL), or Tat (25 ng/mL) and heparin (30 μg/mL) combined sequentially.

the interaction between the Tat-RGD region and integrins in Tat-promoted cellular invasion. As for Tat-induced migration, anti- $\alpha 5\beta 1$ or anti- $\alpha v\beta 3$ antibodies inhibited KS and IC-HUVE cell invasion induced by Tat. Complete inhibition was again observed by the simultaneous addition of antibodies directed against both integrins (Fig 1C). The effect of the antibodies was specific, because they did not inhibit cellular invasion promoted by bFGF (Fig 1C). Consistent with these data, the Tat RGD peptide also promoted collagenase IV 72-kD gene expression at levels comparable with those induced by full-length Tat (Fig 1D), which is known to activate collagenase expression during cell invasion.¹⁵ Thus, Tat-promoted migration and invasion are mediated by the binding of the RGD region of the protein to the $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrins. In contrast, $\alpha v\beta 5$, which is known to bind the basic region of Tat,²⁸ is not involved in these effects.

The basic and the RGD domains of Tat are both required for Tat-induced growth of KS and endothelial cells. Soluble Tat protein promotes KS and IC-HUVE cell growth.^{2,3,6,7,10} To clarify the mechanism(s) of this Tat effect, cell growth experiments were performed with the same Tat peptides employed in the previous experiments. As shown in Fig 2A, the peptides containing the Tat basic region, [46-60] and [48-53] Tat,

induced the growth of both KS and IC-HUVE cells, whereas the RGD-containing peptides, [65-80] and [72-86] Tat, promoted only KS cell growth. In addition, both [46-60] Tat and [65-80] Tat promoted KS cell growth in a dose-dependent fashion (Fig 2A, right panel). Consistent with these results, when the RGD and the basic Tat peptides were added together, KS cell growth increased and reached levels similar to those observed with the Tat protein. Differently from what was observed with KS cells, the combination of [46-60] Tat and [65-80] Tat did not augment HUVE cell proliferation induced by [46-60] Tat alone. Moreover, FN, an RGD-containing molecule,²⁷ promoted KS cell growth at levels similar to those observed with RGD peptides but had no effect on endothelial cells.

Peptide competition experiments were then performed to clarify these data. As shown in Fig 2B, Tat-promoted KS and endothelial cell growth were inhibited only partially when the cells were preincubated with an excess of [46-60] Tat or [65-80] Tat, and complete inhibition of KS and HUVE cell growth was observed only when cells were preincubated with an excess of the two peptides added together to the cells. Thus, both the RGD and the basic region of Tat appear to participate in Tat-promoted cell proliferation, suggesting that more than one pathway is

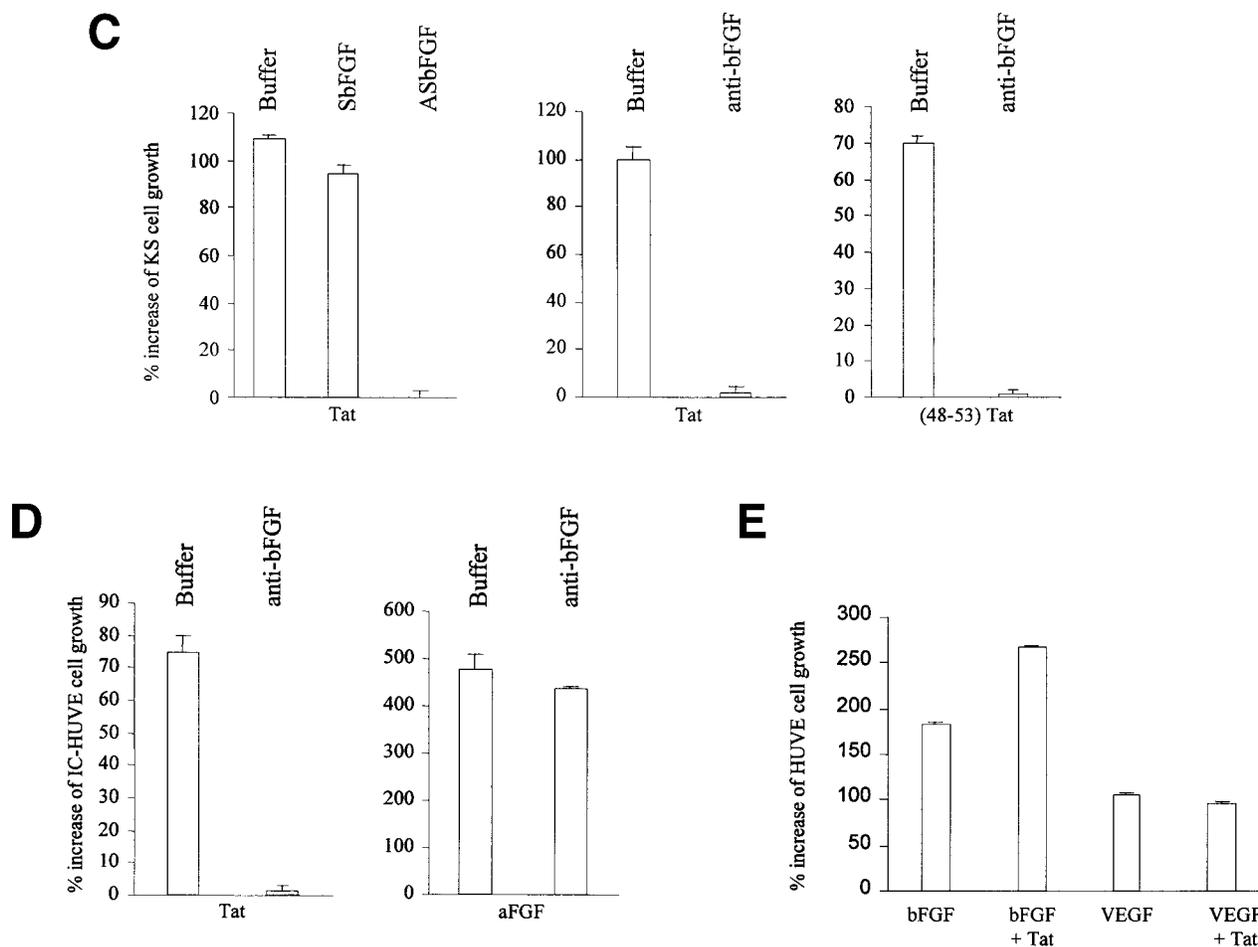


Fig 4 (Cont'd). (C) The left panel shows proliferative assays with KS cells cultured for 48 hours with 0.5 $\mu\text{mol/L}$ antisense bFGF (ASbFGF) or sense bFGF (SbFGF) oligomers.²⁴ Tat (1 ng/mL) and ^3H -thymidine were then added to the cells, and growth was monitored after 48 hours. Results (from 3 experiments, in 5 replicates) are expressed as the percentage increase of ^3H -thymidine uptake after the addition of Tat as compared with basal cell growth (0% increase, which was $1,874 \pm 20$ cpm). In the middle and right panels, anti-bFGF antibodies (10 $\mu\text{g/mL}$) were added to KS cells before the addition of Tat (1 ng/mL) or [48-53] Tat (0.1 ng/mL). The antibody buffer (PBS-0.1% BSA) was the negative control. Results (from 4 experiments, each in duplicate) were obtained by the cell counting method and expressed as the percentage increase of cell growth over the number of KS cells grown in the absence of mitogens (basal cell growth), which was 1 ± 10^4 cells/well. (D) shows proliferative assays with IC-HUVE cells induced to proliferate with 1 ng/mL of Tat or 20 ng/mL of aFGF, in the presence or absence of anti-bFGF antibodies (10 $\mu\text{g/mL}$). (E) shows HUVE cell growth experiments with bFGF or VEGF (5 ng/mL each) in the presence or absence of 1 ng/mL of Tat. For (D) and (E), results from 4 experiments performed by the cell counting method refer to the number of cells collected 4 to 5 days after the addition of bFGF, aFGF, VEGF, or Tat. They are expressed as a percentage increase of cell growth over the number of cells grown in the absence of mitogens (basal cell growth).

involved in this process. Therefore, the role of these two regions of Tat on cell growth was further analyzed.

Tat-RGD region provides KS and endothelial cells with the adhesion signal required for cell proliferation in response to mitogens. To investigate the role of the RGD region and of the Tat-integrin interaction in cellular growth, proliferative assays were performed with Tat in the presence of integrin competitors, such as RGD peptides,²⁷ or with anti- $\alpha 5\beta 1$, anti- $\alpha v\beta 3$, or anti- $\alpha v\beta 5$ antibodies. RGD peptides, but not the KGE-mutated peptides that were used as controls, and anti- $\alpha 5\beta 1$ or anti- $\alpha v\beta 3$ antibodies, but not anti- $\alpha v\beta 5$ or control antibodies, blocked Tat-induced KS and IC-HUVE cell growth (Fig 3, upper left panel). These results were obtained at concentrations of antibodies or peptides that did not cause cell detachment or affect basal cell growth (Fig 3, upper right panel). Similarly, anti- $\alpha 5\beta 1$ or

anti- $\alpha v\beta 3$ antibodies inhibited KS cell growth induced by $\text{TNF-}\alpha$, a mitogen for these cells,⁶ and HUVE cell proliferation induced by ECGS (Fig 3, lower left panel). Thus, as with other growth factors, Tat-induced cell growth requires integrin engagement.

This suggested that, by binding to $\alpha 5\beta 1$ and $\alpha v\beta 3$, Tat may provide endothelial cells with the same adhesion signal that is normally provided by ECM molecules and is required by the cells to proliferate in response to mitogens.^{35,36} In fact, anti- $\alpha 5\beta 1$ and anti- $\alpha v\beta 3$, but not anti- $\alpha v\beta 5$, antibodies inhibited the proliferation of HUVE cells seeded on Tat-coated plates and stimulated to grow with ECGS (Fig 3, lower right panel). Thus, the interaction of Tat-RGD region with $\alpha 5\beta 1$ and $\alpha v\beta 3$ provides the adhesion signal required for cell growth in response to mitogens.

Tat basic region retrieves preformed HSPG-bound bFGF that specifically triggers Tat-induced cell growth. Further studies focused to elucidate the mechanism(s) by which Tat basic region induces the growth of KS and endothelial cells. Basic residues similar to those in Tat are also present in many growth factors, and they bind HSPG associated with the cell membrane and the ECM.^{37,38} For this reason, basic sequences can displace extracellular-bound angiogenic factors through a competitive effect for heparin-binding sites.^{38,39} After their release from the cells, both Tat and bFGF bind to cell surface- and ECM-associated HSPG through their basic region, which has a strong binding affinity for heparin.^{4,37,40} The finding that the binding of Tat to heparin is competed out by bFGF⁴ suggested that Tat and bFGF could compete for the same heparin-binding sites.

To determine this, KS cells were grown with IC that further increase production and release of bFGF¹⁶ and then treated with scalar concentrations (from 0.1 ng/mL to 10 µg/mL) of Tat or Tat basic peptide. A gentle trypsin digest or heparin was used as a positive control, because they are known to release HSPG-bound bFGF into a soluble form.^{16,37} Soluble bFGF was then measured by ELISA. As compared with trypsin treatment, angiogenic concentrations of Tat or equimolar concentrations of [46-60] Tat basic peptide released about 26% of cell-bound bFGF and 41% of ECM-bound bFGF produced by KS cells (Fig 4A). These levels of bFGF were similar to those retrieved by heparin (used to evaluate the total retrievable bFGF).^{16,37} In contrast, [56-70] Tat peptide (used as a control) was not capable of releasing bFGF. The increase of soluble bFGF was detected 20 minutes after the addition of Tat or heparin to the cells, and the levels remained elevated for about 24 hours and returned to baseline after 48 hours (data not shown). As observed for heparin, Tat was also able to retrieve and maintain into a soluble form exogenous bFGF added to the cells (Fig 4B). Because the addition of Tat did not increase bFGF mRNA or intracellular bFGF content, as determined by Northern blot analysis and ELISA, respectively (data not shown), the increase of extracellular soluble bFGF by Tat is caused by the release of HSPG-bound extracellular protein. Thus, the growth effect of Tat basic region could be due to its capability of retrieving HSPG-bound bFGF produced by KS and IC-activated endothelial cells. In fact, antisense oligomers directed against bFGF mRNA, previously shown to specifically inhibit bFGF expression²⁴ but not control sense bFGF oligomers, blocked Tat-promoted growth of KS cells that constitutively produce bFGF (Fig 4C). This was associated with a reduction of intracellular- and extracellular-bound bFGF content as determined by ELISA after normalization to total protein content. In fact, intracellular bFGF content was reduced upon antisense treatment from 9,606 µg/100 µg of total protein to 3,840 µg/100 µg. Similarly, extracellular bound bFGF was reduced from 1,162 to 770 pg/mL.

Consistent with this result, neutralizing antibodies directed against bFGF²² blocked KS cell growth induced by Tat or by the Tat basic peptide (Fig 4C). Anti-bFGF antibodies also blocked Tat-induced growth of IC-HUVE cells that produce bFGF (Fig 4D). In contrast, anti-bFGF antibodies did not block endothelial cell growth induced by aFGF, which was used to control the antibody specificity (Fig 4D). In agreement with its capability of maintaining exogenously added bFGF in a soluble form, Tat enhanced bFGF-promoted proliferation of nonactivated endothe-

lial cells that do not produce bFGF (Fig 4E). In contrast, Tat slightly inhibited the mitogenic effect of VEGF on endothelial cells (Fig 4E). These results indicated that bFGF specifically triggers Tat-induced vascular cell growth.

DISCUSSION

In this study, we have analyzed the mechanisms responsible for the angiogenic, KS-promoting effect of Tat. The results indicate that KS and endothelial cell migration and invasion induced by Tat are mediated by the binding of its RGD region to the $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrins (Fig 1). The RGD sequence of Tat is also sufficient to activate the expression of collagenase IV 72-kD, which plays a key role in cell invasion and angiogenesis.^{15,41,42} These data are consistent with results obtained by others with RGD-containing molecules, such as FN or VN,^{34,41-43} and demonstrate that Tat induces KS and endothelial cell locomotion by a molecular mimicry of ECM molecules. They are also in agreement with the fact that Tat induces the synthesis and release of collagenase IV 92 kD in monocytes⁴⁴ and with the recent finding that Tat activates members of the focal adhesion kinase family that are induced by integrin triggering and that play a major role in cellular migration.^{45,46}

Differently from Tat-induced cell locomotion, the basic and RGD region of Tat are both required for Tat-induced cell growth (Fig 2). It appears that the interaction of Tat with $\alpha 5\beta 1$ or $\alpha v\beta 3$ provides KS and endothelial cells with the adhesion signal that is required for their growth in response to mitogens. Consistent with this, endothelial cells adhere to immobilized Tat in a fashion similar to FN or VN,²⁶ and under these conditions, the addition of exogenous bFGF dramatically increases cell growth,¹⁵ as previously described for ECM molecules.^{35,36,47} The involvement of the RGD region in Tat-induced cell growth is also consistent with previous data indicating that $\alpha 5\beta 1$, which recognizes the RGD region of both FN and Tat, mediates cellular growth promoted by FN in other systems,^{48,49} and that the RGD region of Tat promotes in KS cells the expression of the same mitogen-activated protein kinases that are induced by integrin triggering.⁴⁵

However, the adhesion signal is not sufficient to induce growth of normal endothelial cells that also need the presence of an angiogenic factor that triggers cell proliferation.⁵⁰ However, Tat RGD peptide or FN are mitogenic for KS cells (Fig 2). Because this does not occur with normal cells (for review, see Levesque et al⁵¹), these data suggest that KS cells may possess a difference in intracellular signaling through integrins, as indicated by previous studies with tumor cells.⁵²

Differently from the Tat RGD peptide, full-length Tat protein or Tat basic peptide are capable of promoting the growth of both KS and normal endothelial cells, suggesting that Tat provides cells additionally with a cell growth triggering signal.

Previous studies suggested that bFGF, which is a KS and endothelial cell growth factor,^{20,22,24} may mediate Tat-induced vascular cell proliferation. Basic FGF, in fact, is present in all systems in which Tat has a growth effect. Specifically, exogenous bFGF is required to observe the angiogenic effect of Tat *in vivo*.¹⁵ Moreover, bFGF is produced and released extracellularly by KS cells that spontaneously proliferate in response to Tat^{2,3,16,22} and by IC-activated endothelial cells that, after this activation, proliferate with Tat.^{6,7,10,17} After its release, bFGF

binds the cell surface and the ECM-associated HSPG remaining soluble only in a small fraction.^{16,37} The bound bFGF fraction represents a localized storage of the growth factor that is protected from proteolytic degradation and can be retrieved by treatment of the cells with heparin, heparinase, or trypsin.^{16,37}

Several growth factors can bind HSPG through their basic residues (for review, see Raines and Ross³⁸). In contrast, other angiogenic molecules, such as the majority of VEGF isoforms, lack basic residues and, therefore, do not bind the HSPG associated to the surface of producer cells or to ECM, remaining soluble and diffusible.²¹ Similarly to bFGF, Tat binds heparin through its basic sequence and can compete with bFGF for binding to heparin.⁴ Heparin, in fact, can inhibit the mitogenic effect of Tat (data not shown), as previously found for cellular adhesion to immobilized Tat²⁸ and transactivation of HIV-1 gene expression by extracellular Tat.⁴ Our data indicate that the basic residues of Tat can displace preformed HSPG-bound bFGF by competing for cell surface- and ECM-associated heparin binding sites (Fig 4). This leads to an increase of the soluble fraction of bFGF at levels promoting KS and endothelial cell growth, as shown by the inhibition of Tat-induced cell growth by antisense bFGF oligomers (reducing both the intracellular and extracellular content of bFGF) or by neutralizing anti-bFGF antibodies (Fig 4). Consistent with its capability of maintaining exogenously added bFGF in a soluble and highly diffusible form, Tat enhances endothelial cell proliferation promoted by bFGF. In contrast, Tat does not augment the growth effect of VEGF on endothelial cells (Fig 4). Thus, although Tat binds the VEGF receptor Flk-1/KDR,^{19,45} this does not lead to cell growth. This is consistent with the finding that, differently from Tat or bFGF, VEGF does not promote KS cell proliferation.¹⁸ Moreover, Tat promotes the growth of IC-activated endothelial cells that produce bFGF but not VEGF.¹⁸ Furthermore, in primary KS lesions, the VEGF amounts are much higher than that of Tat,^{15,25} making unlikely an action (either activatory or inhibitory) of Tat on the VEGF receptor. Thus, although both VEGF and bFGF are highly expressed in AIDS-KS lesions, Tat synergizes with bFGF, and not with VEGF, in promoting neoangiogenesis and, therefore, KS development and progression.

In conclusion, the results described herein demonstrate that the Tat protein of HIV-1 is not directly angiogenic, but it enhances angiogenesis by mimicking the effects of ECM proteins on cell migration, invasion, and adhesion, and by mobilizing bFGF, a true angiogenic factor, which acts as the final mediator of Tat-induced KS and endothelial cell growth. This explains why Tat needs exogenous bFGF or factors promoting bFGF expression, such as IC, to exert its angiogenic effect.

IC and bFGF are highly expressed in AIDS-KS lesions, where extracellular Tat coexists with the $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrins on both spindle cells and vessels.¹⁵ This suggests that the mechanisms of Tat action described here are operative in vivo and that integrin, bFGF, and Tat competitors should be considered as a therapeutic strategy for AIDS-KS.

ACKNOWLEDGMENT

The authors thank Dr E. Ruoslahti for some of the anti- $\alpha v\beta 5$ antibodies and A. Lippa and F.M. Regini for editorial assistance.

REFERENCES

1. Arya SK, Guo C, Josephs SF, Wong-Staal F: Transactivator gene of human T lymphotropic virus type III (HTLV-III). *Science* 229:69, 1982
2. Ensoli B, Barillari G, Salahuddin SZ, Gallo SZ, Wong-Staal F: Tat protein of HIV-1 stimulates the growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. *Nature* 345:84, 1990
3. Ensoli B, Buonaguro L, Barillari G, Fiorelli V, Gendelman R, Morgan RA, Wingfeld P, Gallo RC: Release, uptake and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral transactivation. *J Virol* 67:277, 1993
4. Chang HC, Samaniego F, Nair BC, Buonaguro L, Ensoli B: HIV-1 Tat protein exits from intact cells via a leaderless secretory pathway and binds to extracellular matrix associated heparan sulfate proteoglycans through its basic region. *AIDS* 11:1421, 1997
5. Safai B, Johnson KG, Myskowsky S, Cunningham-Randles S, Godbold JH, Dupont B: The natural history of Kaposi's sarcoma in the acquired immunodeficiency syndrome. *Ann Intern Med* 103:744, 1985
6. Barillari G, Buonaguro L, Fiorelli V, Hoffman J, Michaels F, Gallo RC, Ensoli B: Effects of cytokines from activated immune cells on vascular cell growth and HIV-1 gene expression: Implication for AIDS-Kaposi's sarcoma pathogenesis. *J Immunol* 149:3727, 1992
7. 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* 95:1723, 1995
8. 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* 92:4836, 1995
9. Fiorelli V, Gendelman R, Sirianni MC, Chang HK, Colombini S, Markham PD, Monini P, Sonnabend J, Pintus A, Gallo RC, Ensoli B: Interferon-gamma produced by CD8⁺ T cells infiltrating Kaposi's sarcoma induces spindle cells with angiogenic phenotype and synergy with HIV-1 Tat. *Blood* 91:956, 1998
10. Fiorelli V, Barillari G, Toschi E, Sgadari C, Monini P, Sturzl M, Ensoli B: Interferon-gamma induces endothelial cells to proliferate and to invade the extracellular matrix in response to HIV-1 Tat. *J Immunol* 162:1165, 1999
11. Regezi SA, MacPhail LA, Daniels TE: Human immunodeficiency virus-associated Kaposi's sarcoma: An heterogeneous cell population dominated by spindle-shaped endothelial cells. *Am J Pathol* 43:240, 1993
12. Hober DA, Haque A, Watre P, Beaucaire G, Mouton Y, Capron A: Production of TNF α and IL-1 in patients with AIDS. Enhanced level of TNF α is related to higher cytotoxic activity. *Clin Exp Immunol* 78:329, 1989
13. Oxholm A, Oxholm P, Permin H, Bendtzen L: Epidermal tumour necrosis factor alpha and interleukin 6-like activities in AIDS-related Kaposi's sarcoma. An immunohistological study. *APMIS* 97:533, 1989
14. Sirianni MC, Vincenzi L, Fiorelli V, Topino S, Scala E, Uccini S, Angeloni A, Faggioni A, Cerimele D, Cottoni F, Aiuti F, Ensoli B: γ -Interferon production in peripheral blood mononuclear cells (PBMC) and tumor infiltrating lymphocytes from Kaposi's sarcoma patients correlation with the presence of human herpesvirus 8 in PBMC and lesional macrophages. *Blood* 91:968, 1998
15. Ensoli B, Gendelman R, Markham PD, Fiorelli V, Colombini S, Raffeld M, Cafaro A, Chang HK, Brady JN, Gallo RC: Synergy between basic fibroblast growth factor and HIV-1 Tat protein in induction of Kaposi's sarcoma. *Nature* 371:674, 1994
16. Samaniego F, Markham PD, Gallo RC, Ensoli B: Inflammatory cytokines induce AIDS-Kaposi's sarcoma derived spindle cells to produce and release basic fibroblast growth factor and enhance Kaposi's sarcoma-like lesion formation in nude mice. *J Immunol* 154:3582, 1995
17. Samaniego F, Markham PD, Gendelman R, Gallo RC, Ensoli B:

Inflammatory cytokines induce endothelial cells to produce and release basic fibroblast growth factor and to promote Kaposi's sarcoma-like lesions in nude mice. *J Immunol* 158:1887, 1997

18. Samaniego F, Markham PD, Gendelman R, Watanabe Y, Kao V, Kowalski K, Ferrara N, Sonnabend JA, Pintus A, Zon G, Gallo RC, Ensoli B: Vascular endothelial growth factor and basic fibroblast growth factor present in Kaposi's sarcoma (KS) are induced by inflammatory cytokines and synergize to induce angiogenesis, vascular permeability and KS lesion development. *Am J Pathol* 152:1433, 1998
19. Albini A, Soldi R, Giunciuglio D, Giraudo R, Benelli R, Primo L, Noonan D, Salio M, Camussi G, Rockl W, Bussolino F: The angiogenesis induced by HIV-1 Tat protein is mediated by the Flk-1/KDR receptor on vascular endothelial cells. *Nat Med* 2:1371, 1996
20. Rifkin DB, Moscatelli D: Recent development in biology of basic fibroblast growth factor. *J Cell Biol* 109:1, 1989
21. Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung D: The vascular endothelial growth factor family: Identification of a fourth molecular species and characterization of an alternative splicing of RNA. *Mol Endocrinol* 5:1806, 1991
22. Ensoli B, Nakamura S, Salahuddin SZ, Biberfeld P, Larsson L, Beaver B, Wong-Staal F, Gallo RC: AIDS-Kaposi's sarcoma derived cells express cytokines with autocrine and paracrine growth effects. *Science* 243:223, 1989
23. Xerri L, Houssoun J, Planche J, Guigou V, Grob JJ, Parc P, Birnbaum D, De Lapeyriere O: Fibroblast growth factor gene expression in AIDS-Kaposi's sarcoma detected by in situ hybridization. *Am J Pathol* 138:9, 1991
24. Ensoli B, Markham PD, Kao V, Barillari G, Fiorelli V, Gendelman R, Raffeld M, Zon G, Gallo RC: Block of AIDS-KS cell growth, angiogenesis and lesion formation in nude mice by antisense oligonucleotides targeting basic fibroblast growth factor. *J Clin Invest* 94:1736, 1994
25. Cornali E, Zietz C, Benelli R, Weninger W, Masiello L, Breier G, Tschachler E, Albini A, Stürzl M: Vascular endothelial growth factor regulates angiogenesis and vascular permeability in Kaposi's sarcoma. *Am J Pathol* 149:1851, 1996
26. Barillari G, Gendelman R, Gallo RC, Ensoli B: The Tat protein of human immunodeficiency virus type 1, a growth factor for AIDS Kaposi's 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* 90:7941, 1993
27. Hynes RO: Integrins: Versatility, modulation and signaling in cell adhesion. *Cell* 69:11, 1992
28. Vogel BE, Lee SJ, Hiedelbrand A, Craig W, Piershbach MD, Wong-Staal F, Ruoslahti E: A novel integrin specificity exemplified by binding of the $\alpha v \beta 5$ integrin to the basic domain of the HIV-1 Tat protein and vitronectin. *J Cell Biol* 121:461, 1993
29. Pintus C, Ransom JH, Evans D: Endothelial cell growth supplement: A cell cloning factor that promotes the growth of monoclonal antibody producing hybridoma cells. *J Immunol Methods* 61:195, 1983
30. Sankey EA, More L, Dhillon AP: QBEND/10: A new immunostain for the routine diagnosis of Kaposi's sarcoma. *J Pathol* 161:267, 1992
31. Mukai K, Rosai J, Burgdorf WHC: Localization of factor VIII-related antigen in vascular endothelial cells using an immunoperoxidase method. *Am J Surg Pathol* 4:273, 1980
32. Nakamura S, Salahuddin SZ, Biberfeld P, Ensoli B, Markham PD, Wong-Staal F, Gallo RC: Kaposi's sarcoma cells: Long term culture with growth factor from retrovirus-infected CD4⁺ T-cells. *Science* 242:427, 1988
33. Kubota Y, Kleinman HK, Martin GR, Lawley T: Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. *J Cell Biol* 107:1589, 1988
34. Zetter BR, Brightman SE: Cell motility and the extracellular matrix. *Curr Opin Cell Biol* 2:850, 1990
35. Gospodarowicz M, Greenburg G, Birdwell CR: Determination of cellular shape by the extracellular matrix and its correlation with the control of cellular growth. *Cancer Res* 38:4155, 1978
36. Ingber DE, Prusty D, Frangioni JY, Cragoe EJ, Lechene C, Schwartz M: Control of intracellular pH and growth by fibronectin in capillary endothelial cells. *J Cell Biol* 110:1803, 1990
37. Folkman J, Klagsbrun M, Sasse J, Wadzinski M, Ingberg D, Vlodavsky I: A heparin-binding angiogenic protein—basic fibroblast growth factor—is stored within basement membrane. *Am J Pathol* 130:393, 1988
38. Raines EW, Ross R: Compartmentalization of PDGF on extracellular binding sites dependent on exon-6-encoded sequences. *J Cell Biol* 116:533, 1992
39. Barillari G, Albonici L, Franzese O, Modesti A, Liberati F, Barillari P, Ensoli B, Manzari V, Santeusano G: The basic residues of PlGF-2 retrieve sequestered angiogenic factors into a soluble form. *Am J Pathol* 152:1161, 1998
40. Albini A, Benelli R, Presta M, Rusnati M, Ziche M, Rubartelli A, Pagliarunga G, Bussolino F, Noonan D: HIV-tat protein is a heparin-binding angiogenic growth factor. *Oncogene* 12:289, 1996
41. Seftor RE, Seftor E, Stetler-Stevenson WG, Hendrix MJC: The 72 kDa type IV collagenase is modulated via differential expression of $\alpha v \beta 3$ and $\alpha 5 \beta 1$ integrins during human melanoma cell invasion. *Cancer Res* 53:3411, 1993
42. Brooks PC, Stromblad S, Sanders LC, Von Schalscha TL, Aimes RT, Stetler-Stevenson VG, Quigley JP, Cheresch DA: Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin $\alpha v \beta 3$. *Cell* 85:683, 1996
43. Humphries MJ, Obara M, Olden K, Yamada KM: Role of fibronectin in adhesion, migration, and metastasis. *Cancer Invest* 7:373, 1989
44. Lafrenie RM, Wahl LM, Epstein JS, Hewlett IK, Yamada KM, Dhawan S: HIV-1 Tat modulates the function of monocytes and alters their interactions with microvessel endothelial cells. *J Immunol* 156:1638, 1996
45. Ganju RK, Munshi N, Nair BC, Liu ZY, Gill P, Groopman JE: Human immunodeficiency virus tat modulates the Flk-1/KDR receptor, mitogen-activated protein kinase, and components of focal adhesion in Kaposi's sarcoma cells. *J Virol* 72:6131, 1998
46. Milani D, Mazzoni M, Zauli G, Mischiati C, Gibellini D, Giacca M, Capitani S: HIV-1 Tat induces phosphorylation of p125^{FAK} and is associated with phosphoinositide 3-kinase in PC12 cells. *AIDS* 12:1275, 1998
47. Brooks PC, Clark RAF, Cheresch DA: Requirement of vascular integrin $\alpha v \beta 3$ for angiogenesis. *Science* 264:569, 1994
48. Davis LS, OppenheimerMarks N, Bednarczyk JL, McYntire BW, Lipsky PE: Fibronectin promotes proliferation of naive and memory T cells by signaling through both the VLA-4 and VLA-5 integrin molecules. *J Immunol* 145:785, 1990
49. Shimizu Y, Van Severen CA, Horgan JR, Shaw S: Costimulation of proliferative responses of resting CD4⁺ T cells by the interaction of VLA-4 and VLA-5 with fibronectin or VLA-6 with laminin. *J Immunol* 145:59, 1990
50. Folkman J, Shing Y: Angiogenesis. *J Biol Chem* 267:10931, 1992
51. Levesque JP, Hatzfeld A, Hatzfeld J: Mitogenic properties of major extracellular proteins. *Immunol Today* 12:258, 1991
52. Schwartz MA, Both G, Lechene C: Effect of cell spreading on cytoplasmic pH in normal and transformed fibroblasts. *Proc Natl Acad Sci USA* 86:4525, 1989