Inflammatory Cytokines Synergize with the HIV-1 Tat Protein to Promote Angiogenesis and Kaposi's Sarcoma Via Induction of Basic Fibroblast Growth Factor and the $\alpha_v\beta_3$ Integrin[1]

Giovanni Barillari,* Cecilia Sgadari,* Clelia Palladino,* Rita Gendelman,* Antonella Caputo,† Cindy Bohan Morris,‡ Bala C. Nair,§ Philip Markham,§ Andrè Nel,¶ Michael Stürzl,‖ and Barbara Ensoli[2]*

The Tat protein of HIV-1, a transactivator of viral gene expression, is released by acutely infected T cells and, in this form, exerts angiogenic activities. These have linked the protein to the pathogenesis of Kaposi's sarcoma (KS), a vascular tumor frequent and aggressive in HIV-1-infected individuals (AIDS-KS). In this study, we show that a combination of the same inflammatory cytokines increased in KS lesions, namely IL-1β, TNF-α, and IFN-γ, synergizes with Tat to promote in nude mice the development of angioproliferative KS-like lesions that are not observed with each factor alone. Inflammatory cytokines induce the tissue expression of both basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), two angiogenic molecules highly produced in primary KS lesions. However, bFGF, but not VEGF, synergizes with Tat in vivo and induces endothelial cells to migrate, to adhere, and to grow in response to Tat in vitro. Tat angiogenic effects correlate with the expression of the $\alpha_v\beta_3$ integrin that is induced by bFGF and binds the arginine-glycine-aspartic acid (RGD) region of Tat. In contrast, no correlation is observed with the expression of $\alpha_v\beta_5$, which is promoted by VEGF and binds Tat basic region. Finally, KS lesion formation induced by bFGF and Tat in nude mice is blocked by antagonists of RGD-binding integrins. Because $\alpha_v\beta_3$ is an RGD-binding integrin that is highly expressed in primary KS lesions, where it colocalizes with extracellular Tat on vessels and spindle cells, these results suggest that $\alpha_v\beta_3$ competitors may represent a new strategy for the treatment of AIDS-KS. ***The Journal of Immunology*****, 1999, 163: 1929–1935.**

Human immunodeficiency virus-1, the etiologic agent of AIDS (1, 2), contains several regulatory genes that coordinate its expression and replication (reviewed in Ref. 3). Among them, the *tat* gene codes for a protein, Tat, which transactivates viral gene expression (4). During acute infection of T cells by HIV-1, Tat is released into the extracellular compartment both in vitro (5–7) and in vivo (8, 9). In this form, Tat enhances angiogenesis, increasing the frequency of development and the aggressiveness of Kaposi's sarcoma (KS)[3] (3), an angioproliferative disease very frequent and aggressive only in association with AIDS (AIDS-KS) (10, 11).

In particular, picomolar concentrations of extracellular Tat promote the locomotion, adhesion, and growth of spindle cells of endothelial cell (EC) origin derived from KS lesions (KS cells) (5, 6, 12, 13) and of normal EC, considered to be the precursors of KS cells (14). However, to become responsive to these effects of Tat, EC require a preactivation with inflammatory cytokines (IC) (12, 13, 15). Among them, IL-1β, TNF-α, and IFN-γ play a major role in inducing EC responsiveness to the in vitro effects of Tat (15–18). These IC are the same increased in lesions and blood of AIDS-KS patients and patients at risk of KS (17–24).

The requirement of other factors for Tat effects is observed also in vivo. In fact, Tat promotes the development of angioproliferative KS-like lesions in mice only when injected with suboptimal (nonlesion forming) amounts of basic fibroblast growth factor (bFGF) (8), an angiogenic molecule highly expressed by KS cells both in vitro and in primary lesions (8, 25–27).

Although angiogenesis is triggered by soluble angiogenic factors, it is modulated by integrin receptors that mediate the adhesive interactions between EC and the extracellular matrix (ECM) (28). Interestingly, Tat basic sequence present in the product of *tat* I exon binds the $\alpha_v\beta_5$ integrin (29), whereas the arginine-glycine-aspartic acid (RGD) region present at the carboxyl-terminal of Tat binds the $\alpha_v\beta_3$ integrin, and by this interaction mediates the adhesion of EC and KS cells to Tat (12).

In this study, we show that the in vivo KS-promoting effect of Tat is triggered by IC expressed in KS lesions and that it requires both the presence of bFGF, but not of vascular endothelial growth factor (VEGF), and the binding of the RGD region of Tat to RDG-binding integrin receptors such as $\alpha_v\beta_3$.

*Laboratory of Virology, Istituto Superiore di Sanità, Rome, Italy; †Department of Experimental and Diagnostic Medicine, University of Ferrara, Ferrara, Italy; ‡Department of Pathology and Laboratory Medicine, Tulane Cancer Center, Tulane University Medical Center, New Orleans, LA 70112; §Advanced BioScience Laboratories, Kensington, MD 20895; ¶Department of Medicine, Division of Clinical Immunology and Allergy, Los Angeles, CA 90024; and ‖Gesellschaft für Strahlung und Umweltforschung-National Research Center for Environment and Health, Institute for Molecular Virology, Neuherberg, Germany

Received for publication February 18, 1999. Accepted for publication May 26, 1999.

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[1] This work was supported by grants from the Italian Association for Cancer Research, Milan, Italy (B.E.); the Italian Ministry of Health (AIDS Project), Rome, Italy (B.E. and G.B.); the Deutsche Forschungsgesellschaft (SFB 464); the German Ministry of Education, Science, Research, and Technology (BMBF, BioFuture Program) (M.S.); The U.S. Public Health Service Grant CA65329 (A.N.); and by the EC-Biomed-2 Concerted Action on the Pathogenesis of AIDS-Kaposi's Sarcoma.

[2] Address correspondence and reprint requests to Dr. Barbara Ensoli, Laboratory of Virology, Istituto Superiore di Sanità, Viale Regina Elena, 299, 00161 Rome, Italy. E-mail address: ensoli@virus1.net.iss.it

[3] Abbreviations used in this paper: KS, Kaposi's sarcoma; bFGF, basic fibroblast growth factor; EC, endothelial cells; ECM, extracellular matrix; GRGDS, glycine-arginine-glycine-aspartic acid-serine; GRGES, glycine-arginine-glycine-glutamic acid-serine; IC, inflammatory cytokines; RGD, arginine-glycine-aspartic acid; RGE, arginine-glycine-glutamic acid; VEGF, vascular endothelial growth factor.

0022-1767/99/$02.00

Materials and Methods

Reagents

rHIV-1 Tat protein (from the IIIB isolate) was obtained and handled as previously described to avoid protein oxidation (5–8). Human rbFGF, VEGF, mouse rIL-1β, TNF-α, and IFN-γ were purchased from Boehringer Mannheim (Mannheim, Germany) or Promega (Madison, WI). Gelatin (from bovine skin) and BSA (fraction V) were from Sigma (St. Louis, MO). Human collagen IV and matrigel, a reconstituted basement membrane derived from a tumor cell line (30), were obtained from Collaborative Research (Bedford, MA). The glycine-arginine-glycine-aspartic acid-serine (GRGDS) peptide (31) and the mutated glycine-arginine-glycine-glutamic acid-serine (GRGES) peptide were from Research Genetics (Huntsville, AL). Rabbit polyclonal anti-bFGF, anti-VEGF, anti-β_5, and anti-β_3 Abs used for immunohistochemistry on mice tissues were from Santa Cruz Biotechnology (Santa Cruz, CA).

Animal experiments and immunostainings

Proteins were injected s.c. into the lower back (right side) of BALB/c *nu/nu* athymic mice. The negative control (PBS-0.1% BSA, protein suspension buffer) was injected into the left side. Proteins or buffer (in 200 μl of final volume) were mixed with 200 μl of Matrigel before inoculation. Matrigel is not essential to observe lesion formation, but it increases the efficiency of the assay (8). Mice were sacrificed 6 days later. At this time, the sites of injection were evaluated for the presence of macroscopic angioproliferative lesions. Tissue samples were taken from all sites, fixed in Formalin, and analyzed microscopically after hematoxylin-eosin staining, as previously described (8).

For the immunohistochemical stainings, frozen tissue sections from the sites of inoculation were stained with the primary Abs by the peroxidase-antiperoxidase method, as reported elsewhere (8). The slides were counterstained with Mayer's hematoxylin solution (Sigma) before evaluation. Animal care was in accordance with institutional and international guidelines.

Cell cultures and cell adhesion, migration, and growth experiments

HUVEC, passages 4–8, were obtained, characterized, and cultured, as described (5, 15).

Cell adhesion assays were performed with HUVEC seeded onto Tat-coated plates and incubated for 1 h at 37°C in a 5% CO_2 atmosphere. After incubation, adherent cells were fixed with 3% paraformaldehyde, stained with Giemsa, and quantitated by light microscopy, as previously described (32), by counting 5 high power fields/well.

Migration assays were conducted at 37°C in 5% CO_2 for 5 h in the Boyden chambers separated in two compartments by polycarbonate filters (12 μm pore; Nucleoprobe, Cabin John, MD) coated with type IV collagen (13). HUVEC were placed in the upper compartment of the Boyden chambers, whereas Tat or its resuspension buffer (PBS-0.1% BSA), which was employed as the negative control, was placed in the lower compartment. Migrated cells were fixed in ethanol, double stained by toluidine-blue and by hematoxylin-eosin, and quantitated by light microscopy by counting 5 fields/filter, as described previously (13).

Cell growth assays were performed with HUVEC seeded onto gelatin-coated plates and grown in the presence or absence of Tat in medium containing 10% FBS. Media and Tat were replaced after 2 days, and cells were counted after 4 days by trypan blue dye exclusion (in triplicate), as previously described (5, 15).

Results

Combined IC synergize with Tat in inducing angiogenesis and the development of KS-like lesions in nude mice

Exposure to a combination of the same IC increased in KS patients induces EC to become responsive to the cell adhesion, growth, migration, and invasion effects of Tat (12, 13, 15–18). Experiments were therefore performed to evaluate the in vivo effect of these IC.

As shown in Table I, injection of Tat alone had no effects on lesion formation, as previously reported (8). Similarly, recombinant IL-1β, TNF-α, or IFN-γ was not capable of inducing angioproliferative lesions in mice when injected either alone or by combining two of them together at 0.1 or 0.5 μg each. Again, the injection of mice with IL-1β, TNF-α, and IFN-γ combined together at 0.1 μg each had no effects, even in the presence of Tat.

Table I. *Combined IL-1β, TNF-α, and IFN-γ synergize with Tat in inducing the development of angioproliferative KS-like lesions in nude mice*[a]

Injection	Vascular Lesions[b] %	*n*
Buffer	0	12
IL-1β (0.1 μg)	0	6
IL-1β (0.5 μg)	0	6
TNF-α (0.1 μg)	0	6
TNF-α (0.5 μg)	0	6
IFN-γ (0.1 μg)	0	6
IFN-γ (0.5 μg)	0	6
IL-1β + TNF-α (0.5 μg each)	0	6
IL-1β + IFN-γ (0.5 μg each)	0	6
IFN-γ + TNF-α (0.5 μg each)	0	6
IL-1β + TNF-α + IFN-γ (0.1 μg each)	0	12
IL-1β, TNF-α, IFN-γ (0.5 μg each)	8	12
Tat (10 μg)	0	12
IL-1β + TNF-α + IFN-γ (0.1 μg each) + Tat (10 μg)	0	12
IL-1β, TNF-α, IFN-γ (0.5 μg each) + Tat (10 μg)	50	12

[a] rIL-1β, TNF-α, IFN-γ, or Tat were injected in nude mice, alone or in combination. The effects resulting from protein inoculation were controlled by the injection of the protein resuspension buffer (PBS-0.1% BSA). The sites of injection were also examined histologically after hematoxylin-eosin staining for alterations typical of KS such as angiogenesis, spindle cell growth, edema and haemorrhagies, as performed previously (8). See *Materials and Methods* for experimental details.

[b] Data shown include the percentage of mice developing macroscopic angioproliferative lesions and the number of mice inoculated.

In contrast, the combination of these cytokines at 0.5 μg each promoted the development of angioproliferative KS-like lesions in 8% of the inoculated mice, which increased to 50% of the inoculated mice in the presence of Tat (Table I). Thus, IL-1β, TNF-α, and IFN-γ are all required to observe the synergy with Tat in inducing macroscopic vascular lesions and histological changes closely resembling early KS in humans. However, the least amount of IC required to exert this angiogenic synergy with Tat is 0.5 μg of each cytokine.

IC induce expression of bFGF and VEGF; however, only bFGF synergizes with Tat to induce KS-like lesions

Combined IL-1β, TNF-α, and IFN-γ induce production and release of bFGF and VEGF in cultured EC and/or KS cells (33–35). These angiogenic factors are highly expressed in KS lesions and mediate autocrine and paracrine KS and EC growth effects and angiogenesis (8, 25–27, 33–36). Therefore, immunohistochemical analyses of the mice tissues inoculated with IC were performed to evaluate the expression of these angiogenic factors. Injection of combined IC at concentrations exerting angiogenic synergy with Tat induced high levels of expression of both bFGF and VEGF (Fig. 1). In contrast, lower IC concentrations, not sufficient to synergize with Tat in promoting angiogenesis (Table I), were not capable of inducing a significant bFGF and/or VEGF expression (data not shown). These findings strongly suggested that IC may synergize with Tat in vivo by promoting bFGF and VEGF expression.

To investigate whether these two angiogenic factors were both involved in the in vivo synergistic effects of Tat and IC, mice were inoculated with suboptimal (nonlesion forming) amounts of bFGF or VEGF alone or combined with Tat. In the presence of suboptimal amounts of bFGF, Tat increased KS-like lesion formation from 7 to 60% of the inoculated mice; on the contrary, no angiogenic synergy was observed when Tat and suboptimal amounts of VEGF were simultaneously injected in nude mice (Table II). Thus,

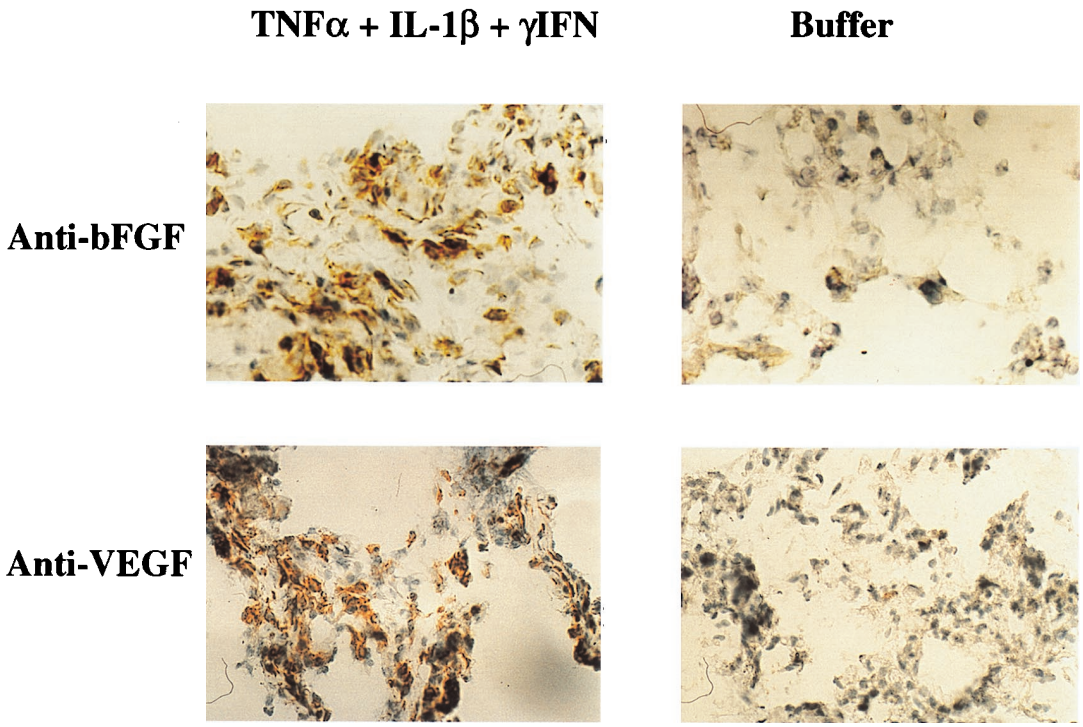

FIGURE 1. Induction of bFGF and VEGF expression in mice inoculated with combined IC. Shown are ×200 magnification of the bFGF staining of tissues from mice injected with 1 μg/each of combined mouse recombinant TNF-α, IL-1β, and IFN-γ (*upper left panel*) or with the protein resuspension buffer (PBS-0.1% BSA, *upper right panel*), and the VEGF staining of tissues from mice injected with 1 μg/each of combined mouse recombinant TNF-α, IL-1β, and IFN-γ (*lower left panel*) or with the protein resuspension buffer (*lower right panel*). Staining experiments were performed on frozen tissues with rabbit polyclonal anti-bFGF and anti-VEGF Abs by the peroxidase antiperoxidase method, as described in *Materials and Methods*. Morphometric quantitative analysis of bFGF and VEGF expression was performed in tissues derived from two to four mice per each experimental condition. The average percentage of bFGF-positive cells (range between minimal and maximal values) was 46% (44–48) in tissues from mice injected with combined IC, and 8% (0–16) in tissues from mice injected with the cytokine resuspension buffer. The average percentage of VEGF-positive cells was 36% (27–42) in tissues from mice injected with combined IC, and 0% in tissues from mice injected with buffer. Each of these values derives from the counting of at least 3 high power microscopic fields per slides per each inoculated mice.

although IC induce expression of both bFGF and VEGF, Tat synergizes with bFGF, but not with VEGF to promote KS-like lesion formation in mice.

bFGF but not VEGF induces endothelial cells to become responsive to the growth, migration, and adhesion effects of Tat

As mentioned above, EC require a preactivation with combined IC to become responsive to the in vitro effects of Tat (12, 13, 15–18). Because IC induce both bFGF and VEGF expression, but bFGF and not VEGF cooperates with Tat in inducing angiogenic lesions in mice, experiments were addressed to verify whether bFGF or VEGF were capable of inducing EC to become responsive to the in vitro angiogenic effects of Tat, as found previously with IC.

Table II. *bFGF, but not VEGF, synergizes with Tat in inducing the development of angioproliferative KS-like lesions in nude mice*[a]

Injection	Vascular Lesions[b]	
	%	*n*
Buffer	0	12
Tat (10 μg)	0	12
bFGF (0.1 μg)	7	12
bFGF (0.1 μg) + Tat (10 μg)	60	12
VEGF (1 μg)	0	8
VEGF (1 μg) + Tat (10 μg)	0	8

[a] rVEGF, bFGF, or Tat were injected in nude mice alone or combined. The effects resulting from protein inoculation were controlled by the injection of the protein resuspension buffer (PBS-0.1% BSA). The sites of injection were also examined histologically after hematoxylin-eosin staining for alterations typical of KS such as angiogenesis, spindle cell growth, edema and haemorrhagies, as performed previously (8). See *Materials and Methods* for experimental details.

[b] Data shown include the percentage of mice developing macroscopic angioproliferative lesions and the number of mice inoculated.

The culture of HUVEC with bFGF induced these cells to adhere onto immobilized Tat, whereas exposure to VEGF had no effects (Fig. 2). Similarly, HUVEC cultured with bFGF, but not after culture with VEGF, migrated and proliferated in response to soluble Tat (Fig. 2). Thus, bFGF, but not VEGF, synergizes with Tat in vivo because only bFGF induces EC responsiveness to the adhesion, migration, and growth effects of Tat.

The in vivo angiogenic effect of Tat correlates with the tissue expression of β_3 but not with β_5 integrin expression

bFGF and VEGF activate different angiogenic pathways that require different integrins. Specifically, bFGF promotes angiogenesis by inducing $\alpha_v\beta_3$ expression, whereas VEGF induces $\alpha_v\beta_5$ expression (37). These integrins modulate angiogenesis by affecting EC invasion, migration, adhesion, and growth (28). As mentioned above, Tat basic sequence binds $\alpha_v\beta_5$ (29), whereas the RGD region of Tat binds $\alpha_v\beta_3$ (12).

Because only IC or bFGF, but not VEGF, synergized with Tat to promote lesions in mice and to induce EC growth, migration, and adhesion to Tat, immunohistochemical stainings were performed to analyze the type of integrins expressed in mice upon injection of bFGF, VEGF, or IC.

As shown in Table III and in Fig. 3, tissues from mice injected with bFGF showed a prevalent β_3 expression, whereas injection of

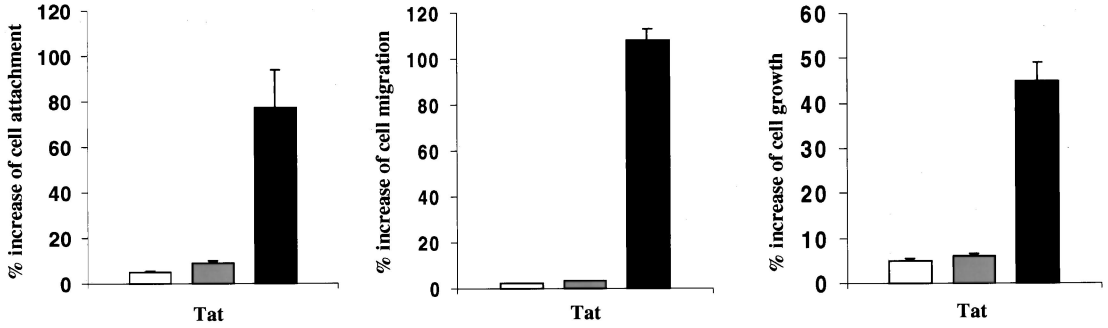

FIGURE 2. Induction of EC responsiveness to the migration, adhesion, and growth effects of Tat by bFGF, but not by VEGF. HUVEC were cultured for 4 days with 5 ng/ml of VEGF (gray bars), bFGF (black bars), or in their absence (negative control, white bars). The adhesion, migration, and growth assays were then performed as described in *Materials and Methods*. For all assays, data are the average from three experiments (±SD). For adhesion assays (*left panel*), the number of adherent cells is expressed as the percentage increase of cell attachment (±SD) to immobilized Tat (10 μg/ml) over the cell adhesion seen with the protein resuspension buffer (PBS-0.1% BSA), which was given a 0% increase value. For the migration assays (*central panel*), results are relative to the number of migrated cells per field in response to 20 ng/ml of Tat (average of 5 fields/filter). Data are expressed as the percentage increase of cell migration (±SD) over the number of cells migrated toward the buffer, which was given a 0% increase value. For the growth assays (*right panel*), results are relative to the number of HUVEC collected 4 days after the addition of Tat (10 ng/ml). Data are expressed as the percentage increase of cell growth (±SD) over the number of cells grown in the absence of Tat (basal cell growth), which was given a 0% increase value.

VEGF induced mostly β_5 expression. Specifically, bFGF injection induced a 14-fold increase of β_3 expression over basal levels and only a 2.4-fold increase of β_5 expression. In contrast, VEGF induced 0- and 7-fold increase of β_3 and β_5 expression, respectively. Consistent with the induction of both bFGF and VEGF (Fig. 1), the injection of combined IL-1β, TNF-α, and IFN-γ promoted the expression of both β_3 and β_5, although the former was more expressed than the latter. In particular, following IC injection, β_3 expression was increased up to 18-fold over basal levels, whereas β_5 was augmented up to 8-fold (Table III). Thus, β_3 expression induced by IC or bFGF correlated with the angiogenic synergy by Tat (Tables I, II, and III). In contrast, the expression of β_5, induced by VEGF, did not correlate with lesion formation. This suggested that the binding of Tat RGD region to $\alpha_v\beta_3$ is required for the synergistic effect of combined bFGF and Tat.

Tat promotes angiogenesis in vivo by engaging RGD-binding integrins

To evaluate whether the in vivo angiogenic effect of combined Tat and bFGF requires the engagement of RGD-binding integrins, in vivo experiments were performed with integrin antagonists. As shown in Fig. 4, competitors of RGD-binding integrins such as RGD peptides (31, 38) blocked the development of KS-like lesions induced by the injection of combined Tat and bFGF in mice. To the contrary, the mutated RGE control peptide had no effects (Fig. 4). Histologic examination of the lesions indicated that RGD peptides, but not RGE peptides, blocked angiogenesis, spindle cell growth, and hemorrhagies (see legend to Fig. 4), the typical features of KS lesions (8, 14). Thus, the engagement of RGD-binding integrins by Tat is required to observe the synergistic angiogenic KS-promoting effect of bFGF and Tat.

Table III. *β_3 and β_5 expression in tissues from mice inoculated with combined IL-1β, TNF-α and IFN-γ, bFGF, or VEGF*

Injection	Average (range), % Positive Cells[a]	
	β_3	β_5
Buffer	3 (0–6)	10 (5–15)
bFGF	42 (28–56)	24 (20–28)
VEGF	1 (1–1)	66 (61–70)
IL-1β, TNF-α, IFN-γ	55 (40–69)	35 (30–40)

[a] Indicates the average percentage and the range (minimal and maximal values) of stained cells in tissues derived from two to four mice per each experimental condition. Each value derives from the counting of at least three high power microscopic fields per slide. Nude mice were inoculated with bFGF (1 μg), VEGF (1 μg), or with combined IL-1β, TNF-α and IFN-γ (0.5 μg/each). The effects resulting from protein inoculation were controlled by the injection of the protein resuspension buffer (PBS-0.1% BSA). Staining experiments were performed on frozen tissues with rabbit polyclonal anti-β_3 or anti-β_5 Abs by the peroxidase antiperoxidase method. See *Materials and Methods* for experimental details.

Discussion

Previous work indicated that the angiogenic KS-promoting effects of Tat are induced in vitro by combined IC, including IL-1β, TNF-α, and IFN-γ (12, 13, 15–18). These IC are increased in tissues and blood of AIDS-KS patients or in patients at risk of KS (17–24).

The results shown herein indicate that these same IC also trigger the angiogenic effects of Tat in vivo. In fact, injection of combined IL-1β, TNF-α, IFN-γ, and Tat promotes in nude mice the development of angioproliferative KS-like lesions in which both bFGF and VEGF are highly expressed (Table I and Fig. 1). This is consistent with IC capability of promoting bFGF and VEGF production in vitro by EC and KS cells (33–35). In addition, it resembles primary human KS lesions, in which both angiogenic molecules are highly expressed (27, 36) and cooperate to mediate angiogenesis, edema, and tumor growth through autocrine (bFGF) and paracrine (bFGF and VEGF) effects (11, 25–27, 33–36). However, IL-1β, TNF-α, and IFN-γ are not capable of inducing angioproliferative lesions in mice when each cytokine is inoculated alone or when two of them are combined. The synergism among these three cytokines is likely to occur because IFN-γ up-regulates TNF receptor expression (39); TNF and IL-1, in turn, increase IFN-γ receptor levels (40); and TNF mimics the effects of IL-1 on EC (41). Nevertheless, to exert angiogenic synergy with Tat, however, IL-1β, TNF-α, and IFN-γ have to be injected at concentrations sufficient to induce a significant level of bFGF and/or VEGF expression (Table I and Fig. 1). These findings confirmed that Tat is not directly angiogenic, but it enhances the effect of true angiogenic factors (8). In fact, our recent work indicates that Tat releases sequestered, extracellular-bound bFGF into a soluble form by competing for its heparin binding sites (42). As a consequence, Tat enhances the mitogenic effect of bFGF on EC (42). On the contrary, Tat does not synergize with VEGF in promoting EC growth (42). Consistent with these in vitro findings, in this study

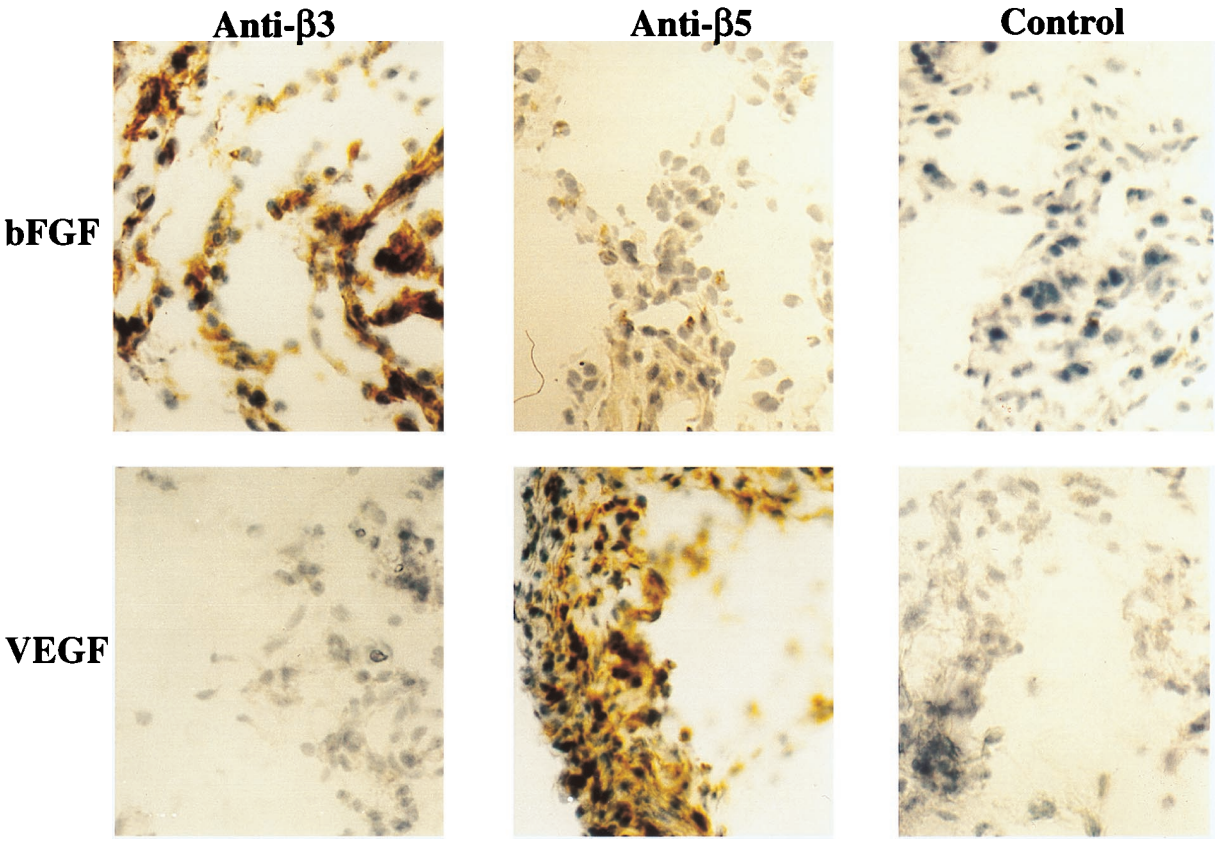

FIGURE 3. Activation of β_3 or β_5 integrin expression in mice inoculated with bFGF or VEGF. Shown are ×200 magnification of β_3 staining (*left panels*), β_5 staining (*middle panels*), or by omission of the primary Ab (*right panels*) of mice tissues injected with bFGF (1 μg, *upper panels*) or VEGF (1 μg, *lower panels*).

we have shown that Tat exerts in vivo synergistic angiogenic effects with bFGF, but not with VEGF (Table II). In agreement with these in vivo findings, the exposure of EC to bFGF induces their adhesion onto immobilized Tat as well as their migration and growth in response to soluble Tat. On the contrary, cell exposure to VEGF has no effects (Fig. 2). These results indicate that bFGF is specifically required for Tat angiogenic effect.

Previous work demonstrated that bFGF and VEGF promote angiogenesis by inducing distinct integrin pathways: VEGF promotes the expression of $\alpha_v\beta_5$ (37), an integrin that binds Tat basic sequence (29), whereas bFGF induces the expression of the $\alpha_v\beta_3$ integrin (37), which binds Tat RGD region (12).

The $\alpha_v\beta_3$ receptor, which binds the RGD region of ECM molecules such as vitronectin (43), is highly expressed by KS cells both in vitro and in primary human KS lesions (8, 12). In addition, $\alpha_v\beta_3$ expression is induced in normal EC by the same IC that induce EC responsiveness to Tat (12, 18).

Immunohistochemical analyses of the mice tissues indicated that, differently from VEGF-induced β_5 expression, the expression of β_3, which is induced by IC or bFGF, correlates with Tat angiogenic effects (Tables I, II, and III, and Fig. 3). These results suggested that the selective angiogenic effect of Tat could be due to the specific interaction of its RGD sequence with the $\alpha_v\beta_3$ integrin, whose expression is triggered by bFGF or IC, but not by VEGF. This is also supported by in vitro results indicating that Abs directed against $\alpha_v\beta_3$, but not anti-$\alpha_v\beta_5$ Abs block Tat-induced migration and invasion of EC and KS cells (42).

Additional experiments confirmed that the in vivo angiogenic effect of Tat requires the engagement of RGD-binding integrins. In fact, the injection of RGD peptides, which are known inhibitors of specific integrin function (31, 38), but not of mutated control RGE peptides, inhibits the development of angioproliferative lesions induced in nude mice by combined Tat and bFGF (Fig. 4).

The involvement of the RGD region in the in vivo angiogenic effect of Tat is consistent with previous in vitro studies showing that, as for the RGD region of ECM molecules (44–47), the binding of Tat-RGD region to $\alpha_v\beta_3$ provides EC with the adhesion signal they require to proliferate in response to mitogens (8, 12, 42), promotes cellular migration, and activates the expression of collagenase IV (8, 13, 42, 48), a protease that plays a key role in angiogenesis and tumor progression (45, 47). Moreover, Tat was found capable of inducing the expression of p125 focal adhesion kinase (p125 FAK) that is activated by integrin triggering (49, 50, and data not shown). This effect is specifically mediated by the RGD region of Tat, because mutations of this Tat region, but not of other Tat sequences, strongly decrease Tat-induced p125 FAK tyrosine phosphorylation (50).

Although Tat was reported to stimulate angiogenesis by the binding of its basic sequence to the VEGF receptor Flk-1/KDR (51), this is unlikely to occur in our experimental system or in primary KS lesions. In fact, Tat has no effect on resting EC (5, 6, 12, 13, 15–18) and it does not promote angiogenesis when it is inoculated alone in mice (8, 17). Furthermore, in primary KS lesions the amount of VEGF is much higher than that of Tat (8, 35, 36), making unlikely a competition of the VEGF receptor by Tat as opposed to its natural ligand. Finally, KS is found in several epidemiologic forms that are not associated with HIV-1 infection, but is more frequent and aggressive in AIDS (10, 11), indicating a role for Tat as a progression and not as an initiating factor.

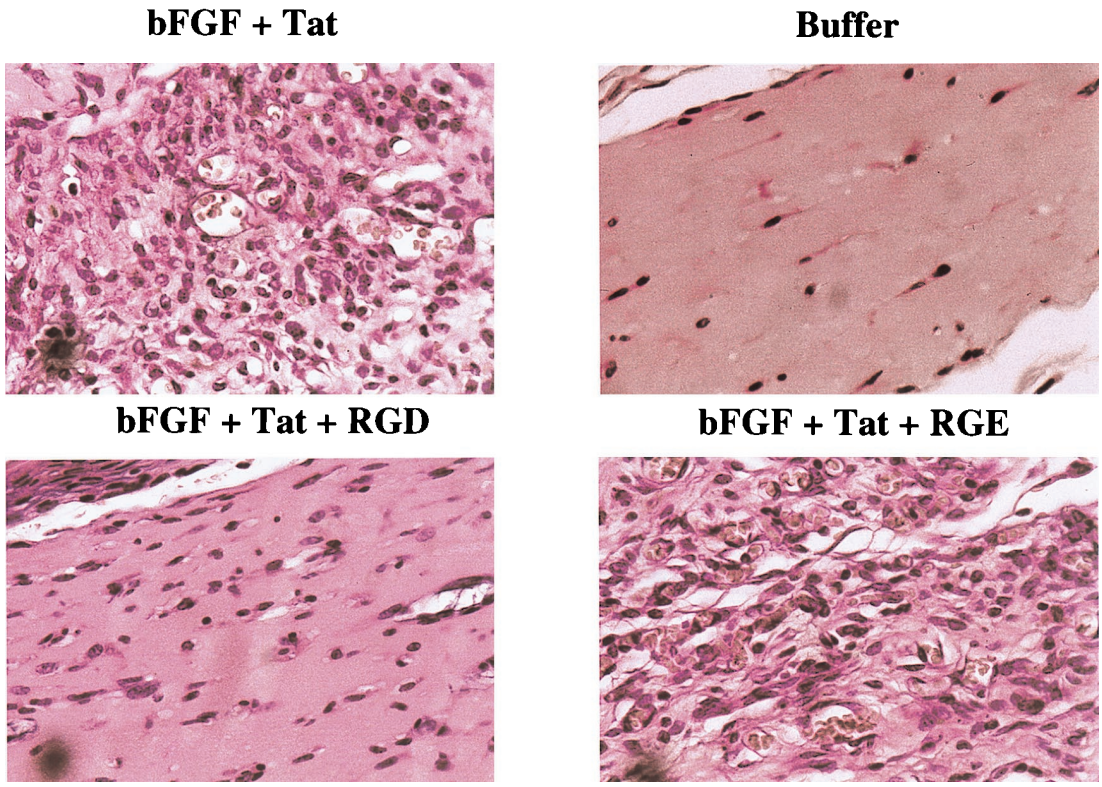

FIGURE 4. Block of the angiogenic KS-promoting effect of combined Tat and bFGF by RGD-integrin antagonists. Shown are ×200 magnification of the hematoxylin-eosin staining of representative tissues from mice injected with bFGF + Tat (*upper left panel*), protein resuspension buffer (*upper right panel*), bFGF + Tat + RGD peptides (*lower left panel*), and bFGF + Tat + RGE peptides (*lower right panel*). Human rbFGF (0.1 μg), recombinant Tat (10 μg), GRGDS, or mutated GRGES peptides (330 μg/each) were injected in nude mice (nine animals per each experimental condition) at day 0. Peptides were inoculated again in the same sites at day 2. At day 6, mice were sacrificed, and the sites of injection were examined microscopically after hematoxylin-eosin staining for histological features typical of KS, such as angiogenesis, spindle cell growth, and hemorrhagies. These were graded according to intensity (from 0 to 10), as previously reported (8). Injection of combined Tat and bFGF induced in all mice angiogenesis (average value of 7), spindle cell growth (average value of 5), and hemorrhagies (average value of 3). These were given a value of 100% for each histological change as compared with the injection of the peptide resuspension buffer (PBS-0.1% BSA) or with the peptides alone, which did not induce histological changes, and was given a value of 0%. When bFGF and Tat were injected in the presence of GRGDS peptides, angiogenesis, spindle cell growth, and hemorrhagies were reduced to 39%, 44%, and 0%, respectively. In contrast, injection of bFGF and Tat with mutated GRGES peptides gave the same values as the positive control (100% per each histological alteration). See *Materials and Methods* for experimental details.

IC and bFGF are highly expressed in AIDS-KS lesions (8, 16, 24, 27), in which extracellular Tat costains with $\alpha_v\beta_3$ on both EC and KS cells (8). This suggests that the mechanisms of Tat action described in this study are operative in vivo and that integrin competitors may be considered as a potential therapeutic strategy for AIDS-KS.

Acknowledgments

We thank Dr. Aurelio Cafaro (Istituto Superiore di Sanità, Rome, Italy) for helpful discussion, and A. Lippa and F. M. Regini for editorial assistance.

References

1. Barrè-Sinoussi, F., J. C. Chermann, F. Rey, M. T. Nugeyre, S. Charmaret, J. Gruest, C. Dauget, C. Axler-Blin, F. Vezinet-Brun, C. Rouzioux, et al. 1983. Isolation of a T lymphotropic retrovirus from a patient at risk for acquired immunodeficiency syndrome. *Science 220:868.*
2. Gallo, R. C., S. Z. Salahuddin, M. Popovic, G. M. Shearer, M. Kaplan, B. F. Haynes, T. J. Palker, R. Redfield, J. Oleske, B. Safai, et al. 1984. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science 224:500.*
3. Cullen, B. R., and W. C. Greene. 1989. Regulatory pathways governing HIV-1 replication. *Cell 58:423.*
4. Arya, S. K., C. Guo, S. F. Josephs, and F. Wong-Staal. 1982. Transactivator gene of human T lymphotropic virus type III (HTLV-III). *Science 229:69.*
5. Ensoli, B., G. Barillari, S. Z. Salahuddin, S. Z. Gallo, and F. Wong-Staal. 1990. Tat protein of HIV-1 stimulates the growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. *Nature 345:84.*
6. Ensoli, B., L. Buonaguro, G. Barillari, V. Fiorelli, R. Gendelman, R. A. Morgan, P. Wingfeld, and R.C. Gallo. 1993. Release, uptake and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral transactivation. *J. Virol. 67:277.*
7. Chang, H. C., F. Samaniego, B. C. Nair, L. Buonaguro, and B. Ensoli. 1997. 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.*
8. Ensoli, B., R. Gendelman, P. D. Markham, V. Fiorelli, S. Colombini, M. Raffeld, A. Cafaro, H. K. Chang, J. N. Brady, and R. C. Gallo. 1994. Synergy between basic fibroblast growth factor and HIV-1 Tat protein in induction of Kaposi's sarcoma. *Nature 371:674.*
9. Westendorf, M. O., R. Frank, C. Ochsenbauer, K. Stricker, K. Dhein, H. Walchzak, K. M. Debatin, and P. H. Krammer. 1995. Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. *Nature 375:497.*
10. Safai, B., K. G. Johnson, S. Myskowsky, S. Cunningham-Randles, J. H. Godbold, and D. Dupont. 1985. The natural history of Kaposi's sarcoma in the acquired immunodeficiency syndrome. *Ann. Intern. Med. 103:744.*
11. Ensoli, B., and M. Stürzl. 1998. M. Kaposi's sarcoma: a result of the interplay among inflammatory cytokines, angiogenic factors and viral agents. *Cytokine Growth Factor Rev. 9:63.*
12. Barillari, G., R. Gendelman, R. C. Gallo, and B. Ensoli. 1993. 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.*
13. Albini, A., G. Barillari, R. Benelli, R. C. Gallo, and B. Ensoli. 1995. Angiogenic properties of human immunodeficiency virus type 1 Tat protein. *Proc. Natl. Acad. Sci. USA 92:4836.*
14. Regezi, S. A., L. A. MacPhail, and T. E. Daniels. 1993. Human immunodeficiency virus-associated Kaposi's sarcoma: an heterogeneous cell population dominated by spindle-shaped endothelial cells. *Am. J. Pathol. 43:240.*

15. Barillari, G., L. Buonaguro, V. Fiorelli, J. Hoffman, F. Michaels, R. C. Gallo, and B. Ensoli. 1992. 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.*
16. Fiorelli, V., R. Gendelman, F. Samaniego, P. D. Markham, and B. Ensoli. 1995. 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.*
17. Fiorelli, V., R. Gendelman, M. C. Sirianni, H. K. Chang, S. Colombini, P. D. Markham, P. Monini, J. Sonnabend, A. Pintus, R. C. Gallo, and B. Ensoli. 1998. Interferon-γ produced by $CD8^+$ T cells infiltrating Kaposi's sarcoma induces spindle cells with angiogenic phenotype and synergy with HIV-1 Tat. *Blood 91:956.*
18. Fiorelli, V., G. Barillari, E. Toschi, C. Sgadari, P. Monini, M. Sturzl, and B. Ensoli. 1999. Interferon-γ induces endothelial cells to proliferate and to invade the extracellular matrix in response to HIV-1 Tat. *J. Immunol. 162:1165.*
19. Lepe Zanuga, J. L., P. W. A. Mansell, and E. M. Hersh. 1987. Idiopathic production of interleukin 1 in acquired immunodeficiency syndrome. *J. Clin. Microbiol. 25:1695.*
20. Lahdervita, J. C., P. J. Maury, A. M. Teppo, and H. Repo. 1988. Elevated levels of circulating cachectin/tumor necrosis factor in patients with acquired immunodeficiency syndrome. *Am. J. Med. 85:289.*
21. Hober, D. A., A. Haque, P. Wattre, G. Beaucaire, Y. Mouton, and A. Capron. 1989. Production of TNFα and IL-1 in patients with AIDS: enhanced level of TNFα is related to higher cytotoxic activity. *Clin. Exp. Immunol. 78:329.*
22. Oxholm, A., P. Oxholm, H. Permin, and L. Bendtzen. 1989. Epidermal tumor necrosis factor α and interleukin 6-like activities in AIDS-related Kaposi's sarcoma: an immunohistological study. *APMIS 97:533.*
23. Fan, J., H. Z. Bass, and J. L. Fahey. 1993. Elevated IFN-γ and decreased IL-2 gene expression are associated with HIV-1 infection. *J. Immunol. 152:5031.*
24. Sirianni, M. C., L. Vincenzi, V. Fiorelli, S. Topino, E. Scala, S. Uccini, A. Angeloni, A. Faggioni, D. Cerimele, F. Cottoni, et al. 1998. 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.*
25. Ensoli, B., S. Nakamura, S. Z. Salahuddin, P. Biberfeld, L. Larsson, B. Beaver, F. Wong-Staal, and R. C. Gallo. 1989. AIDS-Kaposi's sarcoma derived cells express cytokines with autocrine and paracrine growth effects. *Science 243:223.*
26. Ensoli, B., P. D. Markham, V. Kao, G. Barillari, V. Fiorelli, R. Gendelman, M. Raffeld, G. Zon, and R. C. Gallo. 1994. 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.*
27. Xerri, L., J. Houssoun, J. Planche, V. Guigou, J. J. Grob, P. Parc, D. Birnbaum, and O. De Lapeyriere. 1991. Fibroblast growth factor gene expression in AIDS-Kaposi's sarcoma detected by in situ hybridization. *Am. J. Pathol. 138:9.*
28. Folkman, J., and Y. Shing. 1992. Angiogenesis. *J. Biol. Chem. 267:10931.*
29. Vogel, B. E., S. J. Lee, A. Hieldebrand, W. Craig, M. D. Piershbacher, F. Wong-Staal, and E. Ruoslahti. 1993. 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.*
30. Kubota, Y., H. K. Kleinman, G. R. Martin, and T. Lawley. 1988. Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. *J. Cell Biol. 107:1589.*
31. Hayman, E. G., M. D. Piershbacher, and E. Ruoslahti. 1985. Detachment of cells from culture substrate by soluble fibronectin peptides. *J. Cell Biol. 100:1948.*
32. Taraboletti, G., D. Roberts, L. A. Liotta, and R. Giavazzi. 1990. Platelet thrombospondin modulates endothelial cell adhesion, motility and growth: a potential angiogenesis regulatory factor. *J. Cell Biol. 111:765.*
33. Samaniego, F., P. D. Markham, R. C. Gallo, and B. Ensoli. 1995. 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.*
34. Samaniego, F., P. D. Markham, R. Gendelman, R. C. Gallo, and B. Ensoli. 1997. 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.*
35. Samaniego, F., P. D. Markham, R. Gendelman, Y. Watanabe, V. Kao, K. Kowalski, N. Ferrara, J. A. Sonnabend, A. Pintus, G. Zon, et al. 1998. 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.*
36. Cornali, E., C. Zietz, R. Benelli, W. Weninger, L. Masiello, G. Breier, E. Tschachler, A. Albini, and M. Stürzl. 1996. Vascular endothelial growth factor regulates angiogenesis and vascular permeability in Kaposi's sarcoma. *Am. J. Pathol. 149:1851.*
37. Friedlander, H. P., P. C. Brooks, R. W. Shaffer, C. M. Kincaid, J. A. Varner, and D. A. Cheresh 1995. Definition of two angiogenic pathways by distinct αv integrins. *Science 270:1500.*
38. Hammes, H. P., M. Brownlee, A. Jonczyk, A. Sutter, and K. T. Preissner. 1996. Subcutaneous injection of a cyclic peptide antagonist of vitronectin receptor-type integrins inhibits retinal neovascularization. *Nat. Med. 2:529.*
39. Pandita, R., E. Poesik, and B. B. Aggarwal. 1992. Interferon-γ induces cell surface expression of both types of tumor necrosis factor receptors. *FEBS Lett. 312:87.*
40. Krakauer, T., and J. J. Oppenheim. 1993. IL-1 and TNF α each up-regulate both the expression of IFN-γ and enhance IFN-γ-induced HLA-DR expression on human monocytes and on a human monocytic cell line. *J. Immunol. 150:1205.*
41. Pober, J. S., M. A. Gimbronc Jr., L. A. Lapierre, D. L. Mendrick, W. Fiers, R. Rothkin, and T. A. Springer. 1986. Overlapping patterns of activation of human endothelial cells by interleukin 1, tumor necrosis factor and immune interferon. *J. Immunol. 137:1893.*
42. Barillari, G., C. Sgadari, V. Fiorelli, F. Samaniego, S. Colombini, V. Manzari, A. Modesti, B. C. Nair, A. Cafaro, M. Stürzl, and B. Ensoli. 1999. The Tat protein of human immunodeficiency virus type-1 promotes vascular cell growth and locomotion by engaging the $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins and by mobilizing sequestered basic fibroblast growth factor. *Blood. In press.*
43. Hynes, R. O. 1992. Integrins: versatility, modulation and signaling in cell adhesion. *Cell 69:11.*
44. Ingber, D. E., D. Prusty, J. Y. Frangioni, E. J. Cragoe, C. Lechene, and M. Schwartz. 1990. Control of intracellular pH and growth by fibronectin in capillary endothelial cells. *J. Cell Biol. 110:1803.*
45. Seftor, R. E., E. Seftor, W. G. Stetler-Stevensen, and M. J. C. Hendrix. 1993. 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.*
46. Brooks, P. C., R. A. F. Clark, and D. A. Cheresh. 1994. Requirement of vascular integrin $\alpha_v\beta_3$ for angiogenesis. *Science 264:569.*
47. Brooks, P. C., S. Stromblad, L. C. Sanders, T. L. Von Schalscha, R. T. Aimes, V. G. Stetler-Stevenson, J. P. Quigley, and D. A. Cheresh. 1996. Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin $\alpha_v\beta_3$. *Cell 85:683.*
48. Lafrenie, R. M., L. M. Wahl, J. S. Epstein, I. K. Hewlett, K. M. Yamada, and S. Dhawan. 1996. HIV-1 Tat modulates the function of monocytes and alters their interactions with microvessel endothelial cells. *J. Immunol. 156:1638.*
49. Ganju, R. K., N. Munshi, B. C. Nair, Z. Y. Liu, P. Gill, and J. E. Groopman. 1998. 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.*
50. Milani, D., M. Mazzoni, G. Zauli, C. Mischiati, D. Gibellini, M. Giacca, and S. Capitani. 1998. HIV-1 Tat induces phosphorylation of $p125^{FAK}$ and is associated with phosphoinositide 3-kinase in PC12 cells. *AIDS 12:1275.*
51. Albini, A., R. Soldi, D. Giunciuglio, R. Giraudo, R. Benelli, L. Primo, D. Noonan, M. Salio, G. Camussi, W. Rockl, and F. Bussolino. 1996. The angiogenesis induced by HIV-1 Tat protein is mediated by the FlK-1/KDR receptor on vascular endothelial cells. *Nat. Med. 2:1371.*