IFN-γ Induces Endothelial Cells to Proliferate and to Invade the Extracellular Matrix in Response to the HIV-1 Tat Protein: Implications for AIDS-Kaposi’s Sarcoma Pathogenesis

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Previous studies indicated that the Tat protein of HIV functions as a progression factor in Kaposi’s sarcoma (KS), an angioproliferative disease common and aggressive in HIV-1-infected individuals (AIDS-KS). In particular, Tat that is released by infected cells stimulates the growth and invasion of spindle cells of endothelial origin derived from KS lesions (KS cells). Other work suggested that inflammatory cytokines may act as initiating factors in KS since they induce normal endothelial cells to acquire the same phenotype and functional features of KS cells, including the responsiveness to Tat. In this study, we show that among the inflammatory cytokines increased in AIDS-KS lesions, IFN-γ alone is sufficient to induce endothelial cells to proliferate and to invade the extracellular matrix in response to Tat. This is because IFN-γ up-regulates the expression and activity of the receptors for Tat identified as the integrins αvβ3 and αβ6. These results suggest that, by triggering Tat effects, IFN-γ plays a major role in AIDS-KS pathogenesis. The Journal of Immunology, 1999, 162: 1165–1170.

Kaposi’s sarcoma (KS) is a proliferative disease of vascular origin that is more frequent and aggressive in HIV-1-infected homosexual men (AIDS-KS) as compared with classical KS, which is rare and indolent (1–3). In early stages, KS is characterized by endothelial cell activation and proliferation, angiogenesis, and inflammatory cell infiltration (4–9). This is followed by the appearance of the typical spindle-shaped cells (KS cells) that represent a heterogeneous population dominated by activated endothelial cells, which in time become the predominant cell phenotype (5, 8–13).

Previous studies indicated that, at least in early stages, KS is a cytokine-mediated disease and that inflammatory cytokines (IC) and angiogenic factors cooperate in its induction (reviewed in Ref. 14). Specifically, IC such as IFN-γ, TNF, IL-1, IL-6, and others are increased in patients with all forms of KS and in individuals at high risk of KS (15–21). In these patients, IC are produced by activated PBMC and by tissue-infiltrating CD8+ T cells and monocytes/macrophages (8, 9), perhaps in response to (or amplified by) human herpesvirus-8 (8, 9, 14), a new virus that is found in all forms of KS (22, 23).

IC induce endothelial cells to acquire the phenotypic and functional features of KS spindle cells. These include the spindle cell morphology; the down-regulation of factor VIII-related Ag expression; and the up-regulation of ICAM-1, VCAM-1, and endothelial leukocyte adhesion molecule-1 (ELAM-1) expression (9, 24–26). In addition, similar to KS cells, endothelial cells activated by IC become angiogenic in nude mice (9, 25–27). This is because IC induce production and release of basic fibroblast growth factor (bFGF), an angiogenic factor that is highly expressed in all forms of KS (9, 25–31). bFGF, in turn, synergizes with vascular endothelial growth factor, also expressed in KS, to induce angiogenesis, vascular permeability, and edema (32, 33), the typical histologic features of KS. However, since these findings are common to all forms of KS, the data did not explain the higher aggressiveness of AIDS-KS as compared with the other forms of KS.

Other data indicated that the Tat protein of HIV-1 may act as a progression factor in AIDS-KS. Extracellular Tat, released during acute infection of T cells by HIV-1 (34–36), promotes the growth, migration, invasion, and adhesion of KS cells, endothelial cells, and monocytes (34, 35, 37–40). However, normal endothelial cells require a preactivation with the same mixture of IC described above to become responsive to the effects of Tat (9, 25, 37–39). In vivo Tat enhances angiogenesis triggered by bFGF and it synergizes with bFGF to increase endothelial and KS cell growth, invasion, and collagenase IV activation (41, 42).

Thus, IC or bFGF are required to observe Tat activity both in vitro and in vivo. This is because they induce endothelial cells to express the receptors for Tat, identified as the integrins αvβ3 and αβ6 (38) that are constitutively expressed by KS cells (38, 41). Specifically, the RGD sequence present at the carboxyl terminus of Tat binds these receptors and mediates the migration, invasion, and adhesion of KS cells and IC-activated endothelial cells (38, 42, and...

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3 Abbreviations used in this paper: KS, Kaposi’s sarcoma; bFGF, basic fibroblast growth factor; CC, combined cytokines; ELAM, endothelial leukocyte adhesion molecule; FN, fibronectin; GM-CSF, granulocyte-macrophage CSF; IC, inflammatory cytokine; MMP, matrix metalloproteases; OM, oncostatin M; TCM, T cell-conditioned media.
Barillari et al.5. Thus, Tat mimics the action of extracellular matrix molecules such as fibronectin (FN) and vitronectin that bind to the same receptors (38). These mechanisms are likely to be operative in vivo since bFGF and Tat are both present in AIDS-KS lesions and Tat co-stains with β1 and β3 integrins on resident vessels and spindle cells (41).

These observations suggested that cytokines produced by activated immune cells may predispose to KS development and progression by providing initial signals required for KS lesion formation. However, nothing is known about the specific cytokines that, by up-regulating αββ1 and αββ3 expression, induce endothelial cells to invade and proliferate in response to the HIV-1 Tat protein.

Previous data indicated that IFN-γ is the earliest and most abundant IC produced in blood or tissues of patients with all forms of KS (8, 9, 16, 43–45). In this study, we show that IFN-γ, at concentrations similar to those found in HIV-1-infected individuals, is sufficient to induce endothelial cells to become responsive to the mitogenic and invasive effects of extracellular Tat.

Materials and Methods
HIV-1 Tat protein
rHIV-1 Tat protein (from the IIIB isolate) was expressed in Escherichia coli and isolated by either successive rounds of HPLC and ion-exchange chromatography (34, 35) or by heparin-affinity chromatography (36). Analysis of purified Tat by silver staining (Bio-Rad, Hercules, CA) and SDS-PAGE indicated the protein to be more than 95% pure. Protein preparations were negative for endotoxin (37). Tat protein was then lyophilized in small aliquots and resuspended at the moment of the assay in PBS/0.1% BSA in ice and in the dark. These procedures are required to prevent the oxidation of the protein, which leads to the loss of its biologic activities (34–39). To avoid Tat sticking to surfaces, the plasticware was rinsed in PBS/0.1% BSA. Purified Tat was tested for its biologic activity by transactivation of HIV-1 long-terminal repeat (LTR)-directed gene expression, rescue of tat-defective HIV proviruses, and KS cell growth assay, as described elsewhere (34–39). The biologic effects of Tat were blocked by heat inactivation of the protein or by anti-Tat affinity-purified Abs, as reported previously (34–39).

Cell cultures, preparation of conditioned media from T cells, and blocking experiments
HUVEC (passage 5–10) were established and cultured as previously described (25, 37–39, 46). These cells behave as activated immune cells, and TCM contain the same cytokines increased in blood and lesions of KS patients (25, 37–39). The average concentration of these cytokines in different TCM preparations, as determined by ELISA, is: IL-1α (0.5 ng/ml), IL-1β (5 ng/ml), IL-2 (0.3 ng/ml), IL-6 (35 ng/ml), TNF-α (2 ng/ml), TNF-β (50 pg/ml), GM-CSF (0.4 ng/ml), oncostatin M (OM) (0.5–1 ng/ml), and IFN-γ (150 pg/ml, corresponding to 3–4 U/ml of the rIFN-γ utilized in these experiments). Cytokine treatment was performed by culturing HUVEC for 5 to 6 days in the presence of TCM or human recombinant cytokines, alone or combined together at the same concentrations as found in TCM. All recombinant cytokines were purchased from Boehringer Mannheim (Indianapolis, IN), except for OM, which was purchased by R&D Systems (Minneapolis, MN).

For blocking experiments, TCM were preincubated for 12 h (on rotation at 4°C) with 20 μg/ml of neutralizing anti-human IFN-γ mAbs (R & D Systems) before being added to the cells. The Ab resuspension buffer (PBS/0.1% BSA) was employed as control.

Proliferative assays with the HIV-1 Tat protein
Cytokine-treated or untreated HUVEC were seeded at 1 × 103 cells/well in 96-well plates (Costar, Cambridge, MA) coated with gelatin (Sigma, St. Louis, MO). After 18–22 h, Tat (10 ng/ml) or the protein dilution buffer (PBS/0.1% BSA, negative control) and 1 μCi of [3H]thymidine (New England Nuclear, Boston, MA) were added to the cells. Cells were harvested after 72 h, and the cpm of the incorporated precursor was determined with a beta counter.

Invasion assays with Tat
Cytokine-treated or untreated HUVEC were trypsinized, washed with trypsin inhibitors, resuspended in DMEM/0.01% BSA, and placed at 2 × 104 cells/200 μl in the upper compartment of BioCoat invasion chambers (Collaborative Biomedical Products, Bedford, MA). Tat (20 ng/ml) or Tat re-suspension buffer (PBS/0.1% BSA, negative control) was placed in the lower compartment of the chambers diluted in 500 μl of DMEM/0.01% BSA. Assays were conducted in duplicate chambers at 37°C in 5% CO2 for 6 h, as previously described (39). After incubation, noninvaded cells (present on the upper surface of the filters) were removed, and invaded cells (present on the lower surface of the filters) were fixed in ethanol, stained with toluidine blue and with hematoxylin-eosin, and quantitated by light microscopy by counting five fields/filter.

Gelatin zymography
Cytokine-treated or untreated HUVEC were grown for 24 h in RPMI/0.05% BSA in the absence or presence of Tat (20 ng/ml). Supernatants were then collected and concentrated by Centricron-10 (Amicon, Beverly, MA). Protein concentration was determined with the Bradford assay (Bio-Rad) by using BSA as a standard. Two micrograms of proteins were diluted into the sample buffer (0.4 M Tris, pH 6.8, 5% SDS, 20% glycerol, 0.03% bromophenol blue) and run onto 9% polyacrylamide gels containing SDS and embedded with 1 mg/ml gelatin. After electrophoresis, gels were incubated for 1 h in 2.5% Triton X-100 and for an additional 18 h in low salt collagenase buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 5 mM CaCl2, and 0.02% Brij-35). Gels were then stained with 2.5% Coomassie blue G-250 and destained in 30% methanol/10% acetic acid to reveal zones of lysis within the gelatin matrix (47). To quantitatively compare the lysis signals, the gels were analyzed with a densitometer (GS-700 Imaging; Bio-Rad), as described previously (47).

Immunostaining of cells
Cytokine-treated or untreated HUVEC were trypsinized, washed with trypsin inhibitors, resuspended at 5 × 104 cells/100 μl PBS/0.01% BSA, and incubated for 30 min in ice with anti-αβ, anti-βα, anti-αα, or anti-ββ mAb (AMAC, Westbrook, ME), followed by incubation for 30 min in ice with goat anti-mouse FITC-conjugated Abs (Becton Dickinson, San Jose, CA), and fixed in 1% paraformaldehyde. All steps were separated by washes in PBS/0.1% BSA. The relative amount of cell surface fluorescence was quantitated by flow cytometry with log amplification utilizing a FACS (Becton Dickinson).

Adhesion assays
Twelve-well plates (Costar) were coated for 2 h at 37°C with Tat (10 μg/ml) or human FN (1 μg/ml) (Sigma). Plates were then incubated for 30 min with PBS/1% BSA to saturate nonspecific binding sites. HUVEC (suspending at 5 × 104/ml in serum-free medium) were added to the wells (in triplicate) and incubated for 1 h at 37°C in a 5% CO2 atmosphere. Plates were washed with PBS, and adherent cells were fixed with 3% paraformaldehyde, stained with Giemsa, and quantitated by light microscopy by counting six high-power fields/well, as described (48).

Results
IFN-γ is necessary and sufficient to induce endothelial cells to become responsive to the growth effect of Tat
KS spindle cells proliferate in response to extracellular HIV-1 Tat protein (34, 35). After a few days of exposure to TCM, normal endothelial cells also become responsive to Tat (37). Several of the cytokines contained in TCM, including IL-1, IL-6, TNF, OM, and IFN-γ, are also produced by PBMC of KS patients or by mononuclear cells infiltrating KS tissues (8, 9, 49–51).

To identify the IC responsible for the induction of the endothelial cell responsiveness to Tat, experiments were performed by pretreating HUVEC with IL-1 (α and β), IL-2, IL-6, TNF (α and β), GM-CSF, OM, or IFN-γ, and by monitoring cell growth after the addition of Tat. HUVEC activated by TCM were used as the positive control (37–39).

4 G. Barillari, C. Sgadari, V. Fiorelli, F. Samaniego, S. Colombini, V. Manzari, A. Modesti, B. C. Nair, A. Cafaro, and B. Ensoli. The basic and the arginine-glycine-aspartic acid region of the human immunodeficiency virus type-1 Tat protein promote growth and locomotion of Kaposi’s sarcoma and endothelial cells by retrieving heparin-bound basic fibroblast growth factor and by engaging the αββ1 and αββ3 integrins. Submitted for publication.
Among these IC, only IFN-γ was capable of inducing endothelial cell to grow in response to Tat (Fig. 1A). This was dose dependent, increasing at higher concentrations of IFN-γ (Fig. 1B). Consistent with these results, Abs directed against IFN-γ inhibited the capability of TCM of inducing endothelial cell growth in response to Tat (Fig. 1B).

To confirm these findings, HUVEC were exposed to the same combination of cytokines (combined cytokines (CC)) contained in TCM, in the presence or absence of IFN-γ (Fig. 1C). In addition, the preincubation of the cells with combined IL-1β, TNF-α, and IFN-γ lowered the amount of IFN-γ required to induce HUVEC responsiveness to Tat. Under these conditions, cell growth to Tat was observed at low concentrations of IFN-γ that were similar to those found in TCM or in HIV-1-infected individuals (16) (Fig. 1C). These data indicated that IFN-γ is necessary and sufficient to induce normal endothelial cells to proliferate in response to Tat. In addition, although little or no proliferative response to Tat is observed after exposure of HUVEC to either IL-1 or TNF alone or combined, these cytokines enhance IFN-γ action most likely because they can augment IFN-γ receptors (52).

IFN-γ is necessary and sufficient to induce endothelial cells to become responsive to the invasive effects of extracellular Tat and to produce the matrix metalloprotease (MMP)-2 and -9

Tat also induces KS and activated endothelial cells to migrate and to degrade and traverse the basement membrane (invasion) (39). To determine whether IFN-γ is capable of inducing endothelial cells to become responsive to these effects of Tat, experiments were performed by using invasion chambers with filters coated with Matrigel, a reconstituted basement membrane that prevents the invasion of noninvasive cells (39). As found for TCM, exposure to rIFN-γ increased (by 70–90%) the number of endothelial cells invading the basement membrane in response to Tat (Fig. 2). Consistent with this result, Tat stimulated IFN-γ-treated HUVEC to synthesize and to release MMP-2 and MMP-9 (see legend to Fig. 2), two enzymes that degrade the basement membrane and confer invasive properties to cells during angiogenesis or tumor growth (53). These data indicated that IFN-γ is also sufficient to induce normal endothelial cells to acquire invasive properties in response to Tat. In addition, as found for Tat-promoted endothelial cell growth, IL-1β and TNF-α lowered the amount of IFN-γ required to induce endothelial cell invasiveness and MMP-2 and MMP-9 release in response to extracellular Tat (Fig. 2).

IFN-γ up-regulates the expression and adhesive activity of the αvβ3 and αvβ5 integrins that function as the receptors for Tat

Previous studies indicated that the effects of Tat on vascular cells are mediated by the αvβ3 and αvβ5 integrins (38). These receptors are highly expressed by cultured AIDS-KS cells and by vessels and spindle cells of primary AIDS-KS lesions (38, 41), and they mediate Tat-promoted migration and invasion of KS and IC-activated endothelial cells (42, and Barillari et al., submitted). Moreover, binding of Tat to αvβ3 and αvβ5 provides endothelial cells with the adhesion signal required by the cells to respond to angiogenic stimuli (41, and Barillari et al., submitted). In fact, the addition of bFGF to endothelial cells plated on Tat promotes a proliferative response much higher than that observed with cells plated on gelatin or BSA and similar to that observed with FN-coated plates (41).

The expression of αvβ3 and αvβ5 integrins is up-regulated by IC or bFGF (54, 55), and this is simultaneous with the acquisition of the cell responsiveness to Tat (9, 25, 37, 41). As shown in Table I, exposure of endothelial cells to IFN-γ increased the levels of αv (by 60%), β3 (by 63%), α5 (by 25%), and β5 (by 79%), as compared with control cells. The expression of αvβ3 and αvβ5 was augmented by IFN-γ at the levels induced by TCM that was employed as the positive control (37–39). Thus, IFN-γ enhances the expression of the Tat receptors. This explains why IFN-γ alone is
supernatants showed a MMP-9 activity value of 1.09 OD/mm², which was 1.35 OD/mm² by gelatin zymography. In the presence of Tat (20 ng/ml), MMP-2 activity in supernatants from control cells was used as the positive control (37–39). Invasion experiments were performed with Tat (20 ng/ml) by using Biocoat invasion chambers, as described in Materials and Methods. Shown are the average results (±SD) from three independent experiments, each performed in duplicate chambers. They are expressed as the percentage increase of invaded cells over the number of cells invaded in response to Tat dilution buffer (10%) increase. Precultivation with IC at the concentrations reported above slightly increased HUVEC capability of invading the basement membrane in the absence of Tat as compared with control cells (data not shown). MMP-2 activity in supernatants from control cells was 1.35 OD/mm² by gelatin zymography. In the presence of Tat (20 ng/ml), this value increased to 1.5 and 2.11 OD/mm² in supernatants from HUVEC treated with IFN-γ alone (100 U/ml) or with combined IFN-γ (4 U/ml), IL-1β (5 ng/ml), and TNF-α (2 ng/ml), respectively. These same supernatants showed a MMP-9 activity value of 1.09 OD/mm², which raised to 1.29 OD/mm² in supernatants from HUVEC treated with IFN-γ (100 U/ml) and Tat and to 1.35 OD/mm² in supernatants from HUVEC treated with combined IFN-γ (4 U/ml), IL-1β (5 ng/ml), and TNF-α (2 ng/ml) after the addition of Tat.

sufficient to induce endothelial cells to become responsive to the mitogenic and invasive effects of extracellular Tat. Again, IL-1β and TNF-α enhanced the effect of IFN-γ on endothelial cell expression of both αβ and αβ. In addition, the up-regulation of integrin expression induced by IC was accomplished by an increase in the function of these receptors. In fact, IL-1β, TNF-α, and IFN-γ combined together at concentrations as found in TCM augmented (by 45%) HUVEC adhesion to FN, a ligand for these integrins, and induced the cells to adhere to Tat (Fig. 3).

Discussion
Several reports have suggested that IC may act as initiating factors in KS. IL-1, IL-6, TNF, and IFN-γ are increased in HIV-1-infected individuals, are produced at high levels by PBMC of patients with KS, and are expressed in KS lesions (8, 9, 15–21, 43–45, 49–51). In particular, IFN-γ is expressed by CD8+ T cells and by CD14+ or CD68+ macrophages infiltrating early AIDS-KS and classical KS lesions (8, 9). This is associated with endothelial cell activation, as indicated by the expression of HLA-DR and ELAM by vessels and spindle cells of KS lesions (9, 41). Interestingly, IFN-γ expression appears to precede the detection of human herpesvirus-8-specific sequences in the lesions (8, 9).

Recent studies from our group have shown that among the IC present in TCM, IFN-γ induces normal endothelial cells to acquire phenotypic features of KS cells such as the typical spindle morphology; the induction of VCAM-1, ICAM-1, and ELAM-1 expression; the down-regulation of factor VIII-related Ag and EN-4 expression; and the angiogenic phenotype (9).

In this study, we have shown that IFN-γ induces normal endothelial cells to proliferate, migrate, and to invade the basement membrane in response to Tat (Figs. 1 and 2). IL-1β and TNF-α, other cytokines expressed in AIDS-KS lesions, enhance these effects most likely by increasing the expression of IFN-γ receptors (52).

Table I. Enhanced expression of αβ and αβ (Tat receptors) in HUVEC by IFN-γ

<table>
<thead>
<tr>
<th>Ab</th>
<th>Specificity</th>
<th>HUVEC*</th>
<th>IFN-γ HUVEC*</th>
<th>CC HUVEC*</th>
<th>TCM-HUVEC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDw49e</td>
<td>FN receptor α chain</td>
<td>60 ± 12</td>
<td>96 ± 3</td>
<td>82 ± 6</td>
<td>78 ± 2</td>
</tr>
<tr>
<td>CD29</td>
<td>FN receptor β chain</td>
<td>48 ± 15</td>
<td>78 ± 16</td>
<td>67 ± 2</td>
<td>62 ± 20</td>
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<tr>
<td>CD51</td>
<td>VN receptor α chain</td>
<td>61 ± 14</td>
<td>76 ± 3</td>
<td>90 ± 12</td>
<td>89 ± 6</td>
</tr>
<tr>
<td>CD61</td>
<td>VN receptor β chain</td>
<td>19 ± 4</td>
<td>34 ± 4</td>
<td>30 ± 3</td>
<td>48 ± 5</td>
</tr>
</tbody>
</table>

* HUVEC: untreated HUVEC; IFN-γ-HUVEC: HUVEC treated with IFN-γ; CC-HUVEC: HUVEC treated with combined IL-1β, TNF-α, and IFN-γ; TCM-HUVEC: HUVEC treated with TCM. HUVEC were cultured in medium without growth supplements or treated for 5–6 days with recombinant IFN-γ (102 U/ml), with combined IL-1β (5 ng/ml), TNF-α (2 ng/ml), and IFN-γ (4 U/ml) or with TCM that was employed as the positive control (37–39). Cells were then stained by FACS analysis as described in Materials and Methods. Results are expressed as percentage of positive cells (average from three independent experiments, ± SD).

* Percentage of positive cells
The induction of the Tat cell responsiveness by IFN-γ is associated with the up-regulation of the αβ3 and αβ1 integrin expression and activity (Table I and Fig. 3). As IC also promote bFGF expression and release (26, 27, 30), it is likely that integrin up-regulation by IC is mediated by both direct and indirect (bFGF-mediated) effects. The αβ3 and αβ1 integrins function as the receptors for Tat (38, 41, 42, and Barillari et al., 43). The involvement of the interaction between the RGD region of Tat and these integrins in Tat-promoted cellular growth is consistent with the fact that αβ3 (which binds the RGD region of both Tat and FN) mediates FN-promoted cellular growth (56).

In addition, the activation of metalloproteases expression and the induction of endothelial cell invasion by Tat are likely to occur through integrin engagement. This hypothesis is in agreement with results obtained by others with RGD-containing proteins such as FN or vitronectin (57), and with the finding that Tat activates members of the focal adhesion kinase family that have a fundamental role in cellular locomotion and that are induced by integrin triggering (58).

Inoculation of KS spindle cells and TCM-treated endothelial cells in nude mice induces the development of vascular lesions of mouse cell origin closely resembling early KS (27, 31, 41, 59). In addition, the activation of metalloproteases expression and activity (Table I and Fig. 3). As IC also promote the induction of endothelial cell invasion by Tat are likely to occur through integrin engagement. This hypothesis is in agreement with results obtained by others with RGD-containing proteins such as FN or vitronectin (57), and with the finding that Tat activates members of the focal adhesion kinase family that have a fundamental role in cellular locomotion and that are induced by integrin triggering (58).

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