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Angiogenic properties of human immunodeficiency virus type 1 Tat protein
(AIDS/Kaposi sarcoma/angiogenesis/vascular cell invasion/morphogenesis)

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ABSTRACT Extracellular human immunodeficiency virus type 1 (HIV-1) Tat protein promotes growth of spindle cells derived from AIDS-associated Kaposi sarcoma (AIDS-KS), an angioproliferative disease very frequent in HIV-1-infected individuals. Normal vascular cells, progenitors of AIDS-KS cells, proliferate in response to Tat after exposure to inflammatory cytokines, whose levels are augmented in HIV-1-infected individuals and in KS lesions. Here we show that Tat also promotes AIDS-KS and normal vascular cells to migrate and to degrade the basement membrane and stimulates endothelial cell morphogenesis on a matrix substrate. These effects are obtained at picomolar concentrations of exogenous Tat and are promoted by the treatment of the cells with the same inflammatory cytokines stimulating expression of the receptors for Tat, the integrins α5β1 and αβ3. Thus, under specific circumstances, Tat has angiogenic properties. As Tat and its receptors are present in AIDS-KS lesions, these data may explain some of the mechanisms by which Tat can induce angiogenesis and cooperate in the development of AIDS-KS.

Kaposi sarcoma (KS) is a tumor of vascular origin, particularly frequent in human immunodeficiency virus type 1 (HIV-1)-infected homosexual men (AIDS-KS) (1–3). Typical features of KS are spindle cell growth, angiogenesis, and inflammatory cell infiltration (1–3). Recent results suggest that the HIV-1 tat gene product (Tat) cooperates with inflammatory cytokines and basic fibroblast growth factor (bFGF) in AIDS-KS pathogenesis (4–15). During HIV-1 infection of T cells, Tat is released into the extracellular medium (4, 5). Extracellular Tat stimulates adhesion and growth of spindle cells derived from AIDS-KS lesions (AIDS-KS cells) (4, 5, 7). These cells have characteristics of activated endothelial cells in situ and in culture (3, 11, 13). In fact, normal endothelial cells become responsive to the mitogenic and adhesive effects of Tat after they have been exposed to conditioned medium from activated T cells (TCM) rich in inflammatory cytokines (6, 7, 13). These cytokines [interleukin 1 (IL-1), tumor necrosis factors (TNFs), and interferon (IFN-γ)] (6, 7, 13) activate endothelial cells to express the receptors for Tat (the α5β1 and αβ3 integrins) and to acquire other phenotypic and functional features of KS spindle cells, including morphology, marker expression, and production of bFGF (6–8, 13). bFGF is highly expressed in primary lesions from AIDS-associated and classical KS (9, 11) and it mediates KS-like lesion formation induced by inoculation of KS cells or cytokine-activated endothelial cells in nude mice (10, 11, 13). Further, the same inflammatory cytokines present in TCM are increased in HIV-1-infected individuals (16–20) and in AIDS-KS lesions (ref. 21; V. Fiorelli and B.E., unpublished data). These results and other data indicating that Tat and its receptors are also present in AIDS-KS lesions (11) suggest that multiple factors cooperate in the induction and progression of KS in HIV-1-infected individuals. In particular, Tat may represent the factor increasing the frequency and aggressiveness of AIDS-KS as compared to the milder classical form of the disease in which only bFGF is present (11). In fact, in vivo studies indicate that Tat synergizes with bFGF in inducing angiogenesis and KS-like lesion formation in nude mice (11) and that, in the presence of heparin, Tat induces angiogenesis (12). This and the fact that 30–40% of tat transgenic male mice develop dermal lesions characterized by angiogenesis (14, 15) suggest that Tat may have angiogenic properties. Angiogenesis requires endothelial cells to migrate, to degrade the extracellular matrix (invasion), to proliferate, and to differentiate into capillaries (22, 23). Our results indicate that picomolar concentrations of extracellular Tat stimulate not only the growth but also the migration of AIDS-KS and cytokine-activated vascular cells, induce these cells to invade the basement membrane, and promote endothelial cell morphogenesis.

MATERIALS AND METHODS

Cell Cultures and Preparation of TCM. AIDS-KS cells (AIDS-KS2; refs. 3 and 4), human umbilical vein endothelial (H-UVE) cells, and human abdominal aorta smooth muscle (AA-SM) cells were cultured as described (4, 6, 8, 24, 25). Murine brain microvascular cells (26) were cultured in Dulbecco’s minimal essential medium (DMEM) with 10% fetal calf serum (FCS), penicillin/streptomycin, 1× MEM/vitamin, and sodium pyruvate. Immortalized EcoRy926 endothelial cells (27) were cultured in DMEM with 10% FCS with penicillin/streptomycin. MCF 10 human breast cells were cultured as described (28).

AA-SM and H-UVE cells were cultured in the presence or absence of TCM from phytohemagglutinin-activated peripheral blood leukocytes or from human T-cell leukemia/lymphotropic virus type II-infected CD4+ T cells (4, 6–8, 24, 25) that contain similar concentrations of the same cytokines (average concentration expressed in ng/ml): IL-1α, 0.5; IL-1β, 3.5; IL-2, 0.3; IL-6, 35; TNFα, 0.2; TNFβ, 0.05; granulocyte monocyte colony-stimulating factor (GM-CSF), 0.4; and IFN-γ, 0.15 (6, 7, 13).

Abbreviations: KS, Kaposi sarcoma; HIV-1, human immunodeficiency virus type 1; TCM, conditioned medium from activated T cells; H-UVE, human umbilical vein endothelial; AA-SM, abdominal aortic smooth muscle; IL-1, interleukin 1; TNF, tumor necrosis factor; IFN-γ, interferon γ; BSA, bovine serum albumin; TNFα and TNFβ, acidic and basic fibroblast growth factor; ECGS, endothelial cell growth supplement; SFM, serum-free medium.

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Reagents. Lyophilized HIV-1 Tat protein was resuspended in degassed buffer [phosphate-buffered saline (PBS)/0.1% bovine serum albumin (BSA)] and tested for activity as described (5-7, 11). Acidic FGF (aFGF) and bFGF were from R & D Systems and Boehringer Mannheim; endothelial cell growth supplement (ECGS) was from Collaborative Research; and heparin was from Sigma. Affinity-purified anti-Tat or anti-bFGF rabbit polyclonal antibodies were raised against recombinant proteins (4, 5, 8, 11). Reconstituted CM consists of the same cytokines found in TCM, combined at the following concentrations (ng/ml): IL-1α, 0.5; IL-1β, 3.5; IL-6, 35; TNFa, 0.2; TNFβ, 0.05; GM-CSF, 0.4; and IFN-γ, 0.15 (R & D Systems and Boehringer Mannheim). Matrigel (Collaborative Research) is a reconstituted basement membrane derived from a tumor cell line (29).

Migration Assay. Cell suspensions (1.5 × 10^5 in 100 μl of DMEM/0.01% BSA) were placed in the upper compartment of a Boyden chamber. Tat, bFGF (positive control), or serum-free medium (SFM) containing the protein buffer (PBS/0.1% BSA, negative control) were placed in the lower compartment. The compartments were separated by 12-μm pore polycarbonate filters coated with collagen IV (30). Duplicate assays were carried out at 37°C in 5% CO₂ for 6 hr. Cells were then removed from the upper surface of the filters, which were fixed in ethanol and stained with toluidine blue and hematoxylin/eosin. Cells migrated on the lower surface of the filter were quantified by light microscopy. Reported are the average of 10 fields. For blocking assays, Tat or bFGF was preincubated at 4°C for 8-12 hr with the anti-Tat or anti-bFGF antibodies (4, 5, 8). For the checkerboard assays, Tat or bFGF was added to the upper and lower chambers at differing concentrations.

 Invasion Assay. This assay is performed as the migration assay except that filters are also coated with 50 μl of Matrigel (25 μg per filter) (31). As controls, filters were coated with the same amount of gelatin or collagen I.

Degradation of collagen IV was monitored by mixing a solution of H-labeled human collagen type IV (NEN/DuPont) with the uncomplexed Matrigel to a final concentration of 0.1 μCi per filter (<0.3% of the total collagen IV present; 1 Ci = 37 GBq). The filters were coated as described above. After 6 hr of incubation the upper chamber contents were collected, mixed with 1 ml of scintillation liquid, and counted in a β detector to evaluate the quantity of released collagen.

In Vitro Morphogenesis Assay. Matrigel (0.5 ml; 10 mg/ml) was pipetted into 13-mm (diameter) tissue culture wells and polymerized for 30 min to 1 hr at 37°C (32, 33). H-UVE cells (5 × 10^5 per ml) were plated, followed by the addition of Tat (20 ng/ml), bFGF (20 ng/ml), ECGS (45 μg/ml), and heparin (30 μg/ml), or medium alone. In blocking assays, Tat was preincubated with the anti-Tat antibodies (1:100 or 1:250) for 12 hr (4°C) and then added to the cells (4, 5, 8). Plates (incubated at 37°C) were photographed at 3 hr, 9 hr, 20-24 hr, and after 2, 3, and 4 days. Results were quantitated by measuring the percentage of the photographic field occupied by endothelial cells by image analysis. Six to nine photographic fields from three plates were scanned for each point.

RESULTS

Tat Induces AIDS-KS Cell Migration. Picomolar concentrations of extracellular Tat promote growth of AIDS-KS cells (4-6). To determine whether Tat also has the property of inducing AIDS-KS cell migration, Tat, bFGF (positive control), or SFM containing the buffer in which these molecules were resuspended (negative control) was placed in the lower compartment of the Boyden chamber, while AIDS-KS cells were placed in the upper compartment (30). Tat induced the migration of all AIDS-KS cell strains examined and at concentrations between 0.1 and 50 ng/ml, with a peak activity at 10-20 ng/ml (Fig. 1). Cell migration was inhibited by prein-
tion assays. Tat induced little migration of untreated H-UVE cells compared to bFGF (Fig. 2A). However, after culture with TCM, Tat induced levels of H-UVE cell migration similar to those of bFGF (Fig. 2A). Tat concentrations inducing maximal effect were again between 10 and 20 ng/ml. Similar results were obtained with microvascular endothelial cells and smooth muscle cells (legend to Fig. 2A). In addition, the preincubation of H-UVE cells with the same combination of cytokines found in TCM (reconstituted CM) induced cell responsiveness to Tat in a fashion similar to TCM (legend to Fig. 2A). This is consistent with previous data indicating that this cytokine mixture is able to mimic the effects of TCM (6).

Cell migration induced by Tat was inhibited by preincubating the protein with anti-Tat antibodies but not with anti-bFGF antibodies, while anti-Tat antibodies did not inhibit bFGF-induced cell migration (Fig. 2B).

To verify whether the effect of Tat was chemokinetic (stimulation of random migration) or chemotactic (directional), checkerboard assays were performed with cytokine-activated H-UVE cells and Tat or bFGF by adding these factors to the upper and lower compartments of the Boyden chambers (Fig. 3 A and B). The results indicated that Tat and bFGF were chemokinetic for cytokine-activated endothelial cells (Fig. 3 A and B), suggesting that cytokine treatment induces random migration in the presence of chemotactic stimuli. To verify this, experiments were repeated with an immortalized endothelial cell line (Eahy926) (27) that did not require cytokine exposure to be responsive to Tat (Fig. 3C). In this case, Tat induced directional migration at concentrations even below those required by normal cells for maximum effect (Fig. 3C). These data indicated that Tat is truly chemotactic.

**Table 1.** Tat induces AIDS-KS and cytokine-treated H-UVE cell invasion

<table>
<thead>
<tr>
<th>Lower compartment</th>
<th>Invasive cells per field</th>
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<tbody>
<tr>
<td>AIDS-KS</td>
<td>H-UVE</td>
</tr>
<tr>
<td>Tat</td>
<td></td>
</tr>
<tr>
<td>10 ng/ml</td>
<td>61 ± 7</td>
</tr>
<tr>
<td>20 ng/ml</td>
<td>95 ± 1</td>
</tr>
<tr>
<td>Medium + BSA</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>bFGF, 20 ng/ml</td>
<td>27 ± 5</td>
</tr>
</tbody>
</table>

Experiments were repeated at least three times for each cell type and confirmed with AIDS-KS2 and -KS4 cells. The Eahy926 cell line reached a maximal invasion at 1 ng of Tat per ml (30 ± 3). MCF 10 is an epithelial cell line used as negative control. Experiments performed with Eahy926 cells on filters coated with collagen I, or Matrigel (25 µg per filter) gave 25 ± 1, 27 ± 1, and 12 ± 3 invasive cells per field, respectively. Data are expressed as mean ± SD.

**Tat Induces AIDS-KS and Cytokine-Activated Endothelial Cells To Degradate and Traverse the Basement Membrane.** To form new blood vessels the migrating endothelial cells must break and traverse their own basement membrane ("invasion") (22, 23, 31). This event is studied with the Boyden chamber and by coating the filters with Matrigel, which prevents migration of noninvasive cells (31). Tat promoted the invasion of AIDS-KS and cytokine-activated endothelial cells at the same concentrations inducing maximal migration (10-20 ng/ml) (Table 1). Similar effects were observed with the Eahy926 cell line, which responded at Tat concentrations even lower (1 ng/ml) (Table 1). However, Tat had no effect on the migration of control MCF 10 epithelial cells (Table 1). Gelatin or type I collagen, used as controls, did not form a barrier to endothelial cell migration (legend to Table 1). In addition, cell invasion to Tat was associated with degradation and release of radiolabeled collagen IV, suggesting that Tat activates type IV collagenase activity (Table 2).

**Table 2.** Degradation of radiolabeled collagen IV by Eahy926 cells in response to Tat (20 ng/ml)

<table>
<thead>
<tr>
<th>Upper compartment</th>
<th>Lower compartment</th>
<th>Radioactivity, cpm per well</th>
</tr>
</thead>
<tbody>
<tr>
<td>No cells</td>
<td>Tat</td>
<td>3836 ± 470</td>
</tr>
<tr>
<td>Eahy926</td>
<td>SFM</td>
<td>5280 ± 138</td>
</tr>
<tr>
<td>Eahy926</td>
<td>Tat</td>
<td>9928 ± 1524</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD.
dition, endothelial cells showed increased morphogenesis after treatment with inflammatory cytokines or when ECGS or bFGF and Tat were added together to the cells (data not shown), as shown previously for endothelial cell growth (6). Similar to ECGS, Tat also maintained the differentiation of the capillary-like network after adding fresh Tat or ECGS to

![Image of network formation](image)

**Fig. 4.** Tat induces H-UVE cells to form tube-like structures (in vitro morphogenesis) and maintains the tube network. (A–C) Tat induces in vitro morphogenesis (4, 3 hr; B, 9 hr; and C, 20 hr after cell seeding). 1. Medium without growth supplements; 2, Tat (20 ng/ml); 3, Tat (20 ng/ml) previously incubated with anti-Tat antibodies (dilution 1:100 or 1:250); and 4, ECGS (45 µg/ml) and heparin (30 µg/ml). (A and B, ×2.5; C, ×2.5 and ×6.) (D) Maintenance of the tube network by Tat 44 hr after cell seeding. (×6.) 1, Medium without growth supplements; 2, addition of Tat (20 ng/ml) a second time; 3, addition of ECGS (45 µg/ml) and heparin (30 µg/ml) a second time. Experiments were repeated four times.

Table 3. Percentage of the photographic field occupied by the endothelial cell network (in vitro morphogenesis) after addition of Tat

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Surface, %</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>20 hr</td>
<td>44 hr</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.1 ± 1.4</td>
<td>3.2 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>Tat, 20 ng/ml</td>
<td>11.0 ± 2.7</td>
<td>11.8 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>+ anti-Tat 1:250</td>
<td>5.2 ± 1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECGS</td>
<td>11.1 ± 2.6</td>
<td>10.5 ± 0.8</td>
<td></td>
</tr>
</tbody>
</table>

Six to nine photographic fields from three different plates were scanned by image analysis for each point at 20 and 44 hr after cell plating. Quantitative differences were also seen at 3 hr and were already maximum at 9 hr (data not shown). Four days after plating, controls had retracted and occupied only one-fifth of the area occupied by Tat-treated or ECGS-treated cells. Data are expressed as mean ± SD.

24-hr-old cell cultures (Fig. 4D and Table 3). In the absence of Tat or ECGS the network underwent disruption from about 40 to 48 hr after cell plating (Fig. 1D). On the contrary, in the presence of Tat or ECGS the tube network was still present at 40–48 hr (Fig. 4D2 and D3 and Table 3) and was only partially degenerating after 4 days (17.5% of the field occupied by cells with Tat versus 3.4%). Thus, Tat induces and maintains the differentiation of endothelial cells into tube-like structures.

**DISCUSSION**

The data reported here indicate that picomolar concentrations of extracellular Tat induce the migration of AIDS-KS and normal vascular cells and stimulate these cell types to acquire invasive properties and to proliferate. As for the cell growth-promoting effect of Tat (6, 13), normal vascular cells require activation with inflammatory cytokines in order to become responsive to Tat. In particular, the effect of TCM was reproduced by adding together recombinant cytokines at the same concentrations as found in TCM, indicating that they are responsible for the induction of the cell responsiveness to Tat. These are the same cytokines that are increased in HIV-1-infected individuals (16–20) or in KS tissues (ref. 21; V. Fiorelli and B.E., unpublished data). The effect of Tat or bFGF on cytokine-activated cells appears to be mostly chemokinetic. However, an immortalized endothelial cell line, EAhy926 (27), which does not require exposure to inflammatory cytokines, showed a true chemotactic response to Tat.

Tat also induced H-UVE cells to differentiate into a network of capillary-like structures and maintained this network. Although different types of stimuli may induce endothelial cells to organize into capillary-like structures and Matrigel itself is known to induce cell differentiation (23, 32, 33), this morphogenesis was limited in the absence of Tat or ECGS (Fig. 4). Taken together with the migration, invasion, and growth-promoting effects of Tat on vascular cells, these data indicate that Tat has angiogenic properties and may explain the mechanisms by which Tat can induce or contribute to angiogenesis in KS and in in vivo model systems (11, 12, 14, 15).

The mechanism(s) of the effects of extracellular Tat on vascular cells is only partially understood. Tat is a transcriptional transactivator of HIV-1 gene expression and also activates the expression of cytokines (i.e., TNF) (34). However, gene activation by extracellular Tat occurs by nanomolar concentrations of the protein (5, 34, 35). The concentrations of Tat promoting vascular effects (picomolar or 0.1–50 ng/ml) do not induce the production of TNF or other cytokines (IL-1α, IL-1β, IL-6) in AIDS-KS cells nor activate HIV-1 gene expression (refs. 5 and 34; B.E., unpublished data). In addition, we found no detectable variation of the mRNA expression for some well-known angiogenic cytokines (including bFGF and bFGF or inflammatory cytokines in endothelial cells exposed to these Tat concentrations (B.E., unpublished observations).
Recent results indicate that Tat induces the attachment of AIDS-KS and normal vascular cells to culture plates, mimicking the effects of adhesion molecules of the extracellular matrix (7, 11). Similar to these proteins, cell adhesion of Tat is due to the RGD sequence present at the carboxyl terminus of the protein, although the basic region of Tat increases this effect (7). The cell migration property of Tat might also be mediated by this region since the RGD sequence is also involved in cell migration (36). This sequence is recognized by integrin receptors (37). In particular, for AIDS-KS and normal vascular cells, the adhesion effect of Tat is mediated by the RGD-recognizing integrins αvβ3 and αvβ5 (7). As these receptors are increased by cell exposure in inflammatory cytokines (7, 13, 38, 39) and all properties of Tat on vascular cells [growth (6, 13), adhesion (7), migration, invasion, and tube formation] are augmented by the same cytokines, it is conceivable that integrins are at least partially involved in the angiogenic properties of Tat, as indicated by recent studies with Tat (ref. 11; G.B. and B.E., unpublished data) or in other systems (40). In addition, integrins are able to mediate cell growth, adhesion, and migration (36, 41, 42) and are essential for angiogenesis (40). The induction of cell invasion by Tat is associated with collagen IV degradation (Table 2) and with activation of type IV collagenase mRNA expression encoding the 72-kDa protein (11). This suggests that binding of Tat to integrins may also be capable of activating the genes involved in the enzymatic degradation of the basement membrane as found in other systems (43, 44).

Recent studies indicate that Tat may represent one major pathogenetic link between HIV-1 and KS (4–7, 11–15), while another may be the increase in production of inflammatory cytokines (6, 7, 13, 16–21), which triggers bFGF expression and release (13, 45). bFGF and Tat are present in AIDS-KS lesions and Tat receptors (αvβ3 and αvβ5) are highly expressed by vessels and spindle cells (9, 11). In vivo Tat synergizes with bFGF in inducing angiogenesis and KS-like lesion formation in nude mice (11) and, in the presence of heparin, Tat can induce angiogenesis (12). In addition, Tat transgenic mice develop KS-like lesions (14, 15). In this study we have shown that Tat has angiogenic properties and increases AIDS-KS cell invasion. These data may explain the mechanisms by which Tat may enhance the frequency and aggressiveness of KS in HIV-1-infected individuals.

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