The Tat protein of human immunodeficiency virus type 1, a growth factor for AIDS Kaposi sarcoma and cytokine-activated vascular cells, induces adhesion of the same cell types by using integrin receptors recognizing the RGD amino acid sequence

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ABSTRACT Spindle-shaped cells of vascular origin are the probable tumor cells of Kaposi sarcoma (KS). These cells, derived from patients with KS and AIDS, proliferate in response to extracellular Tat protein of human immunodeficiency virus type 1. Normal vascular cells, believed to be the progenitors of AIDS-KS cells, acquire spindle morphology and become responsive to the mitogenic effect of Tat after culture with inflammatory cytokines. Such cytokines are increased in human immunodeficiency virus type 1-infected people, suggesting that immune stimulation (rather than immune deficiency) is a component of AIDS-KS pathogenesis. Here we show that (i) Tat promotes adhesion of AIDS-KS and normal vascular cells; (ii) adhesion of normal vascular cells to Tat is induced by exposure of the cells to the same cytokines; (iii) adhesion is associated with the amino acid sequence RGD of Tat through a specific interaction with the integrin receptors $\alpha_\beta_1$ and $\alpha_\beta_2$, although it is augmented by the basic region; and (iv) the expression of both integrins is increased by the same cytokines that promote these cells to acquire spindle morphology and become responsive to the adhesion and growth effects of Tat. The results also suggest that RGD-recognizing integrins mediate the vascular cell-growth-promoting effect of Tat.

Human immunodeficiency virus type 1 (HIV-1) (1, 2), the etiologic agent of AIDS (3), contains the tat gene, which encodes an early trans-activator protein (Tat) necessary for virus replication (4, 5). We suggested that Tat and inflammatory cytokines are key pathogenetic links between HIV-1 infection and the high frequency of Kaposi sarcoma (KS) development (for review, see ref. 6). KS is an angioproliferative disease frequently associated with HIV-1 infection (7) and characterized by proliferating spindle-shaped cells (KS cells) mixed with endothelial and inflammatory cells (6).

During HIV-1 acute infection of T cells, Tat is released into the extracellular fluid (8, 9), promoting growth of spindle cells derived from AIDS-KS lesions (8, 9). Normal vascular cells, both endothelial and smooth muscle cells, probable precursors of AIDS-KS cells (10, 11), acquire spindle morphology and become responsive to the mitogenic effect of Tat after exposure to cytokines present in conditioned medium (CM) from activated immune cells (12). When we tested these cytokines we found that tumor necrosis factor, interleukin 1 (IL-1), and gamma interferon produce the same effects as the CM (ref. 12; and V. Fiorelli, unpublished work). Because these cytokines are increased in HIV-1-infected individuals (13–15) and because previous results have shown that tat-transgenic mice develop KS-like lesions (16), we hypothesized that cytokines and Tat cooperate in KS development (12).

Tat has been shown to induce adhesion of human lymphoid and rat skeletal muscle cells when immobilized on culture plates through the amino acid sequence RGD present in the product of the second exon of rat (17). Others have suggested that the basic region of Tat, present in the product of the first exon of the gene, mediates, at least in part, cell adhesion to Tat (18). However, these studies have not been done with cytokine-activated vascular cells. The finding that cytokines induce endothelial and smooth muscle cells to proliferate with Tat (12) suggests that inducible receptor(s) mediates the cell-growth-promoting effect of the protein. Similarly, cell adhesion involves a specific interaction with cell-surface receptors (19). Adhesion molecules of the extracellular matrix (ECM), contain the RGD sequence, which represents the major cell-attachment domain recognized by integrin receptors (20, 21). Here we show that Tat induces adhesion of vascular cells of the type that constitute the KS lesion and that the adhesion is mediated by the RGD sequence of Tat, although the basic region increases the cell adhesion effect. The expression of RGD-binding integrins and Tat-cell adhesion are induced by exposure of the cells to the same cytokines promoting growth responsiveness to Tat.

MATERIALS AND METHODS

Cell Cultures and Preparation of CM from Activated T Cells. AIDS-KS 3, KS 4, KS 6, and KS 8 cells (22), smooth muscle cells from human aorta (SM cells), and endothelial cells from human umbilical vein (H-UVE cells) were cultured as described (12, 22, 23). CM from phytobemagglutinin-stimulated and enriched T cells or CM from human T-lymphotrophic virus type II-transformed CD4+ T cells, were used to activate normal vascular cells (12). Each CM contains a combination of inflammatory cytokines: IL-1 alpha at 0.5 ng/ml, IL-1 beta at 3.5 ng/ml, tumor necrosis factor alpha at 0.2 ng/ml, tumor necrosis factor beta at 0.05 ng/ml, gamma-interferon at 0.15 ng/ml (12).

Tat, Other HIV-1 Proteins, and Tat Peptides. Recombinant Tat protein (from HIV-1 IIIG isolate), expressed and purified as described (9), was tested for biological activity by cell-growth assays and rescue of Tat-defective HIV-1 proviruses (9). Recombinant purified HIV-1 Rev protein was from Paul Wingfield (National Institutes of Health). Recombinant purified HIV-1 p24 protein and the HIV-1 peptides p15, Tat(6–14), Tat(11–24), Tat(36–50), Tat(46–60), Tat(56–70), Tat(65–80), and Tat(72–86) were from American Biotechnologies (Cambridge, MA). Tat(32–72) and Tat(65–85) pep-

Abbreviations: KS, Kaposi sarcoma; HIV-1, human immunodeficiency virus type 1; CM, conditioned medium; ECM, extracellular matrix; FN, fibronectin; VN, vitronectin; H-UVE cells, human umbilical-vein endothelial cells; SM cells, aortic smooth muscle cells; IL-1, -2, and -6, interleukin 1, 2, and 6, respectively; BSA, bovine serum albumin.

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tides were from W. C. Saxinger (our laboratory). Human fibronectin (FN) and vitronectin (VN) were from Boehringer Mannheim and Sigma, respectively. Linear and cyclic FN peptides GRGDS and the mutated peptides GKGESP were from Research Genetics (Huntsville, AL) and Peptide Technologies (Washington, DC). Proteins and peptides were resuspended in degassed buffer [phosphate-buffered saline (PBS)/0.1% bovine serum albumin (BSA)].

**Antibodies.** The affinity-purified monoclonal antibodies CDw49e (recognizing the α chain of one of the FN receptors), CD29 (recognizing the β chain of one of the FN receptors), CD51 (recognizing the α chain of one of the VN receptors), CD61 (recognizing the β chain of one of the VN receptors), and CDw49b (recognizing the α2 chain of one of the collage receptors) (19, 24) were from AMAC (Westbrook, ME) and Coulter Immunology. The antibodies QBEND/10, directed against CD34, expressed by both endothelial and AIDS-KS cells (25), were from AMAC. Anti-smooth muscle actin antibodies (clone 1A4) were from Sigma; anti-factor VIII and anti-HLA antibodies were from Dakopatts (Carpinteria, CA); anti-CD4 antibodies were from Pierce.

**Immunohistochemistry.** Cells were seeded (5 x 10⁴ per well) in 8-well chamber slides (Nunc), incubated for 48 h at 37°C, washed in PBS, fixed in cold acetone, air-dried, and incubated overnight with antibodies (1:100) at 4°C. Immunostaining was done by both double-indirect immunoperoxidase and alkaline phosphatase anti-alkaline phosphatase methods (9).

**Cell Adhesion Assays.** Ninety-six-well flat-bottom polystyrene plates (Linbro 76-203; Flow Laboratories) were coated overnight at 4°C with Tat, Rev, p15, p24, FN, VN, Tat-(32-76), Tat-(65-85), or BSA. Plates were then rinsed and incubated for 3 h at room temperature with PBS/15% BSA. One hundred microliters of the cell suspension (5 x 10⁴ per ml in serum-free medium) was added to the wells (in quadruplicate), and plates were incubated for 1 h at 37°C in a 5% CO₂ atmosphere. For some experiments, plates were incubated at 4°C. Plates were extensively washed with PBS, and adherent cells were fixed, stained, and quantitated as described (17). The results were expressed relative to the control buffer given a unit value.

**Competition/Blocking of Cell Adhesion.** Cells were resuspended at 5 x 10⁴ cells per ml in cold binding buffer (0.05% BSA/RPMI 1640) and incubated on a rotator (90 min, 4°C) with serial dilutions (μg/ml) of the competitor proteins or peptides, or with antibodies and assayed for adhesion to immobilized proteins. The number of adherent cells was expressed as the percentage of cell adhesion compared with cells preincubated with the buffer (assumed as 100%).

**RESULTS**

**Tat Induces the Adhesion of AIDS-KS Cells.** We have shown that Tat stimulates the growth of AIDS-KS cells (8, 9, 12), and others have shown that Tat induces the adhesion of lymphoid and skeletal muscle cells (17, 18). To evaluate whether Tat was capable of a specific cell-surface interaction with AIDS-KS cells, cell adhesion experiments were done with recombinant purified Tat protein. In these assays, BSA and the HIV-1 proteins p15, p24, and Rev were used as negative controls. FN and VN were used as positive controls because they contain the RGD sequence and their receptors are expressed in mesenchymal- and sarcoma-derived cells (19, 26). Tat induced the adhesion of AIDS-KS cells in a dose-dependent manner, mimicking the effects of FN and VN (Fig. 1). To the contrary, no cell adhesion was seen with BSA or HIV-1 p15, p24, and Rev at any of the concentrations used or with oxidized Tat (Fig. 1 and data not shown), which is also inactive in inducing cell growth and HIV-1 gene expression.

**Normal Vascular Cells Adhere to Immobilized Tat; This Effect Is Induced by Cell Exposure to Inflammatory Cytokines.** When normal vascular cells are cultured under standard conditions, they show little or no growth response to Tat (8, 12), but after exposure to inflammatory cytokines from CM of activated T cells, they become responsive (12). This resembles IL-2 and normal T cells. IL-2 has no growth effect until cells are activated and express IL-2 receptors (27). Similarly, without prior exposure to inflammatory cytokines, immobilized Tat promoted attachment of SM cells but had little or no effect on the adhesion of H-UVE cells (Fig. 2). After exposure to cytokines, SM cell adhesion to the protein was increased, and H-UVE cells became adherent to Tat (Fig. 2). For both cell types, adhesion reached values similar to AIDS-KS cells (Fig. 1). These cytokines also increased vascular cell adhesion to FN and VN (Fig. 2), suggesting that they increase the expression and/or the affinity of the receptors for both Tat and ECM proteins.

**The Vascular Cell Adhesion to Immobilized Tat Is Specifically Mediated by the RGD Region of the Protein.** Previous studies concluded that Tat-induced cell adhesion is mediated by the RGD sequence (17) and/or by the basic region of Tat.

**RESULTS**

**Tat Induces the Adhesion of AIDS-KS Cells.** We have shown that Tat stimulates the growth of AIDS-KS cells (8, 9, 12), and others have shown that Tat induces the adhesion of lymphoid and skeletal muscle cells (17, 18). To evaluate whether Tat was capable of a specific cell-surface interaction with AIDS-KS cells, cell adhesion experiments were done with recombinant purified Tat protein. In these assays, BSA and the HIV-1 proteins p15, p24, and Rev were used as negative controls. FN and VN were used as positive controls because they contain the RGD sequence and their receptors are expressed in mesenchymal- and sarcoma-derived cells (19, 26). Tat induced the adhesion of AIDS-KS cells in a dose-dependent manner, mimicking the effects of FN and VN (Fig. 1). To the contrary, no cell adhesion was seen with BSA or HIV-1 p15, p24, and Rev at any of the concentrations used or with oxidized Tat (Fig. 1 and data not shown), which is also inactive in inducing cell growth and HIV-1 gene expression.
To determine the Tat domain mediating the attachment of AIDS-KS cells, adhesion assays were done with two Tat peptides spanning the products of tat exon 1 ([32–72], containing the basic region) and exon 2 ([65–85], containing the RGD region). Tat and FN were used as positive controls. Both peptides induced a 2- to 3-fold increase of AIDS-KS cell adhesion (data not shown), suggesting that both portions of Tat are involved in cell attachment. When the same experiments were done at 4°C, the adhesion to Tat-(32–72) peptide was increased up to 6-fold, whereas the adhesion to Tat, FN, and Tat-(65–85) was reduced or lost. This result suggests that the portion of Tat containing the RGD sequence mediates cell adhesion in an energy-dependent manner, probably receptor-mediated, whereas the basic region of Tat induces cell attachment in a non-energy-dependent fashion. The basic region of Tat, in fact, is highly positively charged and likely to interact with the negatively charged cell membranes (ref. 28 and W. C. Saxinger, personal communication). To verify this, competition experiments were done by preincubating AIDS-KS cells with serial dilutions of the peptides Tat-(6–14), Tat-(11–24), Tat-(36–50), Tat-(46–60), Tat-(56–70), Tat-(32–72), Tat-(65–85) and Tat-(72–86) and by seeding the cells on plates coated with Tat (Fig. 3A). Only the peptides containing the RGD sequence [Tat-(65–80), Tat-(65–85), Tat-(72–86)] inhibited the adhesion of AIDS-KS cells to Tat. With these peptides, competition was seen at concentrations as low as 10 μg/ml and increased in a dose-dependent fashion (Fig. 3A and data not shown). In contrast, cell adhesion was not inhibited by any of the other Tat peptides, including those containing the basic region (residues 49–57) at any concentrations used (Fig. 3A). The long peptide Tat-(32–72) inhibited only 20% of AIDS-KS cell adhesion to Tat and did not inhibit in a dose-dependent fashion (data not shown). As shorter peptides (13–14 aa) spanning this region (sequences 36–50, 46–60, and 57–70) did not inhibit Tat–cell adhesion (Fig. 3A), these results suggest that the effect of Tat-(32–72) is nonspecific and perhaps due to the length of this peptide. This result agrees with a previous study showing that a Tat-(38–58) peptide, but not its fragments (sequences 38–47 and 48–58), interacts with the cell membrane (28). In addition, HIV-1 Rev, which contains a basic region functionally interchangeable with that of Tat but not an RGD region (30), did not induce AIDS-KS cell adhesion (data not shown). However, when cells were preincubated with both Tat-(46–60) and Tat-(65–85) peptides, the adhesion to Tat was decreased to a greater extent as compared with Tat-(65–85) peptide alone (data not shown). These data indicate that vascular cell adhesion to Tat is specifically mediated by the portion of the protein containing the RGD region and suggest that the basic region increases the cell-attachment effect.

To confirm that the RGD sequence itself is the Tat domain responsible for cell adhesion, AIDS-KS cells and cytokine-treated H-UVE and SM cells were preincubated with the peptides GRGDSP or GRGDSP and with the mutated peptide GGKESP (20) (Fig. 3B). The effect of these peptides on the cell adhesion to FN was monitored as control. Cell adhesion to both Tat and FN was inhibited only by the RGD peptide but not by the mutated peptide (Fig. 3B). These results indicated that, as for ECM proteins (19, 20, 24), the RGD sequence is the major domain mediating AIDS-KS and vascular cell attachment to Tat.

**AIDS-KS Cells Express High Levels of FN and VN Receptors, and Expression of These RGD-Recognizing Integrins Is Increased on Vascular Cells by Inflammatory Cytokines.** As the RGD sequence of ECM molecules is recognized by integrin receptors (19, 21, 24), the previous data suggested that receptors recognizing Tat through this sequence are responsible for the cell attachment to the protein. As the RGD-recognizing integrins α5β1 and α6β1 are widely distributed on mesenchymal cells (26), their expression was analyzed in AIDS-KS and vascular cells cultured under the same conditions previously used to induce cell growth and attachment to Tat (12) (Table 1). Expressions of HLA I, SM α actin, QBEND/10, and factor VIII were monitored as controls. AIDS-KS cells expressed high levels of both α5β1 and α6β1, whereas the expression of these integrins was lower in vascular cells cultured without cytokines. However, after culture of the normal cells with inflammatory cytokines, the level of integrin expression reached that of AIDS-KS cells (Table 1). The data indicate that the same cytokines inducing vascular cell growth and adhesion to Tat also augment the expression of RGD-recognizing integrins on these cell types. As these cytokines also increase adhesion to FN and VN, the results suggested that the effects of Tat on vascular cells are mediated by the receptors for these proteins.
Table 1. Expression of the α and β chains of FN and VN receptors by AIDS-KS cells and H-UVE or SM cells before and after treatment with inflammatory cytokines.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specifity</th>
<th>SM</th>
<th>H-UVE</th>
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<tbody>
<tr>
<td>CDw49e</td>
<td>α chain</td>
<td>50 ± 0</td>
<td>15 ± 5</td>
</tr>
<tr>
<td>CD29</td>
<td>β chain</td>
<td>50 ± 0</td>
<td>32 ± 15</td>
</tr>
<tr>
<td>CD51</td>
<td>α chain</td>
<td>50 ± 15</td>
<td>25 ± 0</td>
</tr>
<tr>
<td>CD61</td>
<td>β chain</td>
<td>40 ± 0</td>
<td>25 ± 11</td>
</tr>
<tr>
<td>HLA-I</td>
<td>MHC</td>
<td>60 ± 11</td>
<td>60 ± 0</td>
</tr>
<tr>
<td>F.VIII</td>
<td>Endo-theil</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>α-Actin</td>
<td>Smooth muscle</td>
<td>42 ± 5</td>
<td>85 ± 10</td>
</tr>
<tr>
<td>QBEND/10</td>
<td>CD34</td>
<td>33 ± 5</td>
<td>Neg</td>
</tr>
<tr>
<td>NT</td>
<td>55 ± 8</td>
<td>NT</td>
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Results were evaluated in five fields per slide on the colorimetric reaction seen with the positive controls: factor VIII (for H-UVE cells), α-actin (for SM and AIDS-KS cells), QBEND/10 (for H-UVE and AIDS-KS cells), and HLA-I (for all cell types) and expressed as the percentage of positive cells. Results are from four experiments done by immunohistochemistry on cells grown with or without inflammatory cytokines. SDs of the mean are as indicated.

Fig. 4. Preincubation of AIDS-KS cells with Tat inhibits their attachment to immobilized FN or VN. AIDS-KS cells were preincubated with Tat (t) or p24 (a) at 5, 50, or 100 μg/ml or with buffer (c) and seeded on plates coated with FN or VN (10 μg/ml). The results are from three experiments. Preincubation of the cells with the buffer or with p24 did not inhibit their adhesion to immobilized FN and VN. Preincubation of H-UVE and SM cells with Tat at 5 μg/ml, but not with p24, inhibited their adhesion to immobilized FN (40% and 43%) and VN (45% and 30%, respectively).

Fig. 5. The attachment of AIDS-KS, SM, and H-UVE cells to immobilized Tat is specifically inhibited by antibodies directed against the RGD-binding receptors αβ1 and αβ2. AIDS-KS cells and cytokine-treated H-UVE and SM cells were preincubated with affinity-purified monoclonal antibodies (4 μg/ml) directed against α (CDw49e) and β (CD29) chains of the αβ1 receptor (anti-αβ1) or α (CD51) and β (CD61) chains of the αβ2 receptor (anti-αβ2). (A) Antibodies against the αβ1 receptor (t) and the αβ2 receptor (q) were used separately. (B) AIDS-KS cells were preincubated with anti-αβ1 and anti-αβ2 antibodies combined (q). Antibodies directed against the α chain of the collagen receptor αβ1, CD34 antigen (QBEND/10), CD4 antigen, or factor VIII, were used as the negative control (t), c, buffer. After incubation, cells were seeded on plates coated with Tat or FN (10 μg/ml). Results are from four experiments.

DISCUSSION

Previous results suggested that HIV-1 Tat and inflammatory cytokines cooperate in the development of AIDS KS (8, 12-16, 32-34). The induction of vascular cell growth responsiveness to Tat by inflammatory cytokines (12) suggested the presence of inducible Tat receptor(s). The finding that Tat induces AIDS-KS cell adhesion (Fig. 1) confirms the hypothesis of interaction of Tat with receptors expressed by AIDS-KS cells. Experiments with normal vascular cells indicated that the attachment to Tat is induced or increased by exposure of the cells to the same cytokines promoting the Tat-cell-growth response (12) (Fig. 2). The vascular cell adhesion to Tat is mediated by the RGD sequence of the molecule (Fig. 3). Although the basic region of Tat does not inhibit vascular cell adhesion to the protein (Fig. 3A), this region may also contribute to the total cell-attachment effect of Tat. This hypothesis agrees with data indicating that this region of Tat can interact with neural cells (35) and can participate in the
adhesion of lymphocytic cell lines to Tat (18). Recent data indicate that electrostatic interactions of the basic region of Tat with the RNA bulge structure of the Tat-responsive element present in all HIV-1 transcripts increase the RNA-binding and trans-activation ability of Tat through conformational changes that raise the binding affinity of its sequence-specific interactions (36). By analogy, this could also be the role played by the basic region of Tat in cell adhesion.

The RGD sequence is one of the major cell-attachment domains of ECM proteins and is recognized by integrin receptors (20, 21). Among these, $\alpha_{5}\beta_1$ and $\alpha_\beta_5$ (the FN and the VN receptors) are widely distributed on cells of mesenchymal origin and sarcoma-derived cells (26) and are upregulated by inflammatory cytokines (37, 38). When normal vascular cells are cultured with these cytokines, the expression of both $\alpha_5\beta_1$ and $\alpha_\beta_5$ increases to levels detected in AIDS-KS cells (Table I). This result is associated with an increased vascular cell attachment to both Tat and FN or VN (Fig. 2), suggesting that the FN and VN receptors mediate the Tat-induced cell attachment. Tat, in fact, inhibits cell adhesion to either FN or VN (Fig. 4), and antibodies directed against $\alpha_5\beta_1$ or $\alpha_\beta_5$ reduce the cell adhesion to Tat (Fig. 5A). When the antibodies against both receptors are combined, cell adhesion to Tat is further decreased, as for FN (Fig. 5B). These results indicate that both $\alpha_5\beta_1$ and $\alpha_\beta_5$ mediate the Tat-induced cell attachment and agree with previous studies showing that these receptors are functionally interchangeable (39). Similar to FN (19), several RGD-recognition integrins may participate in the effects of Tat on these or, possibly, other cell types. Receptor expression and cell-attachment effects may vary with different cell culture conditions. The fact that SM cells (but not H-UVE cells) attached to Tat before any exposure to cytokines suggests a differential receptor expression and/or affinity by these two cell types when cultured in the absence of any cell-growth inducer—i.e., fibroblast growth factors. However, after cytokine treatment, both cell types adhered to Tat, reaching values similar to AIDS-KS cells.

Because the same cytokines augment expression of RGD-recognition integrins, increase cell attachment to Tat, and induce vascular cells to become responsive to the mitogenic effect of Tat and because FN induces both cell growth and cell attachment through the $\alpha_5\beta_1$ integrin (40–42), we propose that, like cell attachment, the growth-promoting effect of Tat on vascular cells is through the integrins.

Note Added in Proof. Ref. 43 published while our manuscript was in press) shows that a novel integrin ($\alpha_{5}\beta_0$) binds to the basic region of Tat.

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