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HIV-1 Tat Protein Stimulates In Vivo Vascular Permeability and Lymphomononuclear Cell Recruitment

Marco Arrese,* Chiara Ferrandi,* Luca Primo,* Giovanni Camussi, † and Federico Bussolino*

HIV-1 Tat protein released by infected cells is a chemotactic molecule for leukocytes and induces a proinflammatory program in endothelial cells (EC) by activating vascular endothelial growth factor (VEGF) receptors expressed on both cell types. Its potential role in causing vascular permeability and leukocyte recruitment was studied in vivo following its s.c. injection in mice. Tat caused a dose-dependent early (15 min) and late (6 h) wave of permeability that were inhibited by a neutralizing Ab anti-VEGF receptor type 2. Tissue infiltration of lymphomononuclear cells, mainly monocytes (76%), was evident at 6 h and persisted up to 24 h. WEB2170, a platelet activating factor (PAF) receptor antagonist, reduced the early leakage by 70–80%, but only slightly inhibited the late wave and cell recruitment. In vitro, Tat induced a dose-dependent flux of albumin through the EC monolayer that was inhibited by Ab anti-vascular VEGF receptor type 2 and WEB2170, and PAF synthesis in EC that was blocked by the Ab anti-VEGF receptor type 2. Lastly, an anti-monocyte chemotactic peptide-1 (MCP-1) Ab significantly reduced the lymphomononuclear infiltration elicited by Tat. In vitro, Tat induced a dose-dependent production of MCP-1 by EC after a 24-h stimulation. These results highlighted the role of PAF and MCP-1 as secondary mediators in the onset of lymphomononuclear cell recruitment in tissues triggered by Tat. The Journal of Immunology, 2001, 166: 1380–1388.

The host inflammatory response is a key event in early and late HIV-1 infection. Inflammatory cytokine secretion by activated immune cells favors virus replication, regulates lymphocyte traffic, and is instrumental to injury of lymphoid tissue (1–3). Similarly, leukocyte infiltration and abnormal cytokine response are typical features of HIV-1-associated tissue disorders, including tumors, opportunistic infections, and CNS degeneration (4).

In addition to the classic response of the immune system to foreign organisms, the altered host response is directly triggered by HIV-1 proteins, including envelope proteins (5–7), Nef (8), and the transactivating factor Tat, which up-regulates viral gene expression in infected cells (9) and modulates the expression of cellular genes, including those of IL-6 and TNF-α and β (10–12), known to be inductive of HIV replication (13–15). Furthermore, Tat may alter cellular behavior when released by infected cells in the microenvironment (16, 17). Tat easily enters different cell types and contributes to transactivation of the HIV-1 long-terminal repeat promoter in latently infected cells (18, 19). Alternatively, it acts as a soluble mediator acting on T and B cells (20–24) and on those of the CNS (25, 26), thus favoring the progression of AIDS and its associated brain damage. Furthermore, Tat profoundly affects functions of vascular endothelial cells (EC)3 and the monocyte/macrophage system. It is an angiogenic inducer through activation of vascular endothelial growth factor (VEGF) receptor 2 (VEGFR-2) and integrin system (27, 28) and induces a proinflammatory program characterized by the release of proteolytic enzymes (29), the up-regulation of adhesion molecules (30, 31), and vasopermeability (32). These results in the leukocyte extravasation essential for homing of infected lymphomononuclear cells into lymphoid organs and for the tissue injury typical of some features of the progression of AIDS. In monocytes, subnanomolar concentrations of Tat enhance the expression of CCR5 (33) and the release of inflammatory cytokines, TGF-β and metalloproteinase-9 (34–36), and increase chemotaxis, chemokinesis (37, 38), and adhesion to EC (39). These activities are mediated by the activation of VEGFR-1 (38, 39) or by CCR2 and CCR3 (40). Tat also recruits monocytes into tissues when injected in the lateral ventricle of the brain (41). Furthermore, macrophage stimulation by Tat results in the immunosuppressive induction of Fas ligand (42) and impairment of the natural host response by inhibition of NO synthesis (43).

EC are strategically located at the interface between the bloodstream and tissues. They are thus both producers and the targets of autacoids and chemokines that regulate leukocyte traffic and vascular permeability during tissue injury (44).

In light of these in vitro effects of Tat on lymphomononuclear cells and EC, we studied its potential role in causing vascular permeability and leukocyte recruitment in a murine model. We demonstrate that s.c. injection of Tat causes accumulation of lymphomononuclear cells preceded by increased vascular permeability. These activities appear to be mediated by the autacoid platelet-activating factor (PAF) (45–47) and the chemokine monocyte chemotactic peptide-1 (MCP-1) (48).

*Institute for Cancer Research and Treatment and Department of Genetics, Biology and Biochemistry, School of Medicine, University of Torino, Candiolo, Italy; and † Department of Medical and Surgery Sciences, School of Medicine, University of Turin, Turin, Italy

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* Address correspondence and reprint requests to Dr. Federico Bussolino, Institute for Cancer Research and Treatment, strada provinciale 142, kilometer 3.95, 10060 Candiollo, Italy. E-mail address: fbussolino@ircc.unito.it

1 Abbreviations used in this paper: EC, endothelial cells(s); Basic 4 6–8 0 , Tat peptide for aa 46–60; CysL24–51, Tat peptide covering aa 24–51 corresponding to the cysteine and core region; MBP, maltose-binding fusion protein; MCP-1, monocyte chemotactic peptide-1; PAF, platelet-activating factor; snc, murine spleen cells; Tat-MBP, Tat-MBP fusion protein; VEGF, vascular endothelial growth factor; H.end cells, EC from human umbilical cord veins and murine microvascular H.end endothelioma cells.

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Materials and Methods

[1H]Acetyl-CoA (2.2 mCi/mmol), [3H]acetate (2 Ci/mmol), and [3H]Acetyl-CoA (180 mCi/mmol) were obtained from Amersham Pharmacia Biotech (Buchs, U.K.); [125I]albumin (3 μCi/mg) was purchased from NEN (Boston, MA). Human LPS-free serum albumin was obtained from Farma Biogini (Luca, Italy); TLC plates were purchased from Merck (Darmstadt, Germany); PAF (1-O-octadecyl-2-acyl-r)-(g)-lyso-3-phosphocholine and lyso-PAF (1-O-octadecyl-2-lyso-glycerol-3-phosphocholine) were obtained from Bachem Feinkemikalien (Bubendorf, Switzerland); WEB2170 was purchased from Boehringer Ingelheim (Ingelheim, Germany). Plastic materials and products for cell culture were obtained from Falcon (Becton Dickinson, Milano, Italy) and Life Technologies (Paisley, U.K.); VEGF-A, Ag affinity-purified goat polyclonal Ab anti-mouse MCP-1, Ag affinity-purified goat polyclonal anti-mouse VEGFR-2, and mouse MCP-1 were obtained from R&D Systems (Wiesbaden-Nordenstadt, Germany); anti-mouse CD8 (53.6.72 hydridoma, Lyt2, anti-mouse CD4 (CK G 1.5 hybridoma, L3T4), and anti-mouse Mac-3 (M3784, 86, 34 hybridoma) were purchased from PharMingen (San Diego, CA); rabbit anti-rat IgG was purchased from Zymed (San Francisco, CA); and rat peroxidase-antiperoxidase was obtained from Abbot Laboratories (North Chicago, IL). Other reagents were obtained from Sigma (St. Louis, MO). LPS-free Tat, and Tat proteins and Tat peptides covering aa 24–51 corresponding to cysteine and core regions (CysL24–32, NH2-NH2 C KY C KC FCH QV FT KAL GIS YGR KCOOH) (9) was synthesized by Tecnecon (Caserta, Italy), purified by HPLC, and quality controlled by mass spectroscopy (38). Tat peptide for aa 46–60 (Basic46–60, NH2-SYG RRQ RRQ RRQ PPQ COOH) corresponding to the basic region (9) was purchased from Technecon (Caserta, Italy). Two scrambled peptides of CysL24–32 (NH2-NH2 C KY C ISY CFC VIT KAL GCQ CFF RKKCOOH) and Basic46–60 (NH2-KGKRSPQYRQPQQ-COOH) peptides were purchased from Prinl (Milan, Italy). Recombinant wild-type HIV-1 Tat, amino acid length was expressed in Escherichia coli as maltose-binding fusion protein (MBP), indicated throughout the text as Tat-MBP. MBP alone was the control in all experiments. Tat-MBP was purified to homogeneity from bacterial cell lysates by affinity chromatography on amylose resin and used as fusion protein (49). Synthetic and recombinant Tat molecules were selected experiments anti-VEGFR-2 Ab, anti-MCP-1 Ab, goat serum (20%, FCS), or the PAF receptor antagonist WEB2170 (250 ng) was added 1 h before the reaction was stopped. After extraction, labeled phospholipids comigrating with synthetic PAF on the TLC were counted in a Packard beta counter (Packard, Meriden, CT).

In vivo studies

Tat molecules and VEGF-A were s.c. injected into the lower back (right side) of BALB/c mice in PBS for different lengths of time. The negative control was injected into their left side. Thirty minutes before the end of the experiment, [125I]albumin (2 × 106 cpm/mouse) was injected i.v. Mice were then sacrificed, and a skin area of 25 mm2 around the injection point was excised and its radioactivity was counted in a beta counter. Histological skin alterations were studied by omitting the [125I]albumin injection. In selected experiments, VEGFR-2 Ab (clone 1D6), VEGF-C (1H11), and VEGF-D (1H24) were added to the manufacture’s instructions. Human PBMC and monocytes were prepared from buffy coats of healthy human donors as previously detailed (38). Murine splenic cells (spc) and peritoneal macrophages were prepared from BALB/c mice (Charles River, Calco, Italy) according to Refs. 54 and 55.

Enzyme-linked immunosorbent assay measuring the uptake of [3H]acetate into lipids comigrating with synthetic PAF (57). In this case, 3105 human EC were stimulated with medium without serum or with Ab anti-VEGFR-2 or with goat serum (1:50). Cell-associated PAF was extracted, purified by TLC, and measured by washed rabbit platelet aggregation as described (57, 58). PAF was identified from its physicochemical characteristics and sensitivity to lipases as previously described (57, 58). In some experiments PAF synthesis was also quantified by a radioimmun assay measuring the uptake of [3H]acetate into lipids comigrating with synthetic PAF (57). In this case, 3 × 105 human EC were stimulated with medium without serum or with Ab anti-VEGFR-2 or with goat serum (1:50). PAF was extracted, purified by TLC, and measured by washed rabbit platelet aggregation as described (57, 58).

FIGURE 1. Time- and dose-dependent effects of Tat-MBP on skin permeability. Abdominal BALB/c mouse skin was injected with different concentrations of Tat-MBP, MBP (30 ng/30 μl), or Tat-MBP-inactivated (30 ng/30 μl) for 15 min, 90 min, 6 h, and 24 h. Thirty minutes before the end of the incubation time, mice were i.v. injected with [125I]albumin (2 × 106 cpm/mouse). Skin (an area of 25 mm2 around the injection point) was then excised, and the corresponding radioactivity was counted in a beta counter. Results shown are the mean ± SD of five animals. ANOVA gave the following results: mice treated for 15 min, F = 32.06, mice treated for 90 min, F = 6.34; mice treated for 3 h, F = 83.34; mice treated for 6 h, F = 33.1, p < 0.05 vs MBP-injected animals by Student-Newman-Keuls test.

EC permeability was determined by calculating the ratio of [125I]albumin in the upper and lower chambers of a filter chamber assembly (pore diameter 0.2 μm, 24-mm diameter dish) (50). The upper chamber contained 200 μl BSA and 0.2 μCi [125I]albumin in 2 ml of M199 medium. The baseline permeability was determined after a 1-h incubation at 37°C in 5% CO2 (5.6 ± 0.9% of [125I]albumin added to the upper chamber was recovered in the lower chamber, mean ± SD of three experiments performed in triplicate). The baseline permeability of the membrane without EC was 61.0% (mean ± SD of three experiments performed in triplicate). Stimuli were added to the upper chamber, and incubation was continued for 1 h. Radioactivity was counted in the lower chamber, and the results are expressed as follows: Percent change albumin clearance: cpm [125I]albumin after stimulus − cpm [125I]albumin control/cpm [125I]albumin control × 100.

In some experiments EC were preincubated for 15 min with WEB2170 (5 μM), Ab anti-VEGFR-2, or goat serum (1:50).

PAF synthesis

EC were used within V passages without coating the plastic dishes with proteins, and growth medium was reseeded the day before the experiment (57). EC (6 × 105/cm2), human fibroblasts (1.2 × 105/cm2), human keratinocytes (8 × 105/cm2), murine EC line H.7 (7 × 105/cm2), spc (2 × 105/ml), murine macrophages (2 × 105/3.5-cm diameter well), and human PBMC and monocytes (2 × 106/ml) were washed twice with M199 containing 0.25% BSA and incubated in 5% CO2 for different times with Tat molecules. In some experiments cells were preincubated for 15 min at 37°C with Ab anti-VEGFR-2 or with goat serum (1:50). Cell-associated PAF was extracted, purified by TLC, and measured by washed rabbit platelet aggregation as described (57, 58). PAF was identified from its physicochemical characteristics and sensitivity to lipases as previously described (57, 58). In some experiments PAF synthesis was also quantified by a radioimmuno assay measuring the uptake of [3H]acetate into lipids comigrating with synthetic PAF (57). In this case, 3 × 105 human EC were stimulated in DMEM supplemented with 0.25% BSA. [3H]Acetate (100 μM) was added 1 h before the reaction was stopped. After extraction, labeled phospholipids comigrating with synthetic PAF on the TLC were counted in a Packard beta counter (Packard, Meriden, CT).

Assay of acetyl-CoA:lyso-PAF acetyltransferase in human EC

Cell extracts from stimulated or unstimulated EC were prepared as previously described (58). The assay was conducted for 10 min at 37°C in 0.2
ml of Tris-HCl 0.1, pH 6.8, containing 50 μg of lysate protein, 40 mM l-lysine, 100 μM acetyl CoA, and 1 μCi [3H]acetyl-CoA. Lipid purification and calculation of enzymatic activity was performed exactly as previously reported (58).

**Light microscopy and immunohistochemistry**

For microscopy examination mouse skin was fixed in 10% buffered formalin and embedded in paraffin. Sections were cut at 5 μm and stained with hematoxylin and eosin. For immunohistochemistry, samples were embedded in OCT compound (Miles Laboratories, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at −80°C. Five-micrometer cryostat sections were fixed in acetone, preincubated with rabbit serum, and subsequently incubated with rabbit anti-rat IgG and rat peroxidase-antiperoxidase. Each step was performed with optimal dilutions of rat anti-mouse CD8 (1:100), anti-mouse CD4 (1:50), and anti-mouse Mac-3 (1:50). After washes, sections were incubated with optimal dilutions of rat anti-mouse CD8 (1:50), and after 6 h animals were sacrificed. The number of infiltrating lymphomononuclear cells on a 1-mm² grid was given as cells/mm².

**MCP-1 production**

EC (6 × 10⁵/cm²), fibroblasts (1.2 × 10⁶/cm²), keratinocytes (8 × 10⁵/cm²), and monocytes (2 × 10⁶/ml) were starved overnight in M199 supplemented with 2% FCS and 3% human serum albumin. Cells were stimulated at 37°C with Tat molecules in M199 containing 10% FCS for 30 min and PAF cell associated was quantified by a biological assay on washed rabbit platelets.

Results are shown as the mean ± SD of three separate experiments.

* p < 0.001 by Student’s t test.

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**Table I. In vitro and in vivo effects of Tat₆₆ and Tat₁₀₁**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[¹²⁵I]Albumin Leakage at 15 min (cpm)¹</th>
<th>[¹²⁵I]Albumin Leakage at 6 h (cpm)²</th>
<th>Lymphomononuclear Cell Infiltration (cells/mm²)³</th>
<th>PAF (pmol/4 × 10⁵ cells)⁴</th>
<th>MCP-1 (ng/ml)⁵</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.035 ± 456</td>
<td>4.406 ± 976</td>
<td>36 ± 12</td>
<td>0.23 ± 0.08</td>
<td>2.39 ± 0.75</td>
</tr>
<tr>
<td>Tat₆₆</td>
<td>32.450 ± 4.078*</td>
<td>73.224 ± 2.287*</td>
<td>267 ± 45*</td>
<td>4.51 ± 0.78*</td>
<td>16.30 ± 2.45*</td>
</tr>
<tr>
<td>Tat₁₀₁</td>
<td>28.031 ± 2.341*</td>
<td>76.012 ± 6.341*</td>
<td>290 ± 38*</td>
<td>3.88 ± 1.12*</td>
<td>19.24 ± 3.21*</td>
</tr>
</tbody>
</table>

¹ Tat molecules (10 ng/10 μl) were injected s.c. into the lower back of BALB/c mice sacrificed after 15 min or 6 h. Thirty minutes before the end of the incubation time, [¹²⁵I]albumin (2 × 10⁶ cpm/mouse) was injected i.v. Skin (an area of 25 mm² around the injection point) was excised, and its radioactivity was counted in a beta counter. Results shown are the mean ± SD of three animals.

² Mouse skin was injected with Tat molecules (10 ng/10 μl), and after 6 h animals were sacrificed. The number of infiltrating lymphomononuclear cells on a 5-μm hematoxylin and eosin-stained section was determined by light microscopy at ×400 in four fields of a 1-mm² grid. Results shown are the mean ± SD of three animals.

³ EC (6 × 10⁶/cm²) were stimulated with Tat molecules (10 ng/ml) at 37°C for 30 min and PAF cell associated was quantified by a biological assay on washed rabbit platelets. Results are shown as the mean ± SD of three separate experiments.

⁴ EC (6 × 10⁶/cm²) were stimulated with Tat molecules at 37°C for 24 h and released MCP-1 detected by ELISA. Results shown are the mean ± SD of three separate experiments.

⁵ Results shown are the mean ± SD of six animals. ANOVA gave F = 62.50. * p < 0.05 vs irrelevant Ig by Student-Newman-Keuls test.

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**Table II. Effect of Basic 46–80 and CysL 24–51 on [¹²⁵I]albumin leakage in mouse skin**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[¹²⁵I]Albumin Leakage at 15 min (cpm)³</th>
<th>[¹²⁵I]Albumin Leakage at 6 h (cpm)⁵</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>23,421 ± 2,109*</td>
<td>45,210 ± 6,021*</td>
</tr>
<tr>
<td>Scrambled Basic</td>
<td>3,421 ± 1,231</td>
<td>4,321 ± 1,045</td>
</tr>
<tr>
<td>CysL 24–51</td>
<td>5,634 ± 3,288*</td>
<td>3,876 ± 2,321*</td>
</tr>
<tr>
<td>Scrambled CysL</td>
<td>3,256 ± 1,421</td>
<td>6,430 ± 1,098</td>
</tr>
</tbody>
</table>

* Peptides (2 μg/20 μl) were injected s.c. into the lower back of BALB/c mice, and mice were sacrificed after 15 min or 6 h. Thirty minutes before the end of the incubation time, [¹²⁵I]albumin (2 × 10⁶ cpm/mouse) was injected i.v. Skin (an area of 25 mm² around the injection point) was excised, and its radioactivity was counted in a beta counter.

* ANOVA, F = 100.53

* ANOVA, F = 155.77

* Results shown are the mean ± SD of five animals.

* p < 0.05 vs scrambled peptide-treated mice by Student-Newman-Keuls test.

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**FIGURE 2.** Effects of the anti-VEGFR-2 Ab on Tat-MBP- and VEGF-A-induced skin permeability. Anti-VEGFR-2 Ab or an irrelevant Ig (20 μl of 1:50 dilution) were coinjected with Tat-MBP (30 ng/30 μl) or VEGF-A (40 ng/10 μl) in mouse skin for 15 min. Tat-MBP or VEGF-A alone were used as controls. [¹²⁵I]Albumin (2 × 10⁶ cpm/mouse) was injected i.v. 30 min before the end of the incubation time. Skin (an area of 25 mm² around the injection point) was then excised, and the corresponding radioactivity was counted in a beta counter. Results shown are the mean ± SD of six animals. ANOVA gave F = 62.50. * p < 0.05 vs irrelevant Ig by Student-Newman-Keuls test.
synthetic Tat86 and Tat101, and the albumin leakage was monitored. These Tat molecules, too, retained their vasopermeability activity (Table I).

VEGF receptors and CCRs are responsible for several of the extracellular effects of Tat (28, 38–40, 59, 60). To determine the type of receptor involved in albumin leakage, we used two peptides encompassing the basic (Basic46–80) and cysteine-rich and core (CysL24–51) domains of Tat; the former activates VEGFR-2 (28), and the latter CCR2 and CCR3 (40). As shown in Table II, Basic46–80 strongly induced both leakage peaks, whereas CysL24–51 or a scrambled peptide did not. A further experiment with a neutralizing Ab anti-VEGFR2 indicated the operative role of this receptor in our model. The early effect of Tat-MBP and VEGF-A on [125I]albumin leakage was markedly reduced when this Ab was coinjected with the two molecules, whereas the non-immune serum had no activity (Fig. 2). Inhibition of the late leakage by this Ab efficacy was less effective (32 ± 8% inhibition of [125I]albumin leakage induced by 30 ng Tat-MBP, n = 5), suggesting that other activation pathways played a role or that the Ab was degraded.

Two hours after its injection, Tat-MBP induced leukocytes to arrest in capillaries (Fig. 3B, inset). Cell migration into tissue was evident after 2 h, peaked after 6 h, and persisted up to 24 h (Figs. 3 and 4). This effect was dose dependent and maximum with 30 ng/30 µl Tat-MBP (Fig. 4). MBP alone or Tat-MBP inactivated by heating was ineffective (Figs. 3D and 4). Synthetic Tat86 and Tat101 were also active (Table I). Light microscopy indicated that the number of infiltrating polymorphonuclear cells was negligible and that the infiltrate was mainly composed of lymphomononuclear cells (Fig. 3). Skin sections from animals sacrificed after 3 and 6 h after 30 ng/30 µl Tat-MBP were stained with specific rat mAb anti-mouse CD4, CD8, and Mac-3. Mac-3-positive cells were the most abundant (76 ± 18%), whereas CD8 and CD4 accounted for 8 ± 5 and 16 ± 9% of total infiltrating cells, respectively (Fig. 5).

Role of PAF in Tat-induced skin permeability

VEGFR-2 stimulation by VEGF-A triggers PAF synthesis in human EC (61), and PAF induces vasopermeability (62) and alters in vitro EC barrier function (50). Therefore, we investigated the role of PAF in Tat-induced vascular leakage by i.p. injection of the specific PAF-receptor antagonist WEB2170 (56) or its coinjection with Tat-MBP in the skin. Table III shows that WEB2170 reduced the early phase of albumin leakage by 70–80%, but only slightly (0–2%) inhibited the late wave, indicating that other indicators were involved.

A direct effect of Tat on EC toward vascular permeability was demonstrated with an in vitro system in which confluent EC monolayers were cultured on transwell cell culture inserts. [125I]Albumin (2 × 10⁶ cpm) was loaded in the upper chamber of the well along with Tat-MBP at different concentrations. After 1 h at 37°C the radioactivity of the lower well was counted. As shown in Fig. 6, Tat-MBP induced a dose-dependent trans-endothelial flux of albumin that was reduced by 90% by an anti-VEGFR-2 Ab. Irrelevant Ig did not change the response to Tat-MBP, whereas boiled Tat-MBP or MBP alone failed to reduce the flux. Basic46–80 peptide, but not CysL24–51 (Fig. 6) or the scrambled peptides (data not shown), induced an increase of albumin clearance through the EC monolayer. WEB2170 (5 µM) also inhibited albumin flux induced by Tat-MBP and by Tat86, as well as that induced by PAF (Fig. 6).

These data suggest that PAF produced in mouse skin after Tat injection participates in the vasopermeability alteration. To detect the origin of PAF, several human and murine cells were stimulated...
TABLE III. Effect of WEB2170 on Tat-MBP-induced [{\textsuperscript{125}}I]albumin leakage in mouse skin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[{\textsuperscript{125}}I]Albumin after 15 min (cpm)</th>
<th>[{\textsuperscript{125}}I]Albumin after 6 h (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBP</td>
<td>1,534 ± 345\textsuperscript{d}</td>
<td>3,431 ± 561</td>
</tr>
<tr>
<td>Tat-MBP</td>
<td>23,121 ± 3,128\textsuperscript{e}</td>
<td>91,431 ± 8,761\textsuperscript{b}</td>
</tr>
<tr>
<td>WEB2170 locally injected</td>
<td>2,310 ± 670\textsuperscript{a}</td>
<td>4,310 ± 1,226\textsuperscript{b}</td>
</tr>
<tr>
<td>Tat-MBP + WEB2170 locally injected</td>
<td>7,623 ± 1,034\textsuperscript{b}</td>
<td>88,341 ± 5,389\textsuperscript{b}</td>
</tr>
<tr>
<td>WEB2170 i.p.</td>
<td>1,670 ± 452\textsuperscript{g}</td>
<td>4,550 ± 1,077\textsuperscript{b}</td>
</tr>
<tr>
<td>Tat-MBP + WEB2170 i.p.</td>
<td>5,632 ± 1,021\textsuperscript{f}</td>
<td>85,755 ± 4,561\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Tat or MBP (30 ng/30 \mu l) was injected s.c. into the lower back of BALB/c mice sacrificed after 15 min or 6 h. Thirty minutes before the end of the incubation time, \textsuperscript{125}Ialbumin (2 × 10\textsuperscript{6} cpm/mouse) was injected i.v. Skin (an area of 25 mm\textsuperscript{2} around the injection point) was excised, and its radioactivity was counted in a \textbeta counter. WEB2170 (250 ng) was coinjected with Tat-MBP or injected i.p. (30 mg/kg) 30 min before the beginning of Tat-MBP treatment.

\textsuperscript{b} ANOVA, F = 129.75.

\textsuperscript{c} ANOVA, F = 396.55.

\textsuperscript{d} Results shown are the mean ± SD of five animals.

\textsuperscript{e} *p < 0.05 vs Tat-MBP-treated mice by Student-Newman-Keuls test; \textdagger, \textdagger p < 0.05 vs Tat-MBP-treated mice by Student-Newman-Keuls test.

FIGURE 4. Time- and dose-dependent effects of Tat-MBP on leukocyte infiltration in mouse skin. Mouse skin was injected with different concentrations of Tat-MBP, MBP (30 ng/10 \mu l), or Tat-MBP heat-inactivated (30 ng/30 \mu l) for 15 min, 3 h, 6 h, and 24 h. Skin sections were then excised, fixed in 10% buffered formalin, and embedded in paraffin. Then, sections were cut at 5 \mu m and stained with hematoxylin and eosin. The number of infiltrating cells was determined by light microscopy at ×400 in five fields of a 1-mm\textsupersquare grid and given as cells/mm\textsupersquare. Results shown are the mean ± SD of seven animals. ANOVA gave the following results: mice treated for 15 min, F = 3.39; mice treated for 3 h, F = 58.02; mice treated for 6 h, F = 172.23; mice treated for 24 h, F = 159.24. *p < 0.05 vs MBP-injected animals by Student-Newman-Keuls test.

FIGURE 5. Cell-type-specific tissue infiltration elicited by Ta-MBP in mouse skin. Frozen skin sections from animals sacrificed after 3 and 6 h after Tat-MBP injection (30 ng/30 \mu l) were incubated with optimal dilution of rat anti-mouse CD8 (open columns), anti-mouse CD4 (hatched columns), and anti-mouse Mac-3 (filled columns). After washes, sections were incubated with rabbit anti-rat IgG and rat peroxidase-antiperoxidase, and the reaction was developed. The number of positive cells was determined by light microscopy at ×400 in five fields on a 1-mm\textsupersquare grid. Results shown are the mean ± SD of four animals. *p < 0.05 by Student’s \textit{t} test.

with Tat\textsubscript{as6} at 10 ng/ml (an optimal concentration to alter albumin clearance), and their associated PAF was measured by a biological assay on washed rabbit platelets. As shown in Table IV only human and murine EC and monocytes/macrophages produced PAF, whereas human dermal fibroblasts and keratinocytes did not synthesize PAF after Tat\textsubscript{as6} challenge. The small amount of PAF produced by PBMC and sMC may be due to the presence of monocytes. Because in our in vivo model lymphomononuclear cells began to be recruited after 3 h (Fig. 4) and the infiltrating population was almost entirely composed of monocytes (Fig. 5), it is reasonable to speculate that EC are the first to produce PAF. Detailed investigation of PAF production in human EC showed that after 30 min of stimulation 30 ng/ml Tat-MBP induced a peak of PAF synthesis that declined to basal levels within 45 min (Fig. 7). This effect was dose dependent up to 50 ng/ml (Fig. 7). Heat-inactivated Tat-MBP (30 ng/ml) (data not shown) and MBP (Fig. 7) were ineffective. PAF synthesis was also observed when EC were challenged with Basic\textsuperscript{46-80}, whereas CysL\textsuperscript{24-51} and scrambled basic peptide were inactive (pmol PAF after a 30-min stimulation: None, 0.54 ± 0.21; Basic\textsuperscript{46-80} (1 \mu g/ml), 3.65 ± 0.92; scrambled basic peptide (1 \mu g/ml), 0.39 ± 0.12; CysL\textsuperscript{24-51} (1 \mu g/ml), 0.41 ± 0.23; n = 3). Similar results were provided by a radiometric assay of the uptake of labeled acetate into PAF molecule (data not shown). PAF synthesis is preceded by activation of the specific acetyltransferase that is the key enzyme of the remodeling pathway (Fig. 7) (45–47). Preincubation of EC with the anti-VEGFR-2 abolished PAF synthesis triggered by 15-min cell stimulation with 30 ng/ml Tat-MBP (Tat-MBP + goat serum, 5.8 ± 0.5 pmol; Tat-MBP + anti-VEGFR-2, 1.2 ± 0.5 pmol; goat serum, 0.6 ± 0.2 pmol; anti-VEGFR-2, 0.8 ± 0.3 pmol; n = 3).

Role of chemokines in Tat-mediated lymphomononuclear infiltration

The PAF receptor antagonist WEB2170 (administered i.p. or coinjected with Tat-MBP) had a negligible effect on lymphomonocytopenia.
nuclear infiltration induced by Tat-MBP. It delayed the cell recruitment at 3 h, but did not reduce the number of infiltrating cells after 6 h (data not shown).

Because Tat-MBP up-regulates cytokine production in several cell types, we investigated the role of MCP-1 produced by EC. Coinjection of Tat-MBP with a specific blocking Ab anti-VEGFR-2 or goat serum (1:50) and then stimulated with the op-
tained 200 mM BSA and 0.2
added to the upper chamber and incubated for 1 h. The upper chamber also
icated concentrations, heat-inactivated Tat-MBP at 30 ng/ml, Tat 86 at 10
ng/ml, Basic[66–80] and CysL[24–51] at 1 μg/ml, and PAF at 50 nM) were
added to the upper chamber and incubated for 1 h. The upper chamber also
contained 200 mM BSA and 0.2 μCi [125I]albumin in 2 ml of M199 me-
dium. EC permeability was determined by calculating the ratio of [125I]-al-
bumin in the upper and lower chamber of the filter assembly. When indic-
ed, EC were preincubated for 15 min with WEB2170 (5 μM), or with
Ab anti-VEGFR-2 or goat serum (1:50) and then stimulated with the op-
timal concentration of Tat-MBP (30 ng/ml), PAF (50 nM), or Tat[66] (10
ng/ml). Results shown are the mean ± SD of three experiments performed in triplicate. ANOVA gave F = 66.37, *, §, **, #, and %, respectively, indicate p < 0.05 vs MBP, Tat-MBP (30 ng), none, Tat[66], and PAF by the Student-Newman-Keuls test.

Discussion

This paper describes the effects of s.c. injection of Tat in mouse skin. An acute inflammatory response marked by protein leakage and lymphomononuclear cell infiltration is induced.

Subnanomolar Tat concentrations induced an early (15-min) and a delayed (6-h) vascular permeability response. The extent of this effect in the early phase was comparable to that elicited by VEGF-A. This finding is quite remarkable because VEGF-A was originally identified on account of its enhancement of vascular permeability and called “vascular permeability factor”. We then showed that Tat provokes lymphomononuclear recruitment into tissues. This is evident after 3 h and maximum after 6 h. It was mostly evident on monocytes, whereas the number of CD4- and CD8-positive cells was only slightly increased.

Synthesis of acetylated alkyl ethers of phosphorylcholine, namely PAF, is a response of cells stimulated by VEGF-A or Tat. VEGF-A induces rapid PAF synthesis in bovine EC through activation of VEGFR-2 (61, 63). In human monocytes, VEGF-A and Tat trigger the same biological response, but by activating VEGFR-1 (39). Furthermore, VEGF-A-induced protein extravasation in vivo is abolished by a selective PAF receptor antagonist (63). PAF is a mediator of cell-to-cell communication with a broad range of biological activities on inflammatory and noninflamma-
tory cells (45). Vascular endothelium is a main target for PAF that promotes angiogenesis by inducing cell migration (64) and vascu-
lar leakage through modification of cytoskeleton (50, 62, 65, 66). Our results demonstrate that PAF plays a substantial role in the early protein leakage induced by Tat in murine skin and is produced by EC through activation of VEGFR-2. This conclusion is based on the following findings: 1) WEB2170, a PAF receptor antagonist, abrogated Tat-induced in vivo [125I] albumin leakage and 2) Tat-induced increase of [125I]albumin clearance in vitro; 3) by activation of the remodeling pathway, Tat stimulated in vitro PAF synthesis by EC from large vessels or microvasculature; and 4) both [125I]albumin clearance and PAF synthesis were inhibited by EC pretreatment with an Ab anti-VEGFR-2. The second wave of vascular permeability observed in mice injected with Tat was

Table IV. PAF production by human and murine cells stimulated by Tat molecules

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Human EC</th>
<th>Human Fibroblasts</th>
<th>Human Keratinocytes</th>
<th>Human PBMC</th>
<th>Human Monocytes</th>
<th>Murine H. end</th>
<th>spc</th>
<th>Murine Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.23 ± 0.10**</td>
<td>0.32 ± 0.12</td>
<td>0.98 ± 0.31</td>
<td>0.31 ± 0.15</td>
<td>0.44 ± 0.12</td>
<td>1.80 ± 0.71</td>
<td>0.31 ± 0.20</td>
<td>0.25 ± 0.09</td>
</tr>
<tr>
<td>Tat[66]</td>
<td>4.51 ± 0.78</td>
<td>0.38 ± 0.22</td>
<td>1.00 ± 0.23</td>
<td>1.32 ± 0.30</td>
<td>6.32 ± 1.45</td>
<td>10.3 ± 2.22</td>
<td>1.13 ± 0.71</td>
<td>9.20 ± 2.23</td>
</tr>
</tbody>
</table>

* Human EC (6 × 10⁵/cm²), human fibroblasts (2 × 10⁶/ml), murine EC line H. end (7 × 10⁵/cm²), spc (2 × 10⁹/ml), and murine macrophages (2 × 10⁷/35-mm diameter well) were stimulated with Tat[66] (10 ng/ml) for 20 min at 7°C in M199 containing 0.25% BSA. Cell-associated PAF was extracted, purified by TLC, and measured by washed rabbit platelet aggregation.

** pmol PAF/4 × 10⁹ cells

° Results shown are the mean ± SD of three experiments.
not reduced by WEB2170, suggesting that several mediators participate in the late phase. This is not surprising, because PAF triggers and participates in several inflammatory circuits (47).

Our in vitro results indicate that monocytes/macrophages stimulated by Tat also produce PAF, whereas skin fibroblasts and keratinocytes do not. However, the role of monocytes/macrophages as a PAF source seems to be negligible in our model. Monocytes appeared later than the early peak of permeability inhibited by the PAF receptor antagonist, and this antagonist did not interfere with the late wave. Furthermore, WEB2170 did not inhibit lymphomononuclear infiltration in vivo even though PAF receptor antagonists abrogate Tat-induced in vitro monocyte migration (39).

Tat is a powerful in vitro chemoattractant of monocytes (35, 38) and induces them to transmigrate across an EC monolayer (33, 35). Recruitment of lymphomononuclear cells by Tat was specifically inhibited by a mAb anti-MCP-1, a chemokine that potently activates monocyte migration at the site of inflammatory injury (48). EC are a substantial source of MCP-1 (44). Tat increased its baseline production, as already reported for a macrophage-derived cell line and for glial cells (33, 67, 68). Furthermore, Tat up-regulates the cognate receptor of MCP-1 on the surface of human monocytes (33, 69), indicating that it may facilitate the infiltration of monocytes into tissues via endothelium-derived MCP-1 production and by rendering them more susceptible to MCP-1.

Our data do not clarify the relationship between PAF and MCP-1 production. PAF up-regulates the MCP-1 transcript in human EC (70). However, the PAF receptor antagonist WEB2170 did not reduce Tat-induced skin lymphomononuclear cell recruitment and did not block the MCP-1 synthesis in EC stimulated in vitro by Tat, which means that PAF is not a secondary mediator of Tat-induced MCP-1 production in human EC. Furthermore, blocking of its receptor does not inhibit MCP-1 release into plasma during endotoxemia in chimpanzees (71).

Tat exerts its extracellular biological activity by activating two receptor families: the tyrosine kinase VEGF receptors and the G protein-coupled CCRs. Structure-activity relationship studies have...
established that the Tat basic domain binds to and activates VEGF-R-2, and the Cys rich and the core domains are the molecular determinants responsible for CCR2 and CCR5 engagement (28, 38, 40). Our in vitro and in vivo results show that stimulation by Tat of VEGF-R-2, which is primarily expressed on EC (reviewed in Refs. (72, 73)), is crucial for establishment of increased vascular permeability. The dramatic reduction in [%125I]albumin leakage elicited by an anti-VEGF-R-2 Ab in both Tat-MBP- and VEGF-A-treated skin indicates that both molecules activated in vivo VEGF-R-2, as also reported in vitro on EC and Kaposi’s sarcoma cells (60, 74). Similarly, this Ab blocked, in vitro, the augmented albumin clearance and the PAF synthesis triggered by Tat. Mirrored experiments with Basic24–51 and CysL24–51 peptides corroborated the importance of VEGF-R-2. Basic24–51 peptide caused in vivo vasopermeability and increased the albumin clearance through the EC monolayer as well as PAF and MCP-1 production by EC. In contrast CysL24–51 did not share this activity, which may exclude the involvement of chemokines receptors.

These data shed new light on the molecular mechanism through which extracellular Tat may be essential to alter the vascular permeability and recruit monocytes into tissues, including lymphoid organs, where they cooperate in inducing injury and represent a cellular reservoir for HIV replication.

References
TAT ACTIVATES MONOCYTES AND INDUCES VASCULAR PERMEABILITY


