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

Marco Arese,* 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.

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3 Abbreviations used in this paper: EC, endothelial cells(s); Basic 4 6–8 0 , Tat peptide for aa 46–60; CysL 24 –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; spc, 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 endothelium cells.

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Materials and Methods

[14C]acetate (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 obtained 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-acetyl-(R)-gycero-3-phosphocholine) and lyso-PAF (1-O-octadecyl-2-lyso-gycero-3-phosphocholine) were obtained from Bachem Feinchemikalien (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 hybridoma, Lyt 2), anti-mouse CD4 (GK 1.5 hybridoma, L3T4), and anti-mouse Mac-3 (M37/84, 34, 64 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 Tat100 and Tat100 proteins and Tat peptides covering aa 24–51 corresponding to cysteine and core regions (CysL24–51; NH2-NHCY CCK KCC FHC QVC FIT KAL GIS YGR KK-COOH) (9) were synthesized by Tecgene (Castera, Italy), purified by HPLC, and quality controlled by mass spectroscopy (38). Tat peptide for aa 46–60 (Basic46–60: NH2-SYG RKK RRQ RRR PPQ COOH) corresponding to the basic region (9) was purchased from Technogenetics (London, U.K.). Two scrambled peptides of CysL24–51 (NH2-NCY KCC ISY CFC VIT KAL QGC CFG RKK-COOH) and Basic46–60 (NH2-KGG RRQ SQP RYR PQQ-COOH) peptides were purchased from Prinz (Milan, Italy). Recombinant wild-type HIV-1 Tat100 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 stored at –80°C in aliquots of 5 μg/10 μl of PBS containing 0.1% human serum albumin, 0.1 mM ZnCl2, and 1 mM DTT. Biological activity of Tat-MBP was checked as the ability to induce transcriptional activation of the HIV-1-long-terminal repeat in HL3T1 cells containing the bacterial gene of chloramphenicol acetyltransferase directed by this repeat, as previously described (49). In this assay Tat-MBP inactivated by heating (10 min at 90°C) did not activate chloramphenicol acetyltransferase, and was used as negative control in all experiments.

Cells

EC from human umbilical cord veins and murine microvascular H.end endothelioma cells (H.end) were prepared, characterized, and grown as previously described (50, 51). Human umbilical endothelial-cadherin, CD-31, and von Willebrand factor-related Ag, and the ability to respond to inflammatory cytokines IL-1 and TNF-α and to produce chemokines and PAF (51–53). Human dermal fibroblasts and human peritoneal macrophages were prepared from BALB/c mice (Charles River, Calco, Italy) according to Refs. 54 and 55.

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, WEB2170-2 Ab (1:1000 dilution, mice), VEGFR-2 Ab, goat anti-rat Ab (1:1000 dilution), or the PAF receptor antagonist WEB2170 (250 ng) (56) was co-injected with Tat-MBP or VEGF-A. Alternatively, 10 mg/kg WEB2170 in PBS was injected i.p. 30 min before the Tat-MBP treatment.

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 or 300 μg), or Tat-MBP heat-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 six 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 = 3.31. * p < 0.05 vs MBP-injected animals by Student-Newman-Keuls test.
ml of Tris-HCl 0.1, pH 6.8, containing 50 µg of lysate protein, 40 µM lysy-PAF, 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 microscope 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 repeated 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 rabbit anti-rat IgG and rat peroxidase-antiperoxidase. Each step lasted 30 min and was followed by a 10-min wash in TBS (pH 7.4). Sections were then incubated with 0.03% H2O2 and 0.06% 3,3'-diaminobenzidine for 5 min at room temperature washed, and counterstained by hematoxylin. The number of positive cells was determined under a light microscope at ×400 in five fields on a 1-mm² grid and is given as cells/mm².

**MCP-1 production**

EC (6 × 10⁶/cm²), fibroblasts (1.2 × 10⁸/cm²), keratinocytes (8 × 10⁸/cm²), and monocytes (2 × 10⁶/ml) were starved overnight in M199 supplemented with 2% FCS and 3% human serum albumin. Cells 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.

**Results**

**In vivo effects of Tat on vascular permeability and lymphomononuclear infiltration**

Increased vascular permeability is an early event in inflammatory injury and often precedes or parallels leukocyte infiltration of tissues. To study the effects of Tat on vascular permeability and leukocyte traffic from bloodstream to tissues, we set up an in vivo model based on Tat molecules injected into mouse skin and on i.v. [125I]albumin injection 30 min before mice were sacrificed. Fig. 1 shows that Tat-MBP caused a dose-dependent early (15 min) and a late (6 h) peak of albumin leakage. The maximal effect was obtained with 30 ng of Tat-MBP, but a significant leakage was obtained with 10 ng of Tat-MBP (corresponding to 0.7 pmol). MBP alone and heat-inactivated Tat-MBP were devoid of this activity. To further rule out the possibility that the effect observed was due to protein fusion instead of Tat, mice were injected with

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**Table I. In vitro and in vivo effects of Tat86 and Tat101**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[125I]Albumin Leakage at 15 min (cpm)</th>
<th>[125I]Albumin Leakage at 6 h (cpm)</th>
<th>Lymphomononuclear Cell Infiltration (cells/mm²)</th>
<th>PAF (pmol/4×10⁶ cells)</th>
<th>MCP-1 (ng/ml)</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>Tat86</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>

* TAT molecules (10 ng/10 µl) were injected i.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 × 10⁵ 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 β counter. Results shown are the mean ± SD of three animals.

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**Table II. Effect of Basic 46–80 and CysL24–51 on [125I]albumin leakage in mouse skin**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[125I]Albumin Leakage at 15 min (cpm)</th>
<th>[125I]Albumin Leakage at 6 h (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic</td>
<td>23,421 ± 2,109*</td>
<td>45,210 ± 6,021*</td>
</tr>
<tr>
<td>Scrambled</td>
<td>3,421 ± 1,231</td>
<td>4,321 ± 1,045</td>
</tr>
<tr>
<td>CysL</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 i.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 × 10⁵ 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 β 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 co-injected 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 × 10⁵ cpm/mouse) was injected i.v. 30 min before the end of the incubation. 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 Tat$_{86}$ and Tat$_{101}$, 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 (Basic$_{46-80}$) and cysteine-rich and core (CysL$_{24-51}$) domains of Tat; the former activates VEGFR-2 (28), and the latter CCR2 and CCR3 (40). As shown in Table II, Basic$_{46-80}$ strongly induced both leakage peaks, whereas CysL$_{24-51}$ 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 $[^{125}$I$]$albumin leakage was markedly reduced when this Ab was coinjected with the two molecules, whereas the nonimmune serum had no activity (Fig. 2). Inhibition of the late leakage by this Ab efficacy was less effective (32 ± 8% inhibition of $[^{125}$I$]$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 $\mu$L Tat-MBP (Fig. 4). MBP alone or Tat-MBP inactivated by heating was ineffective (Figs. 3D and 4). Synthetic Tat$_{86}$ and Tat$_{101}$ 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 $\mu$L 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. $[^{125}$I$]$Albumin (2 × 10$^6$ 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. Basic$_{46-80}$ peptide, but not CysL$_{24-51}$ (Fig. 6) or the scrambled peptides (data not shown), induced an increase of albumin clearance through the EC monolayer. WEB2170 (5 $\mu$M) also inhibited albumin flux induced by Tat-MBP and by Tat$_{86}$, 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

![FIGURE 3. Hematoxylin and eosin staining of mice skin injected with Tat-MBP. Skin was injected with Tat-MBP for 2 (B) and 6 (C) h or with MBP (A) or heat-inactivated Tat-MBP (D) for 6 h (30 ng/30 $\mu$L) (A). In MBP-injected skin the presence of lymphomononuclear cells was negligible and capillaries contained red cells (A, inset). After 2 h of Tat-MBP treatment, lymphomononuclear cells remained in capillaries (inset) and infiltrated s.c. tissue (B). After 6 h of Tat-MBP treatment, their number dramatically increased (C), whereas few cells were present at the heat-inactivated Tat-MBP injection site (D). A–D, ×200; inset, ×1000.](http://www.jimmunol.org/FIG/3.png)
TABLE III. Effect of WEB2170 on Tat-MBP-induced [125I]albumin leakage in mouse skin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[125I]Albumin after 15 min (cpm)</th>
<th>[125I]Albumin after 6 h (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBP</td>
<td>1,534 ± 3454</td>
<td>3,431 ± 561</td>
</tr>
<tr>
<td>Tat-MBP</td>
<td>23,121 ± 3,128</td>
<td>91,431 ± 8,761</td>
</tr>
<tr>
<td>WEB2170 locally injected</td>
<td>2,310 ± 670</td>
<td>4,310 ± 1,226</td>
</tr>
<tr>
<td>Tat-MBP + WEB2170 locally injected</td>
<td>7,623 ± 1,034*</td>
<td>88,341 ± 5,389</td>
</tr>
<tr>
<td>WEB2170 i.p.</td>
<td>1,670 ± 452</td>
<td>4,550 ± 1,077</td>
</tr>
<tr>
<td>Tat-MBP + WEB2170 i.p.</td>
<td>5,632 ± 1,021*</td>
<td>85,755 ± 4,561</td>
</tr>
</tbody>
</table>

* Tat or MBP (30 ng/30 µl) 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, [125I]albumin (2 × 10^6 cpm/mouse) was injected i.v. Skin (an area of 25 mm^2 around the injection point) was excised, and its radioactivity was counted in a β counter. WEB2170 (250 ng) was injected with Tat-MBP or injected i.p. (30 mg/kg) 30 min before the beginning of Tat-MBP treatment.

ANOVA, F = 129.75
ANOVA, F = 396.55
*p < 0.05 vs MBP-treated mice by Student-Newman-Keuls test; †, 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 µl), or Tat-MBP heat-inactivated (30 ng/30 µl) 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 µm 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^2 grid and given as cells/mm^2. 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 µl) were incubated with optimal dilution of rat anti mice 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^2 grid. Results shown are the mean ± SD of four animals. * p < 0.05 by Student’s t test.

with Tat_{60} 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_{60} challenge. The small amount of PAF produced by PBMC and spleen 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_{46-80} (1 g/ml), 3.65 ± 0.92; scrambled basic peptide (1 g/ml), 0.39 ± 0.12; CysL_{24-51} (1 µg/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 lymphomononuclear infiltration triggered by 15-min cell stimulation with 30 ng/ml Tat-MBP (30 ng/ml Tat-MBP + WEB2170 i.p., 0.2 pmol; 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).
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-MCP-1 reduced the MCP-1 production triggered by Tat-MBP (30 ng/ml), none, Tat-MBP, 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 noninflammatory cells (45). Vascular endothelium is a main target for PAF that promotes angiogenesis by inducing cell migration (64) and vascular 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 detectable after 6 h. At the optimal Tat-MBP concentration of 50 ng/ml, production of MCP-1 by EC was about one-third of that elicited by TNF-α, used as positive control (44). Synthetic Tat86 or Tat101 were also active in terms of MCP-1 production (Table I), as well as Basic24–51 and scrambled basic peptide were ineffective (ng MCP-1/ml after a 24-h stimulation: None, 5.54 ± 2.02; Basic24–51 (1 μg/ml), 18.12 ± 4.03; scrambled basic peptide (1 μg/ml), 4.78 ± 1.15; CysL24–51 (1 μg/ml), 6.32 ± 2.10; n = 3). The presence of 20 μM WEB2170 did not change the MCP-1 production triggered by Tat-MBP (30 ng/ml) after a 12-h stimulation (Control, 3.6 ± 0.4 ng/ml; WEB2170, 4.1 ± 0.9 ng/ml; Tat-MBP, 15.4 ± 0.8 ng/ml; WEB2170 + Tat-MBP, 14.8 ± 1.4 ng/ml; n = 3).

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Human EC Fibroblasts</th>
<th>Human Keratinocytes</th>
<th>Human PBMC Monocytes</th>
<th>Murine H. end peritoneal macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.23 ± 0.10 nM</td>
<td>0.32 ± 0.12</td>
<td>0.98 ± 0.31</td>
<td>0.31 ± 0.15</td>
</tr>
<tr>
<td>Tat86</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>
</tr>
</tbody>
</table>

A Human EC (6 × 10^5/cm²), human fibroblasts (2 × 10^5/ml), human EC line H. end (7 × 10^5/cm²), spc (2 × 10^8/ml), and murine macrophages (2 × 10^7/35-mm diameter well) were stimulated with Tat86 (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.

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 VEGFR-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 VEGFR-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-VEGFR-2 Ab in both Tat-MBP- and VEGF-A-treated skin indicates that both molecules activated in vivo VEGFR-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 Basic6–25 and CysL24–51 peptides corroborated the importance of VEGFR-2. Basic6–25 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 chemokinases.

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


