Plasma From Patients With Idiopathic and Human Immunodeficiency Virus-Associated Thrombotic Thrombocytopenic Purpura Induces Apoptosis in Microvascular Endothelial Cells

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The pathogenesis of thrombotic thrombocytopenic purpura (TTP) is obscure. It is manifested classically by platelet thrombi and localized microvascular endothelial cell (EC) proliferation, in the absence of an inflammatory response. It is statistically associated with human retroviral disease, but pathological studies of TTP lesions have been unable to establish whether perturbation of the endothelium is a primary or secondary event, irrespective of the presence of retroviral infection. We document that plasma from all of four acute TTP patients, with or without human immunodeficiency virus infection, can induce apoptosis in cultured ECs of microvascular but not large vessel origin. This process was documented by three different methods, (1) laser-illuminated light scatter, (2) quantitation of the pre-G1, A1, peak on DNA histograms and direct visualization of chromatin fragmentation by acridine orange and 4′,6-diamidino-2-phenylindole staining, and (3) agarose gel electrophoresis of low molecular weight cellular DNA. Apoptosis was independent of tumor necrosis factor-α secretion or the presence of CD36 on microvascular ECs but was linked to the rapid induction of Fas (CD95) on these cells. Soluble anti-Fas antibody, normal plasma depleted of cryoprecipitate, and low concentrations (≤0.1 μmol/L) of aurintricarboxylic acid were capable of suppressing TTP plasma-mediated EC apoptosis. In conclusion, microvascular EC apoptosis may be of pathophysiological importance in TTP, may be susceptible to interruption by blockade of initiating signals for, or final common enzyme pathways leading to, programmed cell death.

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PATIENTS, MATERIALS, AND METHODS

Patients. Plasma samples were obtained from heparinized or EDTA-treated venous blood of 4 HIV-seronegative and 4 HIV-seropositive asymptomatic controls, 2 HIV-seronegative patients with active disseminated intravascular coagulation (DIC), and 3 HIV-seronegative (TTP-1, -2, and -4) and 1 HIV-seropositive (TTP-3) patients with active TTP. Plasma samples were also available from the HIV- TTP patient after a plasmapheresis-induced remission.

TTP was diagnosed according to the following criteria: fever, unexplained oral temperature greater than 38.0°C; neurologic dysfunction, any new abnormality on general medical neurospsychiatric exam; renal dysfunction, serum creatinine level ≥1.5 mg/dL, or greater than 50% of previous baseline value; and thrombocytopenia.
platelet count less than 150,000 μL. All individuals had evidence for microangiopathic hemolysis on peripheral blood smear.

**EC cultures.** Large vessel ECs were obtained from human umbilical cord veins by digestion with 0.1% collagenase, as previously described. The resulting cells were cultured and maintained in T-75 flasks (Falcon; Becton Dickinson, Lincoln Park, NJ) or were subcultured in 24-well plates in Medium 199 containing 25 mmol/L HEPES, penicillin (80 μg/mL), streptomycin (80 μg/mL), L-glutamine (1.6 mmol/L), amphotericin B (2 μg/mL), 10% pooled human serum, 10% fetal bovine serum (FBS), and heparin (90 μg/mL) at 37°C under 5% to 6% CO2. Cells used in most experiments were in passages 2 to 6. Two different human microvascular ECs of dermal origin were purchased, ie, MVEC-1 (HMVEC 2753; Clonetics, San Diego, CA) and MVEC-2 (DHMVEC 30282; Cell Systems, Kirkland, WA). They were maintained in T-25 flasks (Falcon) in modified MCDB 131 medium containing recombinant human epidermal growth factor (rhEGF); 10 ng/mL hydrocortisone (1 μg/mL), bovine brain extract, 10% rhEGF, amphotericin B (50 mg/mL), gentamicin (50 μg/mL), 5% FBS, and 5% pooled human serum. All cells were positive for expression of von Willebrand’s factor (vWF)/factor VIII antigen and had a doubling time of approximately 36 hours. All subcultures involved a 5- to 10-minute exposure to 0.025% trypsin/0.01% EDTA, followed by washing with phosphate-buffered saline (PBS; pH 7.2).

To insure some uniformity of culture conditions, experiments were performed in “apoptosis culture medium” devoid of human serum: Medium 199 plus 20% FBS, rhEGF, heparin, amphotericin B, and gentamicin (concentrations as provided for microvascular ECs), after overnight preincubation in that medium.

**Cell surface antigens.** Indirect immunofluorescence for CD36 (gp130) and for CD4, the high-affinity receptor for HIV, was performed using murine monoclonal antibodies (MoAbs) 8A6 (IgG2a) and for FasL, No. 13203 (5’-CAGCTCTTCCACCTACATCC) and No. 13206 (5’-TCATGCTTCTCCCTCTTCACATGG). The trisodium salt of ATA interacted with the metachromatic dye acridine orange (AO) staining under ultraviolet illumination. Cells were preincubated with trypsin (0.025%) for 10 minutes at 25°C, were exposed to RNase A (1,000 U/mL) for 20 minutes at 4°C, were washed, were resuspended in 0.1 mL PBS, and were transferred onto glass slides by cytocentrifuge. They were fixed overnight at 25°C in a 1:9 solution of glacial acetic acid and absolute ethanol, air-dried, treated with 2 to 3 drops of a 2% solution of Triton X-100 for 2 minutes, and washed with PBS. Then, 2 to 3 drops of an 8 μg/mL solution of AO in 1 N NaOH was added for 1 to 2 minutes, and the cells were then washed. DAPI (1 μg/mL) and sulforhodamide 101 (Molecular Probes, Inc, Eugene, OR) staining was performed as described. Cells were observed and photographed using a fluorescence microscope with a BG-12 excitation filter for AO and an UG-1 filter for DAPI.

**Apoptosis assays.** Cytofluorometric determinations were first performed. ECs were washed with PBS, assessed for viability by trypan-blue dye exclusion, and then plated in macrowells at 0.15 × 106 viable cells/0.5 mL in apoptosis culture medium, alone or with dilutions of various plasmas. Cells were harvested 18 to 72 hours later, fixed in 70% cold ethanol, and incubated for 20 minutes at 4°C with propidium iodide (PI; 50 pg/mL) in the presence of RNAse A (300 U/mL), as previously described, and 2.5 to 5 × 106 cells were analyzed in the cytofluorograph.

Apoptosis was recognized by quantitated in viable cells by two flow cytometric methods, (1) detection of depressed forward scatter and increased side (right angle) scatter characteristic of apoptotic cells and (2) computer-assisted DNA histogram analysis of PI-labeled cells with calculation of pre-G1 An peaks. All experiments involved at least two separate determinations, with A(, values for each TTP plasma sample and cell type of similar passage number varying by ±15%.

Parallel cultures with larger cell numbers were analyzed for fragmentation of genomic DNA. Low molecular weight DNA was prepared by lysis of 2 × 106 cells per condition in 0.4 mL of lysis buffer, consisting of 10 mmol/L Tris-HCl (pH 7.5), 0.2% Triton X-100, and 1 mmol/L EDTA, followed by centrifugation and precipitation of the supernatant with 0.1 mL 5 mol/L NaCl and 0.5 mL isopropanol overnight at −20°C. Samples were microcentrifuged, and the pellets were washed with 70% ethanol and air-dried. The DNA was resuspended in Tris-EDTA, treated with RNase A (50 μg/mL) for 1 hour at 37°C, and electrophoresed through 1% agarose gels at 50 V/25 mA for 3 hours. DNA was visualized by ethidium bromide (0.5 μg/mL) staining and photography.

Features of apoptotic cells were also directly visualized. Staining of nucleic acid with the metachromatic dye acridine orange (AO) or with 4′,6-diamidino-2-phenylindole (DAPI) at low pH provides a sensitive assay to discriminate among live, apoptotic, and necrotic cells. AO intercalates into double-stranded DNA and, when bound in this form, fluoresces green, whereas denatured, single-stranded DNA and RNA fluoresce red. DAPI stains nuclear fragments blue under ultraviolet illumination. Cells were preincubated with trypsin (0.025%) for 10 minutes at 25°C, were exposed to RNase A (1,000 U/mL) for 20 minutes at 4°C, were washed, were resuspended in 0.1 mL PBS, and were transferred onto glass slides by cytocentrifuge. They were fixed overnight at 25°C in a 1:9 solution of glacial acetic acid and absolute ethanol, air-dried, treated with 2 to 3 drops of a 2% solution of Triton X-100 for 2 minutes, and washed with PBS. Then, 2 to 3 drops of an 8 μg/mL solution of AO in 1 N NaOH was added for 1 to 2 minutes, and the cells were then washed. DAPI (1 μg/mL) and sulforhodamide 101 (Molecular Probes, Inc, Eugene, OR) staining was performed as described. Cells were observed and photographed using a fluorescein microscope with a BG-12 excitation filter for AO and an UG-1 filter for DAPI.

**Fas and Fas ligand (FasL) expression.** Fas (APO-1, CD95) and FasL are membrane antigens implicated in the pathogenesis of several viral infections, including HIV-associated apoptosis of CD4+ T lymphocytes and influenza virus infection of epithelial cells, as well as in activation-induced programmed T-cell death. Expression of messages for these molecules was evaluated by reverse transcriptase-polymerase chain reaction (RT-PCR), using oligodeoxynucleotide primers capable of amplifying a segment from nucleotides 271 to 820 of Fas cDNA or nucleotides 392 to 1182 of FasL cDNA. These primers are as follows: for Fas, 1 (5′-CAAGTGG- ACTGA CATCAACTCC) and Fas 2 (5′-CTTCTCTTTCTTCTT CTGTC); and for FasL, Nos. 13203 (5′-CAGCTTTTTTCA CAG) and Nos. 13206 (5′-TCATTCTTTCTCCTTCACATG). All cellular RNAs were isolated from 2 × 106 cells/sample by the TriZOL (GIBCO-BRL, Gaithersburg, MD) method. RNAs were treated with RNase-free DNase and then were reverse transcribed into cDNA using 1 μg of RNA and 200 U of murine leukemia virus reverse transcriptase (GIBCO-BRL). cDNA aliquots of equal volume were then amplified by PCR, as described, using the primers capable of recognizing Fas or FasL or primers for β-actin (sense, 5′-TGACCGGTTCACCCACACTG T GCCCATCTA; antisense, 5′-CTGAA GCATTGGCGTGCAGAC GTGAGG; Stratagene, La Jolla, CA). Briefly, 50 μL of reaction volume, including 1× reaction buffer (Perkin-Elmer, Foster City, CA), optimized concentrations of MgCl2, deoxyribonucleotide triphosphates and appropriate primers, and 2 U of Taq polymerase, was used. The cDNAs were denatured for 2 minutes at 97°C before 35 runs in a thermal cycler (denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute in each cycle). A final extension at 72°C for 5 minutes was included. PCR products were separated by electrophoresis in a 1.4% agarose gel, were visualized by ethidium bromide staining under ultraviolet illumination, and were photographed.

**Fas function.** The functional meaning of changes in Fas mRNA was explored using a panel of MoAbs directed to Fas, generated by immunizing mice with a purified fusion protein consisting of the extracellular domain of human Fas and the constant region of human IgG. When added in solution, Fas MoAb M3 blocks Fas-mediated cell lysis, whereas Fas MoAb M31 binds to the antigen but has no agonistic or antagonistic properties.

**Aurintricarboxylic acid (ATA).** The trisodium salt of ATA (Sigma, St Louis, MO) was dissolved in PBS, pH 7.4, and used at final concentrations of 0.01 to 1 μmol/L. Polymeric ATA interacts with vWF and inhibits the binding of large vWF multimers to platelet GPIb. These polymers, at high concentration (100 μg/mL), also inhibit vWF-mediated shear stress-induced platelet aggregation asso-
PLASMA-MEDIATED ENDOTHELIAL CELL APOPTOSIS

MVEC-1

UVEC

Fig 1. DNA histograms and light scatter plots of MVECs and UVECs exposed to normal human plasmas. Human dermal (MVEC-1) and UVECs were cultured for 72 hours in human serum-free apoptosis culture medium in the presence of a 1:20 dilution of plasma from an HIV- control donor. DNA histograms of ethanol-fixed, RNase-treated, and PI-stained cells (A), as well as laser-illuminated light scatter plots (forward scatter [FS] versus side scatter [SS]) (B) were obtained.

Associated with acute TTP plasmas. At much lower concentrations (0.01 to 0.1 μmol/L), ATA is a potent suppressor of apoptosis in many cell types, which is presumably related to its ability to block both proteases and Ca²⁺-dependent endonucleases. Its capacity to inhibit thrombosis formation in animals has also led to the experimental use of ATA in the treatment of intractable TTP.

RESULTS

Apoptosis data. We first examined the baseline DNA histogram patterns of ethanol-fixed, RNase-treated, and PI-stained large vessel (umbilical vein; UVECs) and microvascular (two types; MVEC-1 and MVEC-2) ECs. ECs were plated at 0.15 × 10⁶ cells/0.5 mL apoptosis culture medium in uncoated macrowells and were exposed for 72 hours to buffer, control plasma, or plasma derived from TTP patients. On termination of culture, loosely adherent ECs were obtained by removal of the supernatant, addition of 2 mL PBS, and vigorous pipetting. Adherent ECs were removed by treatment with 0.1% collagenase.

As shown in Fig 1, untreated samples (loosely adherent plus adherent) of UVEC and MVEC-1 cells, grown in apoptosis culture medium, showed similar cell-cycle profiles, with low percentages of cells in the pre-G1 area known as the A0 or apoptotic peak (<1%; Fig 1A) and normal light scatter-grams (Fig 1B). MVEC-2 gave an identical pattern, as did the loosely adherent and adherent cells from both microvascular cultures (not shown). No control plasma or serum samples gave an A0 value greater than 18%, and the majority gave values ≤10%.

In striking contrast, exposure of MVEC-1 to a 1:20 dilution of plasma from 1 acute HIV-seronegative TTP patient (Fig 2) and 1:100 dilutions of plasma from 3 other acute TTP patients (1 HIV⁺; see Table 1), showed classic apoptotic patterns. First, PI labeling show prominent A0 regions, representing low DNA staining, comprising up to 40% of the total cell cycle DNA histogram of MVEC-1 cells exposed to TTP-1 plasma (Fig 2A) and greater than 90% in MVEC-1 exposed to TTP-3 and TTP-4 plasmas (Table 1) when adherent plus loosely adherent populations were examined together. Apoptosis was most prominent among the loosely adherent cells (about 25% of the total population in TTP plasma cultures), with A0 peaks greater than 80% among these cells. Second, the majority of MVEC-1 cells showed markedly depressed forward light scatter, secondary to cell shrinkage, and either no change (Fig 2B) or some elevation in side scatter, because of chromatin condensation with increased granularity.

In contrast to these effects, UVECs retained normal DNA histograms and light scattering, with few nonadherent cells after 72 hours of culture and no evidence for apoptosis on exposure to TTP plasmas (Figs 2A and B). Apoptosis of MVECs was confirmed by direct observation of differential staining of double-stranded versus denatured DNA in cells cultured for 72 hours with a 1:20 dilution of TTP plasma, followed by exposure of fixed cells to AO
Table 1. Induction of Apoptosis in MVEC-1 by TTP Plasma and Its Suppression by Normal Plasma

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Experimental Plasma (1:100 dilution)</th>
<th>Normal Human Plasma (1:20 Dilution)</th>
<th>% A&lt;sub&gt;p&lt;/sub&gt; Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control-1 (HIV&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Control-2 (HIV&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>-</td>
<td>7.0 ± 5.0&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>Control A (HIV&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>-</td>
<td>11.1</td>
</tr>
<tr>
<td>4</td>
<td>DIC-1 (HIV&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>DIC-2 (HIV&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>-</td>
<td>9.5</td>
</tr>
<tr>
<td>6</td>
<td>TTP-1 (HIV&lt;sup&gt;-&lt;/sup&gt;)</td>
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</tr>
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<td>7</td>
<td>TTP-2 (HIV&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>-</td>
<td>58.0</td>
</tr>
<tr>
<td>8</td>
<td>TTP-3 (HIV&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>-</td>
<td>&gt;90</td>
</tr>
<tr>
<td>9</td>
<td>TTP-4 (HIV&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>-</td>
<td>&gt;90</td>
</tr>
<tr>
<td>10</td>
<td>TTP-1 (HIV&lt;sup&gt;-&lt;/sup&gt;)</td>
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<td>0</td>
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<td>11</td>
<td>TTP-2 (HIV&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>+</td>
<td>13.3</td>
</tr>
<tr>
<td>12</td>
<td>TTP-3 (HIV&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>+</td>
<td>10.5</td>
</tr>
<tr>
<td>13</td>
<td>TTP-3 (HIV&lt;sup&gt;-&lt;/sup&gt;), postplasmapheresis</td>
<td>-</td>
<td>15.0</td>
</tr>
</tbody>
</table>

MVEC-1 cells were cultured for 72 hours in apoptosis culture medium along with the indicated reagents. A<sub>p</sub> peak was determined by PI staining and DNA histogram analysis.

<sup>*</sup> Mean of four experiments.

<sup>†</sup> 1:20 Dilution of plasma.

(MVEC-1 and TTP-2 plasma; data not shown) or DAPI (Fig 3 and Table 2; MVEC-2 and TTP-4 plasma). Apoptosis was recognized before disruption of plasma membranes, with trypan blue dye exclusion viability ≥85% in all samples.

DAPI staining was also used to assess the reliability of apoptosis determinations based on quantitation of the A<sub>p</sub> peak. Simultaneous, blinded analysis of the effects of 1 control and 1 TTP (TTP-4) plasma on MVEC-2 cells for DAPI<sup>+</sup> apoptotic cells (Table 2) and PI staining and cytofluorometry (not shown) gave percentages of apoptotic cells within 15% to 20% of the values predicted by DNA histogram analysis.

Control plasma included samples from HIV-seronegative donors who were asymptomatic or had active DIC, as well as from HIV<sup>-</sup> donors. No evidence for induction of apoptosis by DNA histogram criteria was noted (Table 1). These data were replicated using MVEC-2 (Table 3).

These observations were extended by DNA fragmentation analysis involving electrophoresis of low molecular weight DNA extracted from MVECs and UVECs exposed to various plasmas. Oligonucleosomal units were visible as bands whose molecular sizes are approximate multiples of 180 nucleotides in 1 sample (TTP-3-exposed MVEC-1) in which sufficient cell numbers were available to obtain an adequate quantity of DNA (data not shown). In the remaining samples (data not shown), an increase in low molecular weight DNA was observed documenting DNA fragmentation even if distinct laddering could not be visualized. UVECs treated in an identical manner showed neither laddering nor an increase in low molecular weight DNA.

**Impact of normal human plasma on TTP plasma-mediated apoptosis.** An attempt to document an effect of clinical treatment, plasmapheresis with fresh frozen plasma replacement, on the EC apoptosis observed in vitro was performed.

![Image of apoptosis visualization](http://example.com/fig3)

Fig 3. Direct visualization of TTP plasma-induced apoptosis in MVECs. MVEC-2 were cultured for 48 hours in the presence of a 1:100 dilution of a normal plasma (left panel) or TTP-4 plasma (right panel). Cells were harvested and stained with DAPI/sulforhodamine 101. When illuminated by ultraviolet light, apoptotic cells (right) show DNA in two compartments, with intense blue staining of fragmented nuclear material and dispersed DNA outside the nucleus, as inferred from the uniformity of the blue/purple cytoplasmic hue. In contrast, control cultures show intact nuclei, with red cytoplasmic staining.
using two methods. First, a postplasmapheresis sample from patient TTP-3 (HIV') was used in EC cultures, according to the assays described above. Second, normal plasma depleted of cryoprecipitate and, thus, of the largest vWF multimers by freeze-thawing and clearance by centrifugation was included in selected cultures. As shown in Table 1, the postplasmapheresis plasma specimen was incapable of inducing apoptosis in MVEC-1. In addition, inclusion of a 1:20 dilution of normal plasma suppressed TTP plasma-induced apoptosis in MVEC-1 and MVEC-2 (Tables 1 and 3). In the absence of normal human serum, 20% FBS did not block induction of apoptosis.

Fas and FasL expression. mRNA for FasL was not detected by RT-PCR using any EC sample, either from early or late passage large vessel (UV) or microvascular (2 dermal) specimens, in the presence or absence of TTP plasma (Fig 4). It was also not inducible by exposure of these cells to the protein kinase activator phorbol myristate acetate (PMA; 5 ng/mL; see Fig 4). As a control for these assays, FasL was detectable on CD4+ T cells infected with HIV-1 (Fig 4), because HIV markedly upregulates expression of this ligand.

In contrast, Fas was induced by plasma from active HIV+ patients, but only in MVECs. TTP plasma (1:100 dilution) led to expression of Fas mRNA in MVEC-1, both fresh (Fig 5A, passage 3) and aged (Fig 5B, passage 7). Aged UVECs (passage 7) did not express baseline Fas (Fig 5B), nor was this message induced by TTP plasma. Low-passage UVEC samples did have some baseline Fas expression by RT-PCR, but expression was not enhanced by normal or TTP plasma or by PMA (Fig 5C) in multiple experiments with varying initial quantities of EC RNA.

These experiments were repeated with the second MVEC type, MVEC-2. Again, there was no baseline expression of Fas in the presence of normal plasma, whereas HIV+ TTP-3 acute plasma (1:100 dilution) induced high levels of Fas mRNA (Fig 6). In contrast, plasma obtained from this patient postplasmapheresis failed to induce Fas (Fig 6), and Fas expression was not observed in the presence of plasma from a patient with acute promyelocytic leukemia and active DIC (data not shown).

Inhibition of TTP plasma-associated MVEC apoptosis by ATA and antibodies to Fas. It is conceivable that induction of Fas by components of acute TTP plasma may simply represent upregulation of this marker for cell activation on MVECs and may be irrelevant or only peripherally related to the apoptosis we observed. To address this issue, we examined the ability of soluble anti-Fas MoAbs M3 and MoAb M31 to modulate apoptosis when added to TTP-plasma–treated ECs. MoAb M3 but not MoAb M31 could block the extent of directly visualized apoptosis by greater than 60% (Table 2), which is consistent with the differential Fas-binding capacities of these reagents and their activities in activation-induced T-lymphocyte death.

Fas also may be induced on MVECs by exposure to tumor necrosis factor-α (TNF-α)29 and TNF-α at high concentrations may initiate apoptosis in ECs.30,31 Thus, we sought to determine whether anti–TNF-α antibodies could block TTP plasma-mediated apoptosis. A total of 1,000 neutralizing units of a rabbit antihuman TNF-α antibody (Genzyme, Cambridge, MA) was added to MVEC-1 cultures in the presence or absence of TTP plasma. This reagent failed to alter either TTP-plasma-induced Fas expression or the extent of EC apoptosis (Table 2). We next examined the susceptibility of these apoptotic processes to an inhibitor of two putative common pathways for programmed cell death, induction of proteases and Ca2+-dependent endonucleases. At 50 to 100 μmol/L, monomeric and polymeric ATA (C22H14O9) inhibit a variety of proteases as well as enzymes that process nucleic acids, including RNA and DNA polymerases, reverse transcriptase, integrase, exonuclease, and endonuclease, as well as vWF-mediated platelet aggregation.9 At lower concentrations (≤2 μmol/L), ATA continues to suppress certain proteases and endonucleases. As such, it has been proven to be a potent inhibitor of apoptosis in cells as disparate as thymocytes33 and adenocarcinoma cells.34 MVEC-1 cells exposed to TTP plasma in the presence of 0.1 μmol/L ATA showed marked suppression of apoptosis (Table 2). This occurred in the absence of an effect of ATA on TTP-plasma–induced Fas expression (data not shown).

EC phenotype. ECs have been phenotypically character-
IZED BY REACTION PATTERNS WITH OVER 1,000 MoAbs, BUT FEWER THAN 10 OF THESE ANTIBODIES DISCRIMINATE AMONG HISTOLOGICAL TYPES OF ECs. A ONE OF THESE MARKERS, CD36, HAS BEEN POSTULATED TO BE OF RELEVANCE TO TTP FOR THREE REASONS: (1) CD36 IS EXPRESSED ON SUBSETS OF THE TYPES OF MVECs AFFECTED IN TTP, BUT IS NOT FOUND ON THE ENDOTHELIUM OF LARGER VESSELS SUCH AS UV; (2) ANTI-CD36 ANTIBODIES HAVE BEEN IDENTIFIED WITH HIGH FREQUENCY IN THE PLASMA OF PATIENTS WITH TTP; AND (3) ACTIVATION OF SEVERAL CELL TYPES BY CD36 CROSS-LINKING OCCURS VIA PROTEIN KINASES OF THE SRC FAMILY THAT HAVE BEEN ASSOCIATED WITH INDUCTION OF APOPTOSIS. WE SOUGHT TO DETERMINE WHETHER CD36 EXPRESSION WAS ESSENTIAL FOR TTP-PLASMA-MEDIATED APOPTOSIS IN VITRO. CONSISTENT WITH PREVIOUS REPORTS, NEITHER FRESH NOR CULTURED UVECS EXPRESSED MEMBRANE CD36, ALTHOUGH THEY DID EXPRESS CD4 (DATA NOT SHOWN). BOTH TYPES OF MVECs HAD MEMBRANE CD4 (NOT SHOWN); HOWEVER, MVEC-1 WERE STRONGLY CD36+, WHEREAS MVEC-2 SHOWED NO CD36 EXPRESSION, AS DETERMINED BY OUR LAB (DATA NOT SHOWN) AND THE COMMERCIAL CELL SOURCE DATA. DESPITE THIS DIFFERENCE, AS NOTED IN THE EXPERIMENTS ABOVE, CD36 ECs WERE HIGHLY SUSCEPTIBLE TO APOPTOSIS INDUCED BY TTP PLASMA (TABLE 3), A PHENOMENON INHIBITABLE BY CRYOPRECIPITATE-DEPLETED NORMAL PLASMA (TABLE 3) AND ASSOCIATED WITH FAS UPRGULATION (FIG 6).

DISCUSSION

Many groups have speculated that primary EC injury is common and fundamental to TTP, and virtually all properties of normal MV endothelium are altered in TTP. ECs synthesize many substances involved in coagulation and fibrinolysis, including vWF, thrombomodulin, tissue-type plasminogen activator, plasminogen activator inhibitor, protein S, prostacyclin, and nitrous oxide. Alterations in the levels of these substances have been reported in TTP; they may simply be reflective of EC injury or may be of pathophysiological importance. Loss of prostacyclin and nitrous oxide, increases in vWF, and so on could lead to platelet aggregation in vascular beds throughout the body, creating a cycle of vasoconstriction with platelet and fibrin deposition and further thrombus formation. However, the underlying mechanism for this EC disruption is an open issue.

The literature relating to EC lysis secondary to anti-EC antibodies plus complement or to other toxic factors is problematic. Some direct evidence for primary vascular injury comes from immunofluorescent studies of involved vessels, by which IgG, IgM, and complement were shown to be present in vascular lesions. Although the specificity of these tissue-associated immune complexes is uncertain, complement-dependent antibodies cytotoxic for ECs have been shown in the serum and plasma of TTP patients. Ultrastructural changes became apparent within 30 minutes after exposure of ECs to TTP sera and fresh complement, and virtually every cell soon developed numerous cytoplasmic inclusions, followed by complete cytoplasmic and nuclear degeneration. However, these experiments all used UVECs, presumably because of the ease of access to material and consistency of growth conditions. The lack of involvement of large vein EC in TTP renders the relevance of this model to the situation in vivo quite questionable, as does the failure of others to document such anti-EC activity. Large vessel ECs respond differently than do MVECs to a variety of injuries, reflected by differences in proliferation, migration, cytokine production, phenotype, and so on. In addition, complement is not depleted in acute TTP. This phenomenon also would not account for the pathological changes in ECs observed in vivo. Vessel walls lack a prominent cellular infiltrate or other signs of inflammatory changes, which would be expected to accompany the type of EC destruction observed in the in vitro lysis models. We failed to see any rapid TTP-plasma-mediated UVEC or MVEC lysis, although, unlike previous reports, we used
Fig 5. RT-PCR for Fas expression in MVEC-1 ECs. (A) Cultures of MVEC-1 were established in the presence of control and TTP plasmas (1:100 dilution). β-actin mRNA controls are shown in the lower panel. Lane 1, buffer; lane 2, PMA (5 ng/mL); lane 3, control-1 (HIV-); lane 4, control-2 (HIV+); lane 5, TTP-1 (HIV+); and lane 6, TTP-3 (HIV+). (B) RT-PCR for Fas was performed using RNAs extracted from late passage UVECs or MVEC-1 in the presence or absence of TTP plasma (1:100 dilution). Lane 1, UVECs; lane 2, MVEC-1; lane 3, MVEC-1 + TTP-2 plasma (HIV+); and lane 4, MVEC-1 + PMA (5 ng/mL). (C) RT-PCR for Fas was performed using RNAs extracted from activated, early passage UVECs in the presence or absence of control and TTP plasma (1:100 dilution). β-Actin controls are shown. Two different experiments with TTP-3 plasma-exposed cells are shown. Lane 1, buffer; lane 2, PMA (15 ng/mL); lane 3, control-1 (HIV-); lane 4, TTP-1 (HIV-); lane 5, control-2 (HIV+); lane 6, TTP-3 (HIV+); lane 7, control-2; and lane 8, TTP-3.

Freeze-thawed plasma rather than serum and did not add fresh complement.

In contrast, we document accelerated apoptosis of MVECs in the presence of acute plasmas from all of 4 patients with TTP, regardless of their HIV status. This phenomenon shows some specificity, with no evidence for such cell death in the presence of plasmas from patients with DIC or asymptomatic HIV infection. Apoptosis was shown by three different methods. Multiple confirmatory assays for apoptosis are critical because there may not be a direct correlation between any single assessment and all the morphological and genomic criteria for this type of programmed cell death.55

The physiological nature of our EC culture system must be addressed. MVECs are normally in a resting state, with complete turnover on the order of 500 to 3,000 days, depending on the tissue examined. However, in TTP, marked MVEC proliferation is typical, and our ability to discriminate between MVEC and large vessel EC apoptosis is consistent with the pathology in vivo. In addition, evidence for an in vivo role for apoptosis in TTP comes from four types of data. First, as noted above, TTP is accompanied by inappropriate EC proliferation, with ECs often covering platelet thrombi. Endothelial hyperplasia may be so profuse that the thrombus appears to be subendothelial in origin.5 In many systems,16,20 cellular proliferation elicited by abnormal signals may preceed apoptosis. Second, TTP is characterized by a striking lack of inflammatory reaction, consistent with an apoptotic process. Third, in some of the microvasculature, enlargement of ECs with vacuoles, lysosomes, and mitochondria; aggregation of rounded cells; and other evidence of intense cellular activation characteristic of the early stages of apoptosis have been noted.46 Finally, an increase in circulating ECs, from a normal level of less than 1, to 5 to 37 cells per milliliter of blood, presumably representing detached but not lysed cells, has been observed during the acute phase of TTP and during relapse.47 A low (0 to 1 cell/mL) circulating
EC count correlated with clinical improvement after plasmapheresis. This may be the peripheral correlate of the description of "physically absent" ECs in involved microvasculature from TTP specimens.

Our observance of apoptosis in CD36+ and CD36- MVECs and its resistance to a TNF-α-neutralizing antiserum render these two factors identified in the blood of TTP patients (ie, anti-CD36 antibodies and elevated TNF-α levels) unlikely primary candidates to mediate apoptosis. Secondary enhancing roles for these factors may still be possible, particularly if TNF-α were elevated to the extremes observed in some HIV+ patients; this requires further study.

The ability of acute TTP plasmas to upregulate Fas in MVECs, but not in large vein ECs, and the capacity of soluble Fas MoAb to partially inhibit these processes, suggest that Fas cross-linking, perhaps related to elevated levels of soluble FasL in the circulation of TTP patients, may be etiologically involved. FasL, a type-II transmembrane protein, can induce apoptosis in Fas+ targets, because shedding of overexpressed ligand triggers cell death after Fas binding.

We are currently establishing an antigen capture system for FasL in an attempt to measure levels in control and TTP plasmas.

The lack of TTP-plasma-induced apoptosis in those UVECs that did express some baseline Fas may relate to threshold levels of Fas protein required for cross-linking, as well as to the need for concomitant sensitivity to induction of an apoptotic cascade by other factors in TTP plasma. This is consistent with the differential susceptibility of normal versus HIV-infected CD4+ T cells (both of which express Fas and FasL, but at different levels) to Fas-mediated apoptosis.

Fig 6. Fas expression in MVEC-2 ECs. RT-PCR for Fas and β-actin was performed on MVEC-2 in the presence or absence of various plasmas (1:100 dilution). Lane 1, control-1; lane 2, TTP-3 (HIV+); and lane 3, TTP-3 (HIV+) postplasmapheresis.

suggests effects on protease and/or Ca2+-dependent endonuclease activation. In this context, it is of interest that activated calpain proteinase has been associated with platelet microparticle formation in TTP plasma and that inhibitors of calpain, including ATA, have suppressed activation-induced apoptosis in T cells. The role of normal plasma in interfering in this process in unclear. It could block a protease or an EC-sensitizing factor in TTP plasma or provide a protective growth factor. Its ability to affect Fas expression is under investigation.

In conclusion, our data suggest that factors in TTP plasma can induce apoptosis in MVECs and that this process may be etiologically important. We postulate that one reason for the restriction of TTP lesions to the microvasculature is the differential response of large vessel ECs versus that of the MVECs to apoptosis-inducing TTP factors, rather than simply the raised fluid shear stresses characteristic of the microvessels. (Large vessels are not inherently resistant to apoptosis, with this process having been observed in in vivo and in vitro models for atherogenesis.) The inciting factor(s) for the TTP-associated process is still unknown but may be long-lived, because the projected recurrence rate for TTP after 10 years is almost 40%. Such a factor(s) appears to be present in both HIV+ and HIV- TTP patients, albeit the possibility of direct HIV infection of ECs in the former may be an additional event contributing to the enhanced development of TTP in HIV+ individuals.

Finally, manipulation of apoptosis is a potential new modality for the treatment of myriad disorders. The ability to remove the Fas-inducing and apoptosis-related factor(s) by plasmapheresis and to suppress the apoptotic process using plasma, anti-Fas MoAb, and ATA-like compounds suggests that inhibitors of EC apoptosis may prove fruitful in the design of new therapeutics for TTP. This type of work has gained new urgency after a recent report from the US Centers for Disease Control (Atlanta, GA) that, "We are left with the disquieting observation that TTP-associated mortality rates [apart from HIV-associated cases] increased approximately 2.5-fold during a period [1968-1991] when effective treatments became available...[and perhaps] by as much as 7.5-fold between 1972 and 1991...and we have no satisfactory explanation..."

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