Differential Regulation of Tissue Factor and Plasminogen Activator Inhibitor by Human Mononuclear Cells

By Bradford S. Schwartz and Jeffrey D. Bradshaw

Fibrin is a hallmark of immune-mediated tissue lesions. The presence of fibrin in such lesions implies both the formation of fibrin via coagulation and the accompanying restriction of fibrinolysis, allowing fibrin to persist. Previous work has shown that human monocytes exposed to an inflammatory stimulus such as lipopolysaccharide (LPS) produce both tissue factor (TF) and plasminogen activator inhibitor—type 2 (PAI-2). These two proteins favor fibrin deposition, and evidence implies that cellular production of these two molecules may be linked. Another proinflammatory process pertinent to immune-mediated tissue damage and fibrin deposition is the response to alloantigen. Peripheral-blood mononuclear cells (PBM), consisting of lymphocytes and monocytes together, responded to alloantigen stimulation with differential expression of TF and PAI-2. PBM exposed to alloantigen developed high levels of TF activity, with no concomitant increase in PAI-2 activity or antigen. Alloantigen-stimulated PBM did not accumulate intracellular PAI-2, nor did they degrade PAI-2 added to cultures. This lack of PAI-2 production was not due to inadequate stimulation, as tritiated thymidine uptake and TF production demonstrated recognition of, and a vigorous reaction to, alloantigen. The divergent TF and PAI-2 responses of PBM exposed to alloantigen was maintained over 5 days and was reflected by mRNA profiles. These results imply that under specific physiologically relevant conditions, the procoagulant and antifibrinolytic effectors of inflammatory mononuclear cells can be independently regulated. This would imply more flexibility to monocyte mechanisms that favor fibrin deposition than previously thought.

There are other processes wherein the inflammatory stimulus is more restrictive—ie, specific recognition of the stimulus by responding cells is required. Perhaps most notable is primary graft rejection, tissue lesions of which demonstrate variable amounts of fibrin deposition. There is an "inflammatory environment" among the mononuclear cells that mediate the reaction, and a number of effector molecules, including TF, have been found in such lesions, or in the in vitro correlate, the mixed lymphocyte culture (MLC). In contrast to the previously mentioned inflammatory mediators, which affect a broad population of responding cells, alloantigen stimulation requires specific recognition by a restricted population of T cells. The T cells then instruct monocytes to produce a specific effector—ie, TF. We used the MLC, since it is a good measure of immune recognition of alloantigens, to elucidate the regulation of expression of TF and PAI-2 by monocytes among peripheral-blood mononuclear cells (PBM) following a specific recognition event. We demonstrated that under the influence of this physiologically pertinent stimulus, the expression of TF and PAI-2 by human monocytes departs from previously described patterns and is uncoupled.

METHODS

Cell isolation and culture. Mononuclear cells were isolated from the peripheral blood of healthy donors as previously described. For experiments wherein TF or PAI-2 was to be measured, PBM were cultured in polypropylene tubes (Falcon, Lincoln Park, NJ) at the indicated concentrations in Roswell Park Memorial Institute (RPMI) Medium 1640 (GIBCO Laboratories, Grand Island, NY) containing 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, 25 mmol/L Hepes (Sigma Chemical Co, St Louis), pH 7.4, and 10% heat-inactivated fetal calf serum (FCS) (Hyclone Labs, Logan, UT). The PBM were cultured in 10% FCS until 24 hours before assay, at which time the publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

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Supported by Public Health Service Grants Nos. AI-20427 and HL 01870, and by a Grant-in-Aid from the American Heart Association and its Indiana Affiliate to Dr Schwartz.

Dr Schwartz is a recipient of a Research Career Development Award from the National Heart, Lung, and Blood Institute.

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This work was presented in abstract form at the American Federation for Clinical Research, May 1988, in Washington, DC.

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Blood Vol 74, No 5 (October), 1989: pp 1644-1650
cells were washed with, and resuspended in, an equal volume of RPMI 1640 without serum for an additional 24 hours. Culture supernatant was then removed, clarified by two minutes' centrifugation in a microfuge (Eppendorf, Brinkmann Instruments, Westbury, NY), brought to 0.01% in Tween 80 (Sigma), and either assayed for PAI immediately or frozen at −80°C until assayed. The cells that had been pelleted were washed and resuspended in 0.5 volume of RPMI 1640 for immediate assay of TF activity. Two-way MLCs to be subsequently assayed for TF or PAI-2 were established by combining equal numbers of PBM from each of two unrelated donors. Controls consisted of twice the number of PBM from each donor, cultured separately (ie, if the MLC had 5 × 10⁶ PBM from each donor, a control had 1 × 10⁶ PBM from a single donor). Hence, the total number of cells was the same in each culture. Tritiated thymidine ([³H]Tdr) uptake, as a measure of alloantigen recognition by T lymphocytes, was determined by incubating 1 × 10⁶ PBM from each of two donors together in 0.2 mL of RPMI 1640 plus 10% FCS in 96 well-round bottom plates for 5 days at 37°C (5% CO₂). Control cell cultures contained 2 × 10⁶ PBM per 0.2 mL from each donor, cultured separately. One μCi of [³H]Tdr (NEN/Dupont, Wilmington, DE) was added to each well. Eighteen hours later, the cells were harvested on a multiple automated sample harvester (MASH) device and assayed by liquid scintillation counting for [³H]Tdr uptake. All media were prepared with pyrogen-free water and stored in acid-washed, high-temperature baked glassware, and contained less than 0.01 ng/mL LPS, as assayed by the generation of monocyte TF activity and as compared with known quantities of LPS. This method is as sensitive as the Limulus assay (Sigma) for detecting LPS40 and has been calibrated in our laboratory against the Limulus assay. Such a monitoring method also provides an internal control for LPS contamination in our experiments.

**Tissue factor assay.** TF was measured as procoagulant activity and converted to milliunits of activity, exactly as previously described.14,20 Monoclonal anti-TF antibody in cell culture medium was kindly supplied by Dr James Morrissey of the Research Institute of Scripps Clinic.14 An isotype-matched monoclonal antibody raised against the large T antigen of SV40 virus (American Type Culture Collection [ATCC] No. TIB115)20 was used as a control. The antibodies were adjusted to equal protein concentrations. For neutralization of procoagulant activity by these antibodies, PBM were cultured for two days, either alone, with 1 μg/mL of LPS, or in a two-way MLC. The cells were washed and resuspended in RPMI and then incubated for 30 minutes at 37°C with the indicated dilutions of antibodies. The one-stage recalcification assay was carried out, and residual procoagulant activity was determined.

**PAI-2 assays.** PAI-2 activity was assayed according to the inhibition of known amounts of urokinase-type plasminogen activator (u-PA) (55 Kd human urinary u-PA was kindly supplied by Dr Gene Murano of the Bureau of Biologics) or 33 Kd human u-PA (Abbott, North Chicago, IL). An 121I-fibrin−coated tissue culture plate assay was carried out as previously described.42 PAI-2 activities using this assay method are given as percent inhibition of a fixed amount of u-PA. We also measured u-PA activity using the chromogenic substrate pyrogly-gly-arg-p-nitroanilide (Sigma).42 A 50 μL sample of cell culture supernate or cell extract, suitably diluted with 0.14 mol/L NaCl, 0.05 mol/L imidazole (pH 7.4), 1 mg/mL bovine serum albumin (BSA; Sigma), and 0.01% Tween 80, was mixed with 0.1 units of u-PA in 50 μL. After a 30-minute incubation at 37°C, 100 μL of 0.6 mmol/L pyrogly-gly-arg-p-nitroanilide in 0.1 mol/L Tris-HCl (pH 8.8) was added. The absorbance at 410 nmol/L was measured with an ELISA reader, and the results were compared with those of a standard curve constructed with known amounts of u-PA. The inhibitor activity of the sample was defined as the difference between the original activity of u-PA and the remaining activity found after mixing with PAI-2, with any dilutional factor accounted for. One milliunit (mU) of PAI-2 inhibits one mU (international units) of u-PA.

A radioimmunoassay (RIA) for PAI-2 was carried out according to Kruihof et al.23 Dr Kruihof was kind enough to supply us with purified PAI-2, as well as suitable antisera monospecific for PAI-2.24 PAI-2 was iodinated by the iodogen method.24 Standard curves for the RIA were constructed using PAI-2, and PAI-2 complexed to u-PA. Complete u-PA-PAI-2 complex formation for the samples used in generating the standard curve was verified by sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) and autoradiography of iodinated proteins. The culture supernatant samples to be tested were diluted appropriately in 0.2 mol/L NaCl, 10 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 7.4), and 0.1% albumin and incubated four hours at room temperature with an equal volume of rabbit antihuman PAI-2 IgG, 9 μg/mL. Fifty microtiter dishes of 121I-PAI-2 containing 2,100 cpm was added, and the mixtures were incubated with rotation overnight at 4°C. Antigen antibody complexes were precipitated by a 30-minute incubation at room temperature with 100 μL of a 30% suspension of goat-antirabbit IgG linked to agarose beads (Sigma). The mixtures were centrifuged, the agarose beads were washed two times with 3 mL of 0.15 mol/L NaCl, and radioactivity bound to the agarose beads was counted in a gamma counter.

Immuno-dot-blotting analysis was carried out as previously described,4 using rabbit anti-PAI-2 kindly supplied by Dr T-C Wun of Monsanto.42 As noted, SDS-PAGE destroyed the antigenic reactivity of PAI-2 derived from native human monocytes; hence, dot blotting was required.4

For analysis of intracellular PAI-2, a lysis buffer consisting of 0.1 mol/L NaCl, 0.01 mol/L Tris-HCl (pH 7.4), and 0.5% NP-40 was used. Preliminary experiments showed that this buffer did not inhibit either PAI-2 functional activity or antigenic detection of PAI-2 using the dot-blotting procedure.

**Northern blot analysis of mRNA.** PBM were cultured at 2.0 × 10⁶/mL in RPMI 1640 with 10% FCS for the times indicated. PBM were then centrifuged, and total cellular RNA was isolated using the guanidinium thiocyanate-phenol-chloroform extraction method of Chomczynski et al.43 Five μg of total cellular RNA per lane was electrophoresed through formaldehyde-agarose, blotted-transferred to Zeta-probe membranes (BioRad, Richmond, CA), and immobilized by exposure to ultraviolet irradiation. Equal loading and transfer of RNA in the cells was verified by intensities of the 28S and 18S ribosomal bands under ultraviolet light. The blots were analyzed by probing with random primer-labeled 24 cDNA probes to TF and PAI-2 (kindly supplied by Drs James Morrissey45 and Evan Sadler,41 respectively) and washed at high stringency in 40 mmol/L NaHPO₄, 1 mmol/L EDTA, and 1% SDS. Filters were exposed to x-ray film at −70°C without an intensifying screen. The observed mRNA levels were indicative of the content of mRNA in monocytes among the PBM, as separation of PBM into monocytes and lymphocytes demonstrated that only monocytes contained mRNA for TF and PAI-2.

**RESULTS**

MLCs were monitored for the fibrinolytic inhibitor PAI-2. As seen in Table 1, no PAI-2 activity above control levels was produced by PBM exposed to alloantigen. This was true regardless of the PBM donors or pairings (nine experiments, seven donor pairings). This lack of PAI-2 production was not due to poor alloantigen stimulation, as the MLCs demonstrated robust H-TdR incorporation, a known indicator of response to alloantigen (Table 1). The PBM also expressed high levels of TF in response to alloantigen (Table 1), further
suggesting an adequate stimulatory signal. To rule out the possibility that the PBM were unable to produce PAI-2, cells from each donor were cultured with LPS, a stimulus known to concomitantly induce TF and PAI-2 production in human PBM.4 Cells from all donors demonstrated increased release of PAI-2 in response to LPS (Table 1), indicating their ability to produce PAI-2 if properly stimulated.

The above PAI-2 assays were based on inhibitory activity alone. It was possible, therefore, that increased amounts of PAI-2 were being produced but were inactive, either through complex formation with u-PA or through some other mechanism. We therefore constructed an RIA for PAI-2 that was able to detect PAI-2 either free or complexed with u-PA (Fig 1). No increase in PAI-2 antigen was seen in culture supernatants from alloantigen-stimulated cells as compared with control cells (Fig 1). In contrast, supernatants from LPS-stimulated PBM contained PAI-2, which generated a parallel curve and complete inhibition of antibody binding to purified PAI-2 in the RIA, indicating the ability of this assay to detect the molecule if it is present.

Since monocytes and U-937 cells have been shown to accumulate appreciable PAI-2 levels intracellularly,12 we measured both intracellular and released PAI-2. Whereas LPS-stimulated PBM both released, and contained appreciably more PAI-2 than control PBM, alloantigen-stimu-

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Table 1. PAI-2 and Tissue-Factor Responses of PBMs After Five Days in a Two-Way Allogeneic MLC

<table>
<thead>
<tr>
<th>PAI-2 (%) Inhibition of 50 mU u-PA/0.1 mL 10^6 PBMs</th>
<th>Tissue Factor (mU Activity/10^6 PBMs)</th>
<th>^3H-TdR Uptake (CPM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor A 1 ± 1.7</td>
<td>5.7 ± 2.2</td>
<td>1312 ± 602</td>
</tr>
<tr>
<td>Donor B 1 ± 1.3</td>
<td>5.0 ± 4.6</td>
<td>1943 ± 1383</td>
</tr>
<tr>
<td>Donor A + Donor B 1 ± 1.6</td>
<td>148 ± 72</td>
<td>30,168 ± 10,621</td>
</tr>
<tr>
<td>Donor A + LPS 70 ± 11</td>
<td>63 ± 31</td>
<td>—</td>
</tr>
<tr>
<td>Donor B + LPS 57 ± 19</td>
<td>71 ± 48</td>
<td>—</td>
</tr>
</tbody>
</table>

Data are x ± SD. N = 9 experiments.
*2 x 10^6 PBM in 0.2 mL RPMI 1640 + 10% FCS for the entire 120 days.
†Total of 1 x 10^6 PBM/mL, 96 hours in RPMI + 10% FCS, then 24 hours in RPMI alone.
‡1 µg/mL LPS added at initiation of culture.

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![Fig 1](https://via.placeholder.com/150)

**Fig 1.** Levels of PAI-2 antigen are not increased in culture supernatants of alloantigen-stimulated PBM. An RIA (see Methods section) demonstrated that free PAI-2 (0—0), PAI-2 complexed with u-PA (O—O), and supernatant of LPS-stimulated PBM (x—x) inhibited precipitation of ^125I-PAI-2 by anti-PAI-2 in a like manner (the x—x curve was generated with supernatants of PBM that had been cultured at 5 x 10^6/mL with 1 µg/mL LPS for 24 hours to demonstrate that PAI-2 produced by native monocytes would completely inhibit ^125I-PAI-2 binding to the antibody in this assay). Hence, this RIA was suitable for detecting PAI-2 of human PBM, either free or complexed with u-PA. Supernatants of 2 x 10^6 nonstimulated (B—B), alloantigen-stimulated (O—O), or LPS (1 µg/mL) stimulated (△—△) PBM (5-day cultures). Values for PBM of the other donor for the mixed lymphocyte reaction were quite similar but were left out for the sake of clarity of the figure.

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![Fig 2](https://via.placeholder.com/150)

**Fig 2.** Alloantigen stimulation does not result in increased levels of either intracellular or released PAI-2. Supernatants and cell lysates of 2 x 10^6 PBM, cultured for 5 days under the indicated conditions, were assayed for their ability to inhibit u-PA activity. Inhibition was determined using a chromogenic substrate assay, as described in Methods.

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labeled cells showed no such increase in either pool (Fig 2). Paralleling the results of the activity assays, alloantigen-stimulated PBM demonstrated no increase in intracellular PAI-2 antigen as assessed by immunoblotting. Hence, the lack of PAI-2 response to alloantigen stimulation was not due to failure to detect the molecule because of impaired secretion or release.

The lack of measurable PAI-2 in supernatants of PBM that were by other indicators responding to alloantigen (ie, proliferating, producing TF) raised the possibility that these cells were in some manner degrading or inactivating PAI-2. To test for this, a known amount of PAI-2 was added to either conditioned media from an MLC or the MLC itself. In neither case was there any evidence of degradation or inactivation of PAI-2.

Since a response can vary depending on the intensity of stimulus, lymphoblastoid cell lines were established and used as stimulators for an alloantigen response.13 These cells expressed no TF activity or PAI-2 activity or antigen over five days of culture, nor did they inactivate PAI-2. However, they stimulated a dose-dependent mitogenic response from allogeneic PBM. Hence, they were ideal for use as stimulator cells in this system. Increasing numbers of irradiated stimulator cells were incubated with a constant number of PBM for five days, at which time PAI-2 and TF activities were assayed. The responding PBM demonstrated an increase in TF that was dependent on the "dose" of alloantigen they were exposed to (Fig 3). In contrast, there were no increases in PAI-2 antigen or activity, either secreted or cell-associated.

To determine the temporal aspects of TF and PAI-2 production, time-course experiments were carried out using PBM stimulated with LPS, alloantigen, or RPMI alone (controls). Figure 4A shows the PAI-2 response, and 4B the TF response, over 5 days. PBM demonstrated a robust TF response to alloantigen; hence, there was recognition of the stimulus. The virtual absence of PAI-2, however, strongly suggests that production of the two molecules is not linked. Both effectors were increased in PBM cultures exposed to LPS, suggesting that the cells were able to produce PAI-2 and TF at the same time if appropriately stimulated. However, the different temporal patterns of production after LPS stimulation also suggested different regulatory pathways.
Northern blot analysis of steady-state mRNA levels for TF and PAI-2 demonstrated mRNA patterns similar to the profiles seen for expression of the proteins (Fig 5). LPS-stimulated PBM demonstrated early, transient expression of TF mRNA. In contrast, the increase in PAI-2 mRNA was maintained over 5 days, as was PAI-2 protein production (compare Figs 4 and 5). In alloantigen-stimulated PBM, TF mRNA increased from 24 to 96 hours and decreased by 120 hours, a pattern that was reflected by expression of the protein. PAI-2 mRNA, on the other hand, was evident at four and 14 hours but was undetectable by 48 hours. Interestingly, we were unable to detect PAI-2 antigen or activity among these PBM. Monocytes among PBM cultured in RPMI alone for prolonged times (two to five days) expressed low levels of TF mRNA without added stimulus; no PAI-2 mRNA was seen at these times.

To be certain that the coagulant activity being monitored throughout the above experiments was indeed TF, we employed two different neutralizing monoclonal antibodies to human TF. As seen in Fig 6, alloantigen-stimulated PBM demonstrated coagulant activity that was progressively inhibited by increasing doses of antibody to TF. Use of a second monoclonal anti-TF antibody gave similar results. Likewise, the coagulant activity of LPS-stimulated PBM was completely inhibited. An isotype-matched irrelevant monoclonal antibody did not inhibit the coagulant activity of such stimulated PBM. In addition, the coagulant activity of alloantigen-stimulated PBM was dependent on plasma coagulation factors II, X, and VII but was independent of factors VIII and IX. Hence the measurements of coagulant activity were indeed measurements of TF activity.

DISCUSSION

The present study was undertaken to explore the concomitant regulation of the prothrombotic molecules TF and PAI-2 by monocytes taking part in an immune response to alloantigen. The response to alloantigen first requires T cells to perceive a difference between the type II histocompatibility molecules of themselves and the cells to which they are exposed. T cells thus stimulated elaborate a number of molecules, among which are those that instruct monocyte/macrophages to produce TF. It was notable that monocytes among PBM exposed to alloantigen
did not produce increased amounts of PAI-2 concomitantly with TF. The fact that 3H-TdR incorporation had taken place showed that the T cells among the PBM did indeed recognize the “nonself” alloantigen. The elaboration of high levels of TF by these PBM corroborated that recognition and demonstrated that T-cell communication to monocytes also occurred. The fact that monocytes among LPS-stimulated PBM produced PAI-2 signifies that the monocytes were capable of producing this effector if properly stimulated.

Although monocytes are the cells that produce both PAI-2 and TF in response to LPS,4-6 we have used whole PBM for several reasons. First, monocytes and lymphocytes are both present at scenes of alloantigen stimulation.37 Second, monocyte TF production in response to alloantigen is strictly T-cell–dependent.36-38 Exploring the comparative regulation of these two monocyte-derived molecules in the setting of alloantigen stimulation therefore required the presence of lymphocytes.

The initial observation of TF induction in the absence of a concomitant PAI-2 response was confirmed by an RIA. Immunologic quantitation is important, as PAI-2 can form complexes with u-PA (which is also produced by monocytes) and be undetectable in activity assays.55 The RIA for PAI-2 antigen was able to detect the molecule complexed to u-PA, yet it did not detect PAI-2 among alloantigen-stimulated PBM. As both activity and antigen assays were in agreement, it is extremely unlikely that PAI-2 was present but not detected.

It has been shown that there are two pools of PAI-2, intracellular and extracellular.32 However, assays for both pools consistently demonstrated no increase in PAI-2 among PBM shown to be producing TF, implying there was not an undetected, or “cryptic,” PAI-2 response. In addition, there was no evidence that PAI-2 was being inactivated or metabolized by PBM exposed to alloantigen. This was not, then, a case of accelerated PAI-2 turnover masquerading as lack of production.

Further evidence of differential regulation of TF and PAI-2 by monocytes was seen in the differing temporal patterns of responses to LPS, a stimulus known to induce increases of both molecules.4 In addition, the profiles of steady-state mRNA levels were clearly different for TF and PAI-2, regardless of the stimulus. If production of these two molecules were indeed linked, a concordance of temporal patterns at both the mRNA and protein levels would be expected.

Several aspects of the mRNA profiles deserve further attention. First, alloantigen-stimulated PBM exhibited appreciable levels of PAI-2 mRNA early in the MLC. However, no PAI-2 protein could be detected. This may be due to failure to transport the mRNA from the nucleus, or lack of translation for another reason. This implies a control mechanism at the step of PAI-2 translation, as has been described for tumor necrosis factor, another inducible monokine.56 Second, LPS-stimulated PBM demonstrated PAI-2 mRNA levels that stayed elevated for at least 5 days, whereas TF mRNA levels decreased rapidly after an early elevation. This is of interest because both PAI-2 and TF mRNAs have several repeats of an AU-rich sequence that lends relative instability to mRNAs in their 3’ non-translated regions.30,40,41 The correlation of TF protein to mRNA does not, from these limited data, suggest a regulatory step at translation. However, the more gradual decline in TF protein compared to its mRNA suggest that the TF gene product is a relatively long-lived protein.

That production of TF and PAI-2 can be uncoupled in response to a physiologic stimulus would be expected to lend flexibility to the hemostatic aspect of the immune response. This observation is also consistent with the lesser amount of fibrin persistence seen in the early lesions of primary allograft rejection, as opposed to the lesions of disseminated intravascular coagulation secondary to endotoxemia.33,42 Elucidation of the regulatory pathways for TF and PAI-2 should shed light on their relative roles in the fibrin deposition seen as part of the immune response.

ACKNOWLEDGMENT

We thank Marcy McCormick for her expert and patient secretarial help.

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