FDP D-Dimer Induces the Secretion of Interleukin-1, Urokinase-Type Plasminogen Activator, and Plasminogen Activator Inhibitor-2 in a Human Promonocytic Leukemia Cell Line

By Motohiro Hamaguchi, Yoshihisa Morishita, Isao Takahashi, Michinori Ogura, Junki Takamatsu, and Hidehiko Saito

We studied the effect of fibrinogen degradation products D, E, and D-dimer on a human promonocytic leukemia cell line, NOMO-1. After exposure to a 10⁻⁵-mol/L fragment D or D-dimer, the cells displayed macrophage-like characteristics, such as adherence to plastic surfaces, and showed approximately a twofold increase in response to the nitroblue tetrazolium reduction test. The secretion of interleukin-1α (IL-1α) into the medium was markedly stimulated by a 10⁻³-mol/L fragment D, E, and D-dimer, whereas a significant increase in IL-1β secretion was observed only in D-dimer-stimulated cells. In addition, D-dimer induced a rapid increase in urokinase-type plasminactivator on day 1 (0.52 ± 0.02 ng/mL vs 0.07 ± 0.01 ng/mL in the control culture) and a slow increase in plasminogen activator inhibitor-2 on day 5 (3.9 ± 1.6 ng/mL vs 1.2 ± 0.2 ng/mL in the control culture). An increase in tissue factor (TF) was also demonstrated on the cell surface of NOMO-1 cells exposed to fragment D or D-dimer by indirect immunofluorescence using an anti-TF monoclonal antibody. Scatchard plot analysis showed that fragment D and D-dimer bound to the NOMO-1 cells with a kd of 33 nmol/L and 2.7 nmol/L, respectively. These results suggest that fragment D-dimer specifically stimulates cells of monocyte-macrophage lineage to secrete key substances that regulate blood coagulation, fibrinolysis, and inflammation.

© 1991 by The American Society of Hematology.

MATERIALS AND METHODS

Purification of F(g)DPs. Fragments D, E, and D-dimer were prepared from human fibrinogen (Kabi Co, Stockholm, Sweden) as previously described.¹ Purified fragments D, E, and D-dimer were dialyzed against 0.15 mol/L physiologic saline (pH 7.4), containing 5 mmol/L epsilon amino caproic acid (EACA) (Sigma Chemical Co, St Louis, MO) and 5 mmol/L CaCl₂. The purity of the fragments was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were run on 7.5% polyacrylamide gels in PhastGel with PhastSystem (Pharmacia LKB, Uppsala, Sweden). Single bands were observed at 85 Kd, 5 mmol/L epsilon amino caproic acid (EACA) (Sigma Chemical Co, St Louis, MO) and 5 mmol/L CaCl₂. The purity of the fragments was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were run on 7.5% polyacrylamide gels in PhastGel with PhastSystem (Pharmacia LKB, Uppsala, Sweden). Single bands were observed at 85 Kd, 50 Kd, and 188 Kd for fragments D, E, and D-dimer, respectively, after staining with Coomassie brilliant blue G-250 (Fluka AG, Buchs, Switzerland).

Cell culture. NOMO-1 cells (1 x 10⁶/mL) were grown in a chemically defined, serum-free medium, COSMEDIUM-001 (Cosmo Bio Co, Tokyo, Japan) supplemented with 1% bovine serum albumin (BSA) in Falcon 3047 plastic tissue culture flasks (Falcon, Division of Becton Dickinson, Oxnard, CA). No fetal calf serum (FCS) was added to the culture medium. The cells were incubated for 1, 3, and 5 days with or without F(g)DPs at a final concentration of 10⁻⁴ mol/L. At the indicated times, 2 mL of medium was removed from each flask and centrifuged. The supernatants were stored at −80°C until they were analyzed.
Endotoxin. Contamination by endotoxin was measured using the Toxicolor test (Seikagakukogyo, Ltd, Tokyo, Japan). Lipid A, LA-15-PP(506) (Daichikagaku Pharmaceutical, Co, Ltd, Tokyo, Japan), was used as the endotoxin.

Morphologic studies. Morphologic observations of live cultured cells were performed with an Olympus (Tokyo, Japan) inverted microscope. Cytospin slide preparations of 0.2-mL aliquots of cell suspensions were prepared by using a Shandon Cytospin 2 centrifuge (Shandon Southern Products, Ltd, Cheshire, England) and stained with May-Grünwald-Giemsa (MGG).

Nitroblue tetrazolium (NBT) reduction test. NBT reduction test was performed according to the method of Park et al. Briefly, the cells were washed and resuspended in RPMI 1640 (GIBCO, Grand Island, NY) containing 10% FCS (Flow Laboratories, Stanmore, New South Wales, Australia) and 1% NBT. The cells were then incubated with $10^{-7}$ mol/L TPA (Paesel GmbH Co, Frankfurt, West Germany) or 0.5 mg/mL opsonized zymosan (Sigma) for 30 minutes at 37°C. The cells were then spun on slides and examined under a microscope. The opsonized zymosan was prepared as described above.19

Determination of IL-1α, IL-1β, u-PA, PAI-1, and PAI-2 antigens. Immunoreactive IL-1α was determined by an enzyme-linked immunosorbent assay (ELISA) kit (Ohutaka Pharmaceutical Co, Tokushima, Japan) according to the manufacturer’s protocol. Immunoreactive IL-1β, u-PA, PAI-1, and PAI-2 were also measured by ELISA kits (Biopool, Stockholm, Sweden).

Immunofluorescence study. The reactivity of NOMO-1 cells with a monoclonal antitissue factor (TF) antibody A1-312 (kindly supplied by Dr F.R. Rickles, Department of Medicine, The University of Connecticut Health Center School of Medicine, Farmington) was examined by indirect immunofluorescence.14

F(g)DP binding assay. Radioionation of F(g)DP was performed by the solid-state lactoperoxidase method.19

NOMO-1 cells were grown in RPMI-1640 with 10% FCS. Cells were grown to confluency, trypsinized (trypsin-EDTA, 1 g/L), and pelleted by centrifugation at 500g for 5 minutes at 25°C. Cells were washed and resuspended in Tyrode’s buffer (NaCl at 8 g/L, KCl at 0.2 g/L, NaHCO$_3$ at 1.0 g/L, NaH$_2$PO$_4$ at 0.05 g/L, MgCl$_2$·6H$_2$O at 0.2 g/L, dextrose at 1.0 g/L, and 2% BSA). Aliquots of 250 μL containing 0.5 × 10$^6$ cells were added to Eppendorf tubes. Ten microliters of human F(g)DP binding assay.

Morphologic changes of NOMO-1 cells after exposure to F(g)DPs. NOMO-1 cells usually proliferate as a suspension of single cells without forming clumps. But by the fifth day of exposure to fragments D, E, or D-dimer (10$^{-5}$ mol/L), the growth of the cells decreased by approximately 15%, 0%, and 25%, respectively, as compared with control cultures. However, the cell viability remained high (determined to be 82% to 92%) during the 5-day incubation period. Examination of the cells with an inverted microscope showed that the control NOMO-1 cells grew as a single cell suspension, but the cells exposed to fragment D-dimer were loosely clumped and weakly attached to the plastic surface of the incubation flasks (data not shown).

Effect of F(g)DPs on superoxide anion production and phagocytosis. To examine the effect of F(g)DPs on superoxide anion production, NBT reduction tests were performed using NOMO-1 cells cultured with 10$^{-5}$ mol/L F(g)DPs for 3 days (Table 1). Without exposure to F(g)DPs the cells had no ability to reduce NBT. After a 30-minute incubation with 10$^{-7}$ mol/L TPA, 9.3% ± 2.3% of NOMO-1 cells reduced NBT. Cultures that had been incubated with fragment D or D-dimer (for 30 minutes) showed approximately a twofold increase in the number of cells that reduced NBT. Fragment D did not stimulate superoxide anion production in NOMO-1 cells. Similar results were obtained using opsonized zymosan as a stimulator. Incubation of NOMO-1 cells with fragment D or D-dimer did not influence the phagocytic activity of the cells (data not shown).

F(g)DPs stimulate the production of IL-1α and IL-1β. To determine whether NOMO-1 cells produce or secrete various cytokines on F(g)DP stimulation, the concentration of IL-1 in the supernatant of culture incubated with F(g)DPs was determined. In the control culture, IL-1α increased slightly from 1.4 ± 0.4 pg/mL on day 1 to 5.4 ± 1.2 pg/mL on day 5. The release of IL-1α was significantly stimulated after 1, 3, and 5 days of incubation with all three F(g)DPs at 10$^{-3}$ M ($P < .01$) (Fig 2A). Fragment D-dimer was the most potent stimulator, increasing IL-1α secretion more than six times the level secreted by control cells. When fragment D was added at concentrations of 10$^{-8}$, 10$^{-7}$, and 10$^{-6}$ mol/L, the concentration of IL-1α in the medium on day 5 was 7.0, 10.5, 15.6, and 23.7 pg/mL, respectively. However, 10$^{-5}$ mol/L fibrinogen had no effect on the secretion of IL-1α (7.0 ± 1.3 pg/mL).

In contrast to the stimulation of IL-1α secretion, IL-1β secretion was increased only by the addition of fragment D-dimer to the culture medium (37.7 ± 1.2 pg/mL) ($P < .01$). Neither of F(g)DPs increased IL-1β secretion at
all during the 5-day incubation period (Fig 2B). Note that the control cells secreted more IL-1β than IL-1α, just as in normal blood monocytes.16

Endotoxin is known to stimulate IL-1 release from monocytes,17 and small amounts of endotoxin are usually present in many reagents. In fact, about 80 ng/mL endotoxin (final concentration) was found to contaminate our purified F(g)DPs. Although 80 ng/mL endotoxin should be too low to induce IL-1 release based on the results reported in reference 16, it seemed possible that the results reported above might have been due to the presence of endotoxin in F(g)DPs. To exclude this possibility we tested the effect of 0.2 and 2 µg/mL (final concentration) lipid A on IL-1α secretion in NOMO-1 cells. No increase in the secretion of IL-1α by day 5 (5.4 ± 0.9 pg/mL) was found in cells treated with either concentration of lipid A as compared with control cultures.

To show that the increase in IL-1 was specifically induced by D-dimer and not some other compound in the D-dimer preparation, we passed the D-dimer preparation over columns of sepharose 4B coupled to anti-FSP D IgG or anti-FSP E IgG. The increase in IL-1α and IL-1β induced by D-dimer was completely abolished by pretreating the D-dimer preparation with an anti-FSP D IgG column. In contrast, treatment of the D-dimer preparation with anti-FSP E IgG column did not inhibit the effect of D-dimer on IL-1 levels (Table 2).

D-dimer–mediated induction of IL-1α appears to require functional protein synthesis. NOMO-1 cells were incubated with D-dimer in the presence of the protein synthesis inhibitor cycloheximide (C-6255) (Sigma). D-dimer–induced IL-1α secretion was inhibited (>90%) by the 10 µg/mL cycloheximide in the culture medium.

**Table 1. NBT Reduction Test**

<table>
<thead>
<tr>
<th>Stimulator</th>
<th>TPA (10⁻⁷ mol/L)</th>
<th>Opsonized Zymosan (0.5 mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.3 ± 2.3</td>
<td>6.9 ± 4.3</td>
</tr>
<tr>
<td>Fragment D</td>
<td>22.0 ± 8.3</td>
<td>17.7 ± 2.6</td>
</tr>
<tr>
<td>Fragment E</td>
<td>12.3 ± 4.2</td>
<td>11.3 ± 1.2</td>
</tr>
<tr>
<td>Fragment D-dimer</td>
<td>20.5 ± 4.3†</td>
<td>16.2 ± 4.1</td>
</tr>
</tbody>
</table>

*Mean ± SD, three separate experiments.  
†P < .05 v control.

**Differential effect of F(g)DPs on the secretion of u-PA and PAI-2.** u-PA and PAI-2 have opposite biologic activities. However, both are considered to be markers of activation or differentiation of monocytes and macrophages.18 The presence of these two substances was determined in culture supernatants of NOMO-1 cells incubated with 10⁻⁵ mol/L F(g)DPs. D-dimer enhanced the secretion of u-PA within 24 hours of its addition to the culture. Subsequently, the concentration of u-PA decreased from 0.52 ± 0.02 ng/mL on day 1 to 0.22 ± 0.07 ng/mL on day 5 (Fig 3A). A similar but less apparent increase was found in the supernatant of cultures incubated with fragment D or E. PAI-2 secretion was also stimulated by D-dimer (Fig 3B). However, fragments D and E did not influence PAI-2 secretion. The concentration of PAI-2 was much higher than that of u-PA during F(g)DPs-stimulation.

PAI-1 was not detected in the supernatants of cells treated with F(g)DPs.

**Immunofluorescence study of TF.** By indirect immunofluorescence using a monoclonal antibody, TF antigen was shown to be on the cell surface of NOMO-1 cells cultured with fragment D or D-dimer (Fig 4). TF antigen was exhibited by 20.7% ± 1.2% of the control cells, whereas 56.7% ± 4.2% and 69.3% ± 7.0% of cells, which had been cultured for 3 days with 10⁻⁵ mol/L fragment D or D-dimer,
respecitively, expressed measurable levels of TF antigen ($P < .01$). It is also noteworthy that the fluorescent intensity of these cells was stronger than that of control cells. Fragment E did not increase the percentage of the TF-positive cells nor their fluorescent intensity.

**F(g)DP binding assay.** The results of binding studies, where human $^{125}$I-labeled fragment D or $^{125}$I-labeled fragment D-dimer was mixed with NOMO-1 cells, are shown in Fig. 5. Scatchard analysis showed that $^{125}$I-labeled fragment D and $^{125}$I-labeled fragment D-dimer bound with a $k_d$ of 3.3 nmol/L and 2.7 nmol/L, respectively. Under identical conditions $^{125}$I-labeled fragment E binding occurred at low levels, less than 4% of those of the other fragments (data not shown).

**DISCUSSION**

In the present study, we investigated the interaction between fibrinolysis products, F(g)DPs, and a monocyte-macrophage system. We demonstrated that D-dimer specifically stimulates a human promonocytic leukemia cell line, NOMO-1, to induce the secretion of IL-1, u-PA, and PAI-2. An increasing expression of TF was also observed on the surface of cells incubated with F(g)DPs. NOMO-1 cells were used as a model of monocyte function because they have many properties of promonocytes and are available in large amounts without contamination from other cells. The stimulation effect of F(g)DPs does not appear to be due to endotoxin present in the F(g)DP preparations, because the addition of as much as 2 pg/mL lipid A did not significantly increase IL-1α secretion. Additionally, the treatment of D-dimer with anti-FSP D serum before incubation with the cells completely abolished the stimulation effects.

Among three fragments purified from human fibrinogen or fibrin, D-dimer was found to be the most potent stimulator of NOMO-1 cells. Both fragment D and D-dimer had positive effects on IL-1α, u-PA, and TF production and on NBT reduction. However, D-dimer was the only agent capable of stimulating secretion of IL-1β and PAI-2. Fragment E exerted no effect on NBT reduction, TF expression, or the secretion of IL-1β or PAI-2. These observations are consistent with the effects of FDP on hepatocytes where the fragment D, but not fragment E, had the ability to stimulate the production of fibrinogen. These differential effects may be due to the expression of F(g)DP receptors. Fragments D and E are recognized by separate receptor systems in murine macrophages, while fragments D and D-dimer are recognized by the same receptor. To evaluate the interaction of F(g)DPs and NOMO-1 cells, we determined the binding affinity of radiolabeled F(g)DPs. These studies clearly showed that labeled fragment D and
Fig 3. Effects of F(g)DPs on the secretion of u-PA and PAI-2. (A) u-PA; (B) PAI-2. Values are plotted as means $\pm$ SD of three separate flasks.

Fig 4. Immunofluorescent staining of NOMO-1 cells using an anti-TF monoclonal antibody. (A) Control; (B) cultured with fragment D (10$^{-5}$ mol/L); (C) cultured with fragment E (10$^{-5}$ mol/L); (D) cultured with fragment D-dimer (10$^{-5}$ mol/L). (Original magnification $\times$ 400.)
THE INTERACTIONS OF FDP AND MONOCYTES

The production of IL-1 is regulated by TPA, lipopolysaccharide activity in inflammatory and immunologic responses. IL-1 has been identified as a chemical mediator released from monocytes/macrophages and exhibits important biologic activity in inflammatory and immunologic responses. The production of IL-1 is regulated by TPA, lipopolysaccharide (LPS), and tumor necrosis factor-α (TNF-α). The present study demonstrates that F(γ)DPs, especially D-dimer, upregulate IL-1 production in NOMO-1 cells. This regulation appears to be physiologically relevant, because the concentration of D-dimer used in our experiments, 10⁻² mol/L, roughly corresponds to the levels of F(γ)DPs found in the blood of patients with severe disseminated intravascular coagulation (DIC). Because F(γ)DPs may be produced at the site of inflammation, they may represent the regulatory substances that modulate the release of chemical mediators by monocytes.

De novo protein synthesis is required for the secretion of IL-1α and IL-1β by F(γ)DPs, because the stimulatory effect of D-dimer was blocked by cycloheximide. In human peripheral blood monocytes and the monocytic cell line THP-1, pro-IL-1β synthesis was regulated by TPA or LPS at the level of gene transcription. Only minor changes were observed in IL-1α and IL-1β messenger RNAs (mRNAs) in NOMO-1 cells incubated with D-dimer (data not shown). This result may be due to the weak stimulatory effect of D-dimer on monocytes in comparison with TPA or LPS.

It has been reported that the production of u-PA and PAI-2 by human or murine monocytes/macrophages is regulated by TPA or LPS. u-PA and PAI-2 were also found to be produced by the cells of the human histiocytic lymphoma cell line U-937, and a 50-fold molar excess of PAI-2 over u-PA is found in conditioned media of TPA-treated cells. The mechanism controlling u-PA and PAI-2 secretion from monocytes is largely unknown. On treatment with D-dimer, the level of PAI-2 was approximately 10-fold higher than that of u-PA. Therefore, the activation of monocytes or macrophages by D-dimer may result in the inhibition of fibrinolysis.

Resting intact monocytes and endothelial cells express very little TF activity. However, on exposure to a variety of stimuli including LPS or TPA both endothelial cells and monocytes produce TF activity. Unstimulated U-937 cells have also been shown to possess a small amount of TF activity, which after stimulation with LPS or TPA increases several fold. The fact that fragment D and D-dimer enhance the expression of TF is interesting, because the products of fibrinolysis appear to promote blood coagulation.

These interactions suggest another link between blood coagulation, fibrinolysis, and inflammation. Further studies are required to elucidate the signal transduction pathways and the molecular mechanisms underlying the cellular responses to F(γ)DPs. NOMO-1 cells may be a useful system for these studies.

ACKNOWLEDGMENT

We thank Sayoko Sugiura for skillful technical assistance.

REFERENCES

5. Boci V, Pacini A: Factors regulating plasma protein synthes-

s. II. Influence of fibrinolytic products on plasma fibrinogen concentration. Thromb Diath Haemorrh 29:63, 1973


FDP D-dimer induces the secretion of interleukin-1, urokinase-type plasminogen activator, and plasminogen activator inhibitor-2 in a human promonocytic leukemia cell line

M Hamaguchi, Y Morishita, I Takahashi, M Ogura, J Takamatsu and H Saito