DIGESTION of human fibrinogen by plasmin produces a set of terminal degradation products, fragments D and E. Plasmin digestion of cross-linked fibrin, as opposed to fibrinogen, leads to the formation of fragment D-dimer, which consists of two D moieties with cross-linked gamma chains, and fragment E. These fibrin and fibrinogen degradation products (FDPs and FgDPs or collectively referred to as F(g)DPs) appear to exert diverse effects on inflammatory processes and fibrinogen synthesis. It has been shown that elevated plasma F(g)DPs inhibit several functions of polymorphonuclear leukocytes critical to the bactericidal activity of these inflammatory cells. The hepatic synthesis of fibrinogen, an acute-phase reactant, is stimulated by plasmin-derived fragments of fibrinogen and fibrin. It has recently been demonstrated that fragment D, but not fragment E, directly and specifically stimulates rat hepatocytes to produce fibrinogen. Other results suggest that fibrinogen production may be controlled by regulatory proteins produced by blood monocytes in response to fragments D and E. Conceivably, F(g)DPs could stimulate monocytes to release hepatocyte-stimulating factors including interleukin-1 (IL-1), which in turn would increase the synthesis of fibrinogen. Therefore, F(g)DPs and blood monocytes (or macrophages) appear to interact and play a critical role in the biologic modulation of coagulation and fibrinolysis.

We have recently established a human promonocytic leukemia cell line designated NOMO-1, which displays macrophage-like characteristics when exposed to 12-O-tetradecanoylphorbol-13-acetate (TPA). Moreover, the phorbol derivative of TPA induced differential secretion of IL-1, urokinase-type plasminogen activator (u-PA), and plasminogen activator inhibitor-2 (PAI-2). The present study was undertaken to evaluate the interactions of F(g)DPs and the monocyte-macrophage system in the NOMO-1 cell line. This cell line was stimulated to produce IL-1, u-PA, and PAI-2 in response to F(g)DPs, and the response was most apparent with FDP D-dimer.

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. 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, 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 10⁻⁸ 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 (Seikagaku Kogyo, Ltd, Tokyo, Japan). Lipid A, LA-15-PP-S06 (Daichi Kagaku 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 tests were 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^{-3} 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 before.

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 (Ohtsuka 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).

Radioiodination of F(g)DPs. Radioiodination of F(g)DPs was 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 E did not stimulate superoxide 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)DP binding assay. Radioiodination of F(g)DP was performed by the solid-state lactoperoxidase method.

NOMO-1 cells were grown in RPMI-1640 with 10% FCS. Cells were grown to confluency, trypsinized (trypsin-EDTA, 1 g/L, 0.25%), and pelleted by centrifugation at 500g for 5 minutes at 25°C. Cells were washed and resuspended in Tyrode’s buffer (NaCl at 8.0 g/L, KCl at 0.2 g/L, NaHCO₃ at 1.0 g/L, NaH₂PO₄ at 0.05 g/L, MgCl₂·6H₂O at 0.2 g/L, dextrose at 1.0 g/L, and 2% BSA). Aliquots of 250 μL containing 0.5 × 10⁶ cells were added to Eppendorf tubes. Ten microliters of human 125I-labeled fragment D, E, or D-dimer (3.6 to 90 nmol/L) was added to the tubes (total assay volume = 260 μL). Specimens were incubated at 4°C for 1 hour and periodically gently agitated to ensure homogeneous mixing. The NOMO-1 cells/fragment D, E, or D-dimer mixture was then centrifuged at 16,000 g for 5 minutes. The cellular pellet was resuspended and washed three times in Tyrode’s buffer. The radioactivity in the final pellet was counted to determine the amounts of 125I-labeled fragment D, E, or D-dimer bound to the NOMO-1 cells. In some experiments, cells were incubated with radiolabeled ligand either alone or in the presence of a 100-fold molar excess of unlabeled ligand to determine nonspecific binding. All experiments were performed in triplicate.

RESULTS

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). The morphologic changes in NOMO-1 cells are shown in Fig 1. The control NOMO-1 cells were mostly round with a very uniform modal diameter (Fig 1A). After exposure to F(g)DPs (Fig 1B), some cells displayed a macrophage-like appearance with small vacuoles and extending pseudopods. TPA-treated NOMO-1 cells displayed dramatic transformation to a macrophage-like appearance (Fig 1C).

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 E 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^{-4}, 10^{-3}, and 10^{-2} 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^{-3} 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
Fig 1. Morphology of NOMO-1 cells that have been cultured for 5 days. (A) Control; (B) in the presence of fragment D-dimer (10^-8 mol/L). Extending pseudopods are evident at the tips of the cytoplasm. (C) In the presence of TPA (10^-7 mol/L). TPA-treated NOMO-1 cells changed to macrophage-like cells. (Original magnification × 1,000.)

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. Endotoxin is known to stimulate IL-1 release from monocytes, 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>
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</thead>
<tbody>
<tr>
<td></td>
<td>% of NBT reduction (+) cells*</td>
<td></td>
</tr>
<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.
THE INTERACTIONS OF FDP AND MONOCYTES

respectively, 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 kd 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 μg/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

Table 2. Specificity of Fragment D-Dimer for the Induction of IL-1 Release From NOMO-1 Cells

<table>
<thead>
<tr>
<th>Addition</th>
<th>IL-1 α pg/mL</th>
<th>IL-1 β pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.0</td>
<td>10.3</td>
</tr>
<tr>
<td>D-dimer</td>
<td>19.4</td>
<td>22.7</td>
</tr>
<tr>
<td>D-dimer treated with anti-FSP D IgG</td>
<td>2.5</td>
<td>12.5</td>
</tr>
<tr>
<td>D-dimer treated with anti-FSP E IgG</td>
<td>18.8</td>
<td>24.2</td>
</tr>
</tbody>
</table>

NOMO-1 cells were cultured for 3 days.

*Mean of two separate experiments.

Fig 2. Effects of F(g)DPs on the secretion of IL-1α and IL-1β. (A) IL-1α; (B) IL-1β. Values are plotted as means ± SD of three separate flasks.
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 ± 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 (10⁻¹ mol/L); (C) cultured with fragment E (10⁻¹ mol/L); (D) cultured with fragment D-dimer (10⁻³ mol/L). (Original magnification × 400.)
THE INTERACTIONS OF FDP AND MONOCYTES

The production of IL-1 is regulated by TPA, lipopolysaccharide (LPS), and tumor necrosis factor-α (TNF-α). The present study demonstrates that D-dimer binds to NOMO-1 cells, whereas labeled fragment D bound at a much lower level than the other fragments. Fragment D-dimer had a slightly lower k_d than fragment D.

IL-1 has been identified as a chemical mediator released from monocytes/macrophages and exhibits important biological 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 FDPs, 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^(-7) mol/L, roughly corresponds to the levels of FDPs found in the blood of patients with severe disseminated intravascular coagulation (DIC). Because FDPs 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 FDPs, 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 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 FDPs. NOMO-1 cells may be a useful system for these studies.

ACKNOWLEDGMENT

We thank Sayoko Sugiura for skillful technical assistance.

REFERENCES


Fig 5. Binding of FDPs to NOMO-1 cells. Scatchard plot analysis of bound (B) 125I-labeled fragment D (□) and D-dimer (○) on the abscissa versus bound/free (B/F) on the ordinate are shown. This plot demonstrates that 125I-labeled fragment D and 125I-labeled fragment D-dimer bind to the NOMO-1 cells with a k_d of 3.3 nmol/L and 2.7 nmol/L, respectively. The results are the mean of triplicate experiments.


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

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