Tuftsin Induces Tissue Factor-Like Activity in Human Mononuclear Cells and in Monocytic Cell Lines

By Abraham Kornberg, Raphael Catane, Shoshana Peller, Suzana Kaufman, and Mati Fridkin

Normal human monocytes and macrophages generate potent procoagulant activity (PCA) resembling tissue factor (TF) in response to various stimuli. In this study we show that tuftsin, a natural stimulator of many functions of monocytes and macrophages, also stimulates a potent PCA in mixed mononuclear cells and monocytes, and a mild PCA in lymphocytes and cell lines of monocytic origin (U937 and THP). No activity was generated by several lymphoid cell lines and HL-60 cells. The PCA resembled TF in that it accelerated clotting through the extrinsic coagulation pathway and was inhibited by concanavalin-A and by monoclonal anti-TF antibodies. The induction of TF-like activity by tuftsin was dose- and time-dependent. It was located in the cell membrane and did not require T cells for expression. Generation of TF-like activity was prevented by actinomycin D, while cytarabine had no effect on this process, suggesting that expression of the activity depends on protein synthesis. Studies with various tuftsin analogs suggest that tuftsin stimulates generation of TF-like activity, as well as other functions of monocytes via the same receptors. The results with the monocytic cell lines show that tuftsin affects mainly mature cells. The induction of TF-like activity in mononuclear cells by tuftsin constitutes an important link between mononuclear cells and the immune and coagulation systems. It may play a major role in the pathogenesis of thrombosis and fibrin deposition in various inflammatory and immunologic disorders.

MATERIALS AND METHODS

Separation and identification of cells. Blood was collected in heparin from normal volunteers. Mononuclear cells were isolated on lymphoprep density gradient centrifugation (Mycobed, AS, Oslo, Norway). The cell layer was centrifuged once at 140g and twice at 100g in phosphate-buffered saline (PBS) at room temperature for 10 minutes to remove the platelets. Monocytes (adherent cells) were obtained by incubation of mixed mononuclear cells for 90 minutes at 37°C in a humidified atmosphere of 5% CO₂ in air, either in large Petri dishes (90 x 15 mm, Sterilin, Feltham, Middlesex, England, 10 mL per dish of 5.0 x 10⁶ cells/mL) or in small dishes (50 x 15 mm, 1 mL per dish of 2.0 x 10⁶ cells/mL) in RPMI 1640 supplemented with L-glutamine (Biological Industries, Beth Haemek, Israel) and 10% heat-inactivated fetal calf serum (HI-FCS) (GIBCO, Paisley, Scotland). Lymphocytes (nonadherent cells) were removed from the dishes by aspiration with a Pasteur pipette. The dishes were then washed twice with warm (37°C) media to remove the nonadherent cells. In part of the experiments the adherent cells were removed by scraping the dishes with a rubber policeman, washed twice in PBS, and used in the incubation studies. In other parts of the experiments the cells were incubated with various compounds in the small Petri dishes, after which they were scraped and used in the PCA assays.

Monocytes were identified by positive staining with fluoride-inhibited nonspecific esterases and phagocytic capability. The percentage of phagocytic cells was determined by quantitation of the number of cells that ingested at least four latex particles, as described by Edwards et al. The mixed mononuclear cell population comprised 15% to 30% monocytes, the adherent cell population 85% to 95% monocytes, and the nonadherent cell population consisted of 1% to 5% monocytes.

To remove T lymphocytes, a suspension of mononuclear cells was incubated with sheep red blood cells (SRBCs) to allow the formation of rosettes. The cell suspension was layered on lymphoprep and centrifuged at 140g for 20 minutes. The T-depleted mononuclear cell population was collected from the interface. The percentage of T lymphocytes was determined by the formation of rosettes with SRBCs. The mixed mononuclear cells and the T-depleted cells contained 72% ± 5% and 3% ± 2% T lymphocytes, respectively.

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The cell lines Raji, HD-Mar, REH, HL-60, U 937, and THP were maintained by subculturing every 3 to 4 days in RPMI supplemented with L-glutamine and 10% HI-FCS in 5% CO₂ in air.

**Incubation of cells.** After separation, cells were washed twice in PBS and incubated at 3.0 × 10⁶ cells/mL in the presence of the following compounds, alone or in various combinations: tuftsin, tuftsin analogs, polymyxin-B (Teva Ltd, Tel Aviv, Israel), cytarabine (Upjohn, Kalamazoo, MI), actinomycin-D (Merck Sharp, Rahway, NJ), and LPS (from *Escherichia coli* 026:B6, Sigma, St Louis, MO). Initially, incubations were performed in either RPMI supplemented with 10% HI-FCS or PBS at 37°C in 5% CO₂ or at room temperature. Because the results were very similar, later studies were performed only in PBS at room temperature. The lymphocytes were incubated in plastic tubes (Sterilin, Fetham) at a final volume of 1 mL/tube. Initially, the monocytes were incubated either in tubes after scraping the Petri dishes or in Petri dishes and then obtained by scraping.⁸ Because the results of the two methods were similar, in later experiments the monocytes were first obtained by scraping and then used in the incubation studies. In all experiments cells were used only if their viability, as determined by trypan blue or eosin exclusion tests, exceeded 90%.

**Assay and characterization of procoagulant activity.** After incubation, cells were washed twice in PBS and resuspended in veronal acetate buffered saline, pH 7.35.¹² Procoagulant activity was determined using either intact cells or cells disrupted by sonification with ultrasonic vibration for 60 seconds in an ice bath (Sonifier B-12, Branson Sonic Power, Danburg, CT). To obtain the membrane-rich fraction, nuclei were sedimented at 40,000g for 20 minutes at 20°C (Sorval, RC-5, DuPont, Newtown, CT). Procoagulant activity was measured by the modified prothrombin time (M-PT)²⁶ through the use of pooled citrated plasma from at least 10 normal donors was incubated with 0.1 mL of pooled citrated plasma from at least 10 normal donors was incubated with 0.1 mL cell suspension at 37°C for 1 minute, 0.1 mL of 0.025 mol/L CaCl₂ was then added, and the clotting time recorded. Each sample was run in duplicate. In some experiments PCA was assayed using plasma deficient in factors VII, VIII, IX (Pacific Hemostasis, Ventura, CA), X (Diagnostica Stago, Ashieres-Sur-Seine, France), or XI (Merz-Dade, Duedingen, Switzerland).

The effect of monoclonal anti-TF antibodies (provided by Dr. S.D. Carson, University of Nebraska Medical Center, Omaha) on monoclonal PCA was studied by determination of M-PT of tuftsin-treated mononuclear cells after incubation with monoclonal anti-TF antibodies for 1 to 2 hours at 37°C.²⁴

**Reagents.** Tuftsin was synthesized and provided by Abic Ltd (Ramat Gan, Israel).²⁵ The tuftsin analogs (Leu')-tuftsin, nitro-tuftsin, and (Des-Thr')-tuftsin were synthesized according to Fridkin et al. The compounds were dissolved in PBS, pH 7.4. Activated TF was purchased from Dade Diagnostica (Agnada, Puerto Rico) and Concavanalin-A (Con-A) from Miles-Yeda (Rehovot, Israel). Statistical analysis was performed using the paired student's t-test.

**Results**

**Effect of tuftsin on procoagulant activity generation by mixed mononuclear cells, lymphocytes, and monocytes.** Tuftsin in concentrations less than 5 µg/mL did not enhance PCA generation, either in MMC or in purified cells (Fig 1). Both monocytes and lymphocytes showed a significant increase in PCA expression after incubation with 5 µg/mL tuftsin (P < .001; P = .007, respectively). However, the effect on monocytes was significantly more prominent (P < .001), increased progressively, and reached peak levels at 500 µg/mL tuftsin. The lymphocyte PCA was not affected by the increasing concentrations of tuftsin. PCA was not increased in either the untreated cells or in the controls with tuftsin without cells during the same periods of incubation (M-PT 142 to 156 seconds).

PCA did not change during the first 6 hours of exposure to a constant concentration of tuftsin (250 µg/mL) (Fig 2). The activity increased significantly with MMC and monocytes to a maximal level at 16 hours (P < .001), thereafter remaining at the same plateau for up to 36 hours of incubation. Lymphocytes generated significantly less PCA during the same incubation period (P < .001).

Artifacts due to LPS contamination of various reagents and solutions² were excluded by incubating MMC and tuftsin with polymyxin B. Although this substance binds to the lipid A region of LPS and inhibits its ability to induce monocyte PCA generation,³⁴ elaboration of PCA was not inhibited by the addition of polymyxin B to the cultures of MMC and tuftsin (M-PT without polymyxin B was 70 ± 12 seconds, and 62 ± 8 seconds with polymyxin B).

**Characterization and localization of procoagulant activity generated by mixed mononuclear cells.** As tuftsin proved to induce a similar increase in the levels of PCA in MMC and monocytes, only MMC were used in further experiments. Both PCA and commercial TF accelerated the M-PT of normal plasma and plasma deficient in factors VIII, IX, and XI, and had no effect on the M-PT of plasma deficient in factors VII or X (Table 1).

Incubation of Con-A with tuftsin-treated mononuclear
Table 2. Generation of Procoagulant Activity by Various Fractions of Tuftsin-Treated Mononuclear Cells

<table>
<thead>
<tr>
<th>Cell Fraction</th>
<th>Modified PT (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole</td>
<td>69 ± 10</td>
</tr>
<tr>
<td>Supernatant</td>
<td>152 ± 14</td>
</tr>
<tr>
<td>Pellet</td>
<td>60 ± 6</td>
</tr>
<tr>
<td>Pellet</td>
<td>146 ± 12</td>
</tr>
<tr>
<td>Culture media</td>
<td>164 ± 15</td>
</tr>
</tbody>
</table>

Mononuclear cells were incubated with 250 µg/mL tuftsin for 24 hours. The cells were disrupted and the supernatant and pellet were obtained after centrifugation at 40,000g as described in Materials and Methods. Results are mean ± SD of 3 to 5 experiments.

*The procoagulant activity was assayed after 24 hours of incubation of the pellet of the disrupted cells with 250 µg/mL tuftsin.

culture media or PBS. No activity was generated on incubation of disrupted MMC with tuftsin.

Effect of T lymphocytes and DNA and protein synthesis inhibitors on the generation of TF-like activity by mixed mononuclear cells. Depletion of T lymphocytes from 72% ± 5% to 3% ± 2% did not prevent the generation of TF-like activity by MMC exposed to tuftsin for 24 hours (Table 3). Incubation of MMC with tuftsin in the presence of 10 µg/mL actinomycin-D reduced the generation of TF-like activity by 93%, while 5 µg/mL cytarabine did not inhibit its expression.

Effect of tuftsin analogs on the generation of procoagulant activity by mixed mononuclear cells. The effect of various tuftsin analogs on the generation of the TF-like activity by MMC is compared with that of tuftsin in Table 4. (Leu')-tuftsin, which stimulates phagocytosis to the same extent as tuftsin,, induces slightly less activity at concentrations of 5 µg/mL and 250 µg/mL. Nitro-tuftsin, a tuftsin analog that does not affect phagocytosis, failed to induce generation of TF-like activity. (Des-Thr')-tuftsin, which has been shown to exert an inhibitory effect on tuftsin-stimulated phagocytosis, also inhibits the generation of TF-like activity by tuftsin.

Table 1. Characterization of Tuftsin-Induced Mononuclear Procoagulant Activity

<table>
<thead>
<tr>
<th>Substrate (dilution 1:16)</th>
<th>Plasma</th>
<th>Inhibitor (µg/mL)</th>
<th>Modified PT (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thromboplastin</td>
<td>Normal</td>
<td>—</td>
<td>53 ± 4</td>
</tr>
<tr>
<td>Mononuclear cells</td>
<td>Normal</td>
<td>—</td>
<td>69 ± 10</td>
</tr>
<tr>
<td>Mononuclear cells</td>
<td>VII deficient</td>
<td>—</td>
<td>143 ± 14</td>
</tr>
<tr>
<td>Mononuclear cells</td>
<td>X deficient</td>
<td>—</td>
<td>162 ± 16</td>
</tr>
<tr>
<td>Mononuclear cells</td>
<td>VIII deficient</td>
<td>—</td>
<td>60 ± 12</td>
</tr>
<tr>
<td>Mononuclear cells</td>
<td>IX deficient</td>
<td>—</td>
<td>68 ± 8</td>
</tr>
<tr>
<td>Mononuclear cells</td>
<td>XI deficient</td>
<td>—</td>
<td>64 ± 10</td>
</tr>
<tr>
<td>Mononuclear cells</td>
<td>Normal</td>
<td>Con-A 16</td>
<td>102 ± 8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>64</td>
<td>116 ± 10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>128</td>
<td>132 ± 10</td>
</tr>
<tr>
<td>Mononuclear cells</td>
<td>Normal</td>
<td>Monoclonal anti-TF antibodies 2</td>
<td>55 ± 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>118 ± 8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
<td>136 ± 6</td>
</tr>
</tbody>
</table>

Generation of mononuclear procoagulant activity was assayed after incubation of 3 x 10⁶/mL cells with 250 µg/mL tuftsin for 24 hours. The effect of Con-A and monoclonal anti-TF antibodies was determined after 1 to 2 hours of incubation with the tuftsin-treated cells at 37°C. The results are the mean ± SD of 3 to 5 experiments.
Effect of tuftsin on generation of procoagulant activity by cell lines. Tuftsin in various concentrations had no effect on the generation of TF-like activity by HL-60 cells and by the lymphoid cell lines Raji (B type), HD-Mar (T type), and REH (pre-B) (Table 5). It induced a modest but significant increase in the expression of the activity in the cell line of monocyte-macrophage origin U937 and THP at concentrations of 5 μg/mL and 250 μg/mL (P = .001).

DISCUSSION

Stimulated monocytes generate PCA after exposure to a wide range of inducers in vitro,\textsuperscript{11,16} while various monocytic functions are activated by tuftsin.\textsuperscript{21,22} Therefore, it is not surprising that the tetrapeptide also stimulates PCA generation in monocytes. Moreover, LPS, the most potent stimulus for the generation of monocyte TF-like activity\textsuperscript{a} as well as other inducers belonging to the immune system, such as complement, lymphokines, and immune complexes, augments different functions of monocytes.\textsuperscript{26,27} It seems that there is a general link between induction of monocyte TF-like activity and activation of a number of functions of monocytes.

The nature of PCA and the process of its induction by tuftsin resembles, in several aspects, the PCA generated in monocytes such as LPS, lectins, and immune complexes all require collaboration of T lymphocytes for optimal effect,\textsuperscript{5,15-17} whereas the action of tuftsin was not prevented by the removal of T cells. However, it has been claimed that there are two pathways for the generation of TF-like activity, one of them being relatively T-cell independent.\textsuperscript{13,18} Second, the activity appears relatively late in tuftsin-treated MMC and monocytes compared with its expression by other inducers.\textsuperscript{3,4,9} For instance, MMC showed significant increase in the activity 4 hours after stimulation with 10 μg/mL LPS (M-PT 122 ± 8 and 82 ± 10 seconds, respectively) that increased after 8 hours (M-PT 64 ± 8 seconds), and reached peak levels after 16 hours (M-PT 44 ± 6 seconds). Despite these differences, the results with the monoclonal anti-TF antibodies prove conclusively that tuftsin-induced PCA resembles TF. Finally, most of the studies attribute the bulk of TF-like activity induced by various stimuli to monocytes and macrophages.\textsuperscript{14,15} In that respect it was found that purified lymphocytes bind less than 5% tritiated tuftsin, as compared with polymophonuclears and monocytes.\textsuperscript{22} In light of these findings, the origin of the activity in the preparations of tuftsin-treated purified lymphocytes is unclear. The possibility of contamination with minute amounts of monocytes in our studies cannot be ruled out.\textsuperscript{14} Nevertheless, it was demonstrated by cytologic assay that lymphocyte-like cells can express TF-like activity after stimulation with immune complexes,\textsuperscript{9} and that pure lymphocytes can bind\textsuperscript{125}I-tuftsinyl-tyrosine,\textsuperscript{29} indicating the existence of a subpopulation of lymphocytes that possess tuftsin's receptor site, and, therefore, can be stimulated.\textsuperscript{14}

The dose-response relationship between the concentrations of tuftsin and the generation of TF-like activity is different from its effect on other functions of monocytes. A significant increase in the activity was noted with 5 μg/mL, which reached peak levels in the presence of 500 μg/mL. In contrast, the in vitro effects of tuftsin on phagocytosis, chemotaxis, migration, and cytotoxicity of monocytes were shown to be evident already at concentrations as low as 10^{-3} to 10^{-2} μg/mL, becoming maximal at 1 to 10 μg/mL and decreasing at higher concentrations.\textsuperscript{22,25} Tuftsin concentra-
tions in the blood range between 0.2 and 0.5 μg/mL, which poses the question regarding the clinical significance of our studies. However, it is plausible that during inflammatory or immunologic disorders the concentration of tuftsin in the blood, and especially in tissue, is increased and reaches levels that induced in vitro the generation of TF-like activity. Moreover, tuftsin was degraded during incubation to free amino acids as well as peptideic fragments as shown by amino acid analysis and by high pressure liquid chromatography (HPLC), respectively. Degradation was usually maximal after 16 hours and amounted to 30% to 95% of the peptide. It is also known that in consequence to its association with specific receptor sites on phagocytic cells, tuftsin is internalized by the cells and degraded by a battery of various peptidases. The results of the experiments with the various tuftsin analogs suggest that tuftsin induces TF-like activity in monocytes, as well as affecting other functions of the cells via the same receptor sites. Because tuftsin is part of the CH₂ domain of the Fc-fragment, and since immune complexes and aggregated Igs induce the activity in monocytes, it is not unlikely that the tuftsin binding sites on monocytes are related in some way to the Fc-receptor.

Our results with the monocyte-macrophage cell lines are in agreement with other studies on the response of such cell lines, including PU 5, WEHI 265, TG-PEG, and J774, to various stimuli. The lack of response of the tuftsin-treated B, T and pre-B type cell lines may support the conclusion that the monocyte-macrophage is the principal cell responsible for the generation of TF-like activity.

Tuftsin, a natural tetrapeptide of the immune system, plays a significant role in the defense of the body and in inflammatory and immunologic phenomena. TF-like activity induced by tuftsin in monocytes is an additional example of the link between the immune and coagulation systems, which may have a significant importance in the pathogenesis of fibrin deposition in inflammatory and immunologic disorders, and in activation of coagulation during septicemia and malignancy.

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