Human Atheromatous Plaque Extracts Induce Tissue Factor Activity (TFα) in Monocytes and also Express Constitutive TFα

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Summary

Tissue factor activity (TFα) is a major activator of the coagulation cascade and may play a role in atheroma-induced thrombosis. Monocyte-macrophages (MO-MF) generate considerable quantities of TFα when stimulated by a variety of inducers. To test the hypothesis that MO could be induced by atheromatous plaque to generate TFα, plaque extracts obtained from patients with obstructive atheromatous disease were used. These extracts were also assayed for constitutive TFα. The constitutive activity was variable from extract to extract but could be very high, up to 250 U TFα. The TFα induced in MO could be also very high, up to 200 U (i.e. 1/5 of the TFα of full strength rabbit brain thromboplastin). These findings point to a major role for MO-MF TFα in the induction or thrombosis by atheromatous plaque.

Introduction

Arterial thrombosis is a well known complication of atheromatous plaques and is triggered by plaque fissure resulting in strokes, myocardial infarction and other thrombotic events (1-5). Reocclusion of a vessel which had been recently permeabilized either by lysis or mechanically, is a vexing and dangerous event which may occur even in the presence of vigorous anticoagulation (6, 7). The origin of this prothrombotic activity is incompletely elucidated. Tissue factor (TF) is a lipitated integral membrane-bound glycoprotein whose activity (TFα) is a powerful promoter of blood coagulation.

TFα complexes with factor VII/VIIa to hydrolytically activate factor X and factor IX leading to the formation of thrombin and subsequently a fibrin clot (8, 9). TF mRNA and TF antigen have been detected in the adventitia of arteries (10). However, the adventitia is isolated from blood circulation. TFα has been induced in vitro in endothelial cells (11), and TF mRNA has been induced in the muscularis after balloon injury of the vessel (12).

Two circulating blood elements, MO and platelets, could be implicated in plaque-induced thrombogenesis. MO-MF may be induced to generate TFα and have a well-established thrombogenic activity (13). MO TFα may be induced by a variety of agents, such as bacterial endotoxin (lipopolysaccharide) (13, 14), antigen-antibody complexes (15), complement fragments (16), and lipoproteins (17, 18). It is noteworthy that MO TFα generation occurs not only in vitro but also in vivo as has been demonstrated not only in peritoneal MF (13) but also in peripheral blood MO in rabbit and human (19, 20). Moreover, it has been recently reported that MO of patients with coronary ischemic syndromes bear TF antigen (21).

Furthermore, Tipping et al. have shown that MF teased out of atheromatous plaques have TFα (22). More recently, TFα has been demonstrated within atheromatous plaques obtained from coronary arteries at the time of surgery (33). Thriruvikraman et. al. have reported TF in human atherosclerotic plaques by digoxigenin-labeled coagulation factors VIIa and X. TF was within the extracellular lipid-rich core, macrophages, smooth muscle cells, adventitia and endothelium (34). Ardissino et al. have shown TF antigen and activity in coronary vessels were lowest in patients with stable angina and became progressively higher in patients with unstable angina and myocardial infarction respectively (38).

Platelets also play a role in atheroma-induced thrombosis. Platelets adhere to atheromatous vessel lesions (3). Furthermore, a monoclonal antibody inhibiting platelet function facilitated repermeabilization of injured vessels in dogs and reduced mortality in patients undergoing angioplasty (23-25).

In this report we provide evidence that atheromatous plaque extracts may contain substantial amounts of TFα, and that the atheromatous plaques may induce TFα generation in MO. Therefore specific inhibitors of TFα and TFα generation by MO may be an appropriate form of anticoagulation during angioplasty.

Materials and Methods

Plaque material was obtained steriley during surgery from atheromas of aorta, carotid or leg arteries and was handled in endotoxin-free containers. Prior to extraction plaques were assessed qualitatively to be either soft or hard (and calcified). Plaques were extracted with 3 ml of 0.9% NaCl in a hand held glass homogenizer with 8-10 strokes under a sterile hood. Non-dispersed material was sedimented for 5 min at 1 × g and discarded. The extracts were either tested at once or frozen at –70° C, and tested at a later date. Freezing did not affect the results. The extracts were tested for constitutive TFα, or studied as inducers of MN-TFα.

Peripheral blood MN were prepared as previously described either from the patient from whom the atheromatous specimen was obtained or a blood group compatible donor; this did not alter the results. Briefly whole blood was anticoagulated with 4% sodium citrate. The blood was diluted by 1:10 with PBS (phosphate buffered saline (PBS) without Ca or Mg (Grand Island Biologicals, NY), layered on Ficoll-Hypaque (Pharmacia, Piscataway, NJ) and centrifuged at 400 × g for 40 min at 14 degrees C. The MN layer was collected and washed twice with PBS, without Ca, Mg; the last wash was with PBS-1% human albumin (26). Cells were then suspended in RPMI 1640 (Grand Island Biologicals N.Y.). MN preparations contained 25% MO on average. For some experiments fetal calf serum (FCS), heated at 60° C for 1h, was added to RPMI to achieve a final concentration of 12% in the incubation mixture.

Reagents and components partaking in incubation mixtures where MO-generated TFα was assessed, were handled steriley and were endotoxin-free. MN (3 × 10^6/tube) were incubated with either atheromatous plaque extract and RPMI 1640, or endotoxin 0.05 μg/ml (Difco, Detroit, Ml) as a standard inducer of TFα, or RPMI 1640 alone as negative control. After 16 h incubation at...
Monocyte TFα Generation Induced by Plaque Extract

Fig. 1 Dose-dependent increase of plaque-induced MN TFα. The symbols represent two separate experiments.

37° C, MN were washed by suspension in 3 ml of 0.9% NaCl, and sedimented at 400 × g for 10 min at 14° C and the supernate discarded. The washing step was repeated and the cells resuspended in initial volume of 0.9% NaCl, were sonically disrupted before being tested for TFα. Testing for TFα was done by the one stage test and the results compared to those of 5 dilution of rabbit brain thromboplastin, which at full strength had an arbitrarily assigned activity of 1000 U/ml (14). MN incubated in RPMI had a TFα, which was always below 0.5 U.

Neutralization of constitutive and induced TFα was carried out with monoclonal anti-TFα (mab TFα) kindly provided by Dr. Yale Nemerson, Mount Sinai School of Medicine (33). Specimens containing TFα were incubated for 30 min at 37° C at antibody concentrations of 8 μg/ml, 42 μg/ml or with control media (PBS-1% albumin) and the residual TFα was then measured.

Platelet aggregation was performed with the platelet Aggregation Profiler, model PAP-4 (Bio/Data Corporation, Horsham, PA). Platelet-rich plasma (PRP), 300,000 platelets/μl, and platelet-poor plasma (PPP) were prepared from blood-group compatible donors and used to test the ability of atheromatous extract to induce aggregation. Epinephrine, ADP and collagen (Bio/Data Corp.) were used at final concentrations of 10 μM, 2 μM and 19 mg/ml respectively, and induced appropriate platelet aggregation in PRP. Fifty μl of saline plaque extract were added to 450 μl of PRP in the cuvette and platelet aggregation was measured.

Total protein, triglycerides, total cholesterol and HDL-C were measured by standard laboratory methods on plaque extracts. The total protein was measured by the biuret method; triglycerides and total cholesterol were quantitated in the Boehringer Mannheim/Hitachi 747 analyzer. HDL-C was quantitated on the Beckman Synchron CX.

Numerical data were expressed as the mean ± standard error of the mean. As the TFα did not follow a normal distribution, non-parametric tests were used. The Mann-Whitney-U test was used for TFα from different samples. Paired data (i.e. TFα activity from the same samples) were evaluated with the Wilcoxon test. The relationships among induced and constitutive TFα and other factors were analyzed with the Spearman rank correlation (Zar, 1984). Results were considered statistically significant if the p values were less than 0.05. Statistical calculations were performed using SPSS for Windows (SPSS Inc., Chicago, Illinois).

Results

Plaque Induced TFα in MO

To determine whether plaque extracts may induce TFα generation in MO, autologous, or blood-group-compatible MN were incubated with plaque extracts and after 16 h incubation the TFα generated was assayed. Some extracts induced considerable activity, while others did not (Table 1). The induced activity was dependent on the concentration of extract added (Fig. 1).

Enrichment of MN culture media with FCS enhanced generation of MO TFα by plaque extract. The generated TFα of 6 consecutive extracts were compared. Addition of 12% FCS in the MN-plaque extract incubation mixture amplified TFα generation in some, but not all of the samples. Enhancement was up to 19-fold and as much as 200 U TFα per ml was generated in the presence of FCS. The enhancement was significant at p = 0.04 level. These results show that under some conditions the induced TFα could be very high, in most instances severalfold higher than the TFα induced by endotoxin. However, absence of enhancement by FCS in 2 atheroma-extract samples, absence of enhancement by FCS in the endotoxin specimen, and absence of enhancement by FCS in RPMI indicate that FCS by itself was devoid of inducer TFα activity (Table 2).

Constitutive TFα

The constitutive TFα of 35 atheromatous plaque extracts was examined. Thirty extracts were obtained from soft plaques. The TFα was quite variable ranging from 0 to 250 U. The mean was 22 U. The activity of the remaining 5 extracts obtained from hard, calcified plaques was lower ranging from 0 to 7 U of TFα (Table 1).

To establish that the procoagulant activity was TFα, plaque extracts were incubated with mabTF and the remaining activity after 30 min was assayed. The neutralization of TFα was dose-dependent (Fig. 2). These results indicate that the major procoagulant activity is indeed due to TFα.

To discriminate between induced and carried-over constitutive TFα in incubations of MN with plaque extract, MN were washed prior to assay for TFα. Constitutive TFα was not carried over to the incubation mixtures of plaque extract with MO. Indeed, when specimens with high

Table 1 Constitutive TFα was determined on plaque extract. Plaque-induced MO TFα was determined on MN (3 × 10⁶) incubated for 16 h with plaque extract. Thereafter, MN were washed, disrupted and tested for TFα.

<table>
<thead>
<tr>
<th>Sample</th>
<th>N Constitutive TFα U</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soft plaque</td>
<td>31</td>
</tr>
<tr>
<td>Hard plaque</td>
<td>7</td>
</tr>
<tr>
<td>RPMI</td>
<td>–</td>
</tr>
</tbody>
</table>

Table 2 Extracts of six consecutive plaques were compared for TFα inducer activity. MNs were incubated with plaque extract-RPMI, or with plaque extract-RPMI enriched with heat-inactivated FCS. Constitutive TFα was also assayed and compared. Note: absence of enhancement of endotoxin-induced TFα by FCS.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Induced (Plaque)</th>
<th>Induced (Plaque + FCS)</th>
<th>Constitutive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18</td>
<td>25</td>
<td>27</td>
</tr>
<tr>
<td>2</td>
<td>33</td>
<td>84</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>75</td>
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<tr>
<td>4</td>
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</tr>
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<td>5</td>
<td>9</td>
<td>130</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>19</td>
<td>9</td>
</tr>
<tr>
<td>Endotoxin</td>
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<td>5</td>
<td>–</td>
</tr>
<tr>
<td>RPMI</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>–</td>
</tr>
</tbody>
</table>
(over 30 U) induced TFα were compared with their corresponding constitutive TFα, the induced activities were up to 52 times greater. Similarly, plaque extracts with the highest constitutive activity also correlated poorly with the induced activity (Table 3). These findings indicate that the induced TFα could not be carried-over TFα.

TFα of plaque extracts were compared with their lipid and protein content. No correlation was found between cholesterol, HDL, and triglyceride concentrations on one hand and TFα, constitutive or induced, on the other. However, a negative correlation was observed between total protein concentration and constitutive TFα (p = 0.03) (Table 4).

**Platelet Aggregation**

Ability to aggregate platelets in PRP was demonstrated by 5 of 9 extracts. Maximum aggregatory activity ranged from 7 to 48%. In comparison, standard agonist-induced aggregations were: epinephrine, 76%; ADP, 70%; and collagen 70%. There was no correlation between plaque-induced platelet aggregation and TFα, whether induced or constitutive (Table 5).

**Discussion**

We have demonstrated constitutive TFα in many atheromatous plaque extracts which were obtained surgically from patients with advanced atheromatous disease. These results were recorded in abstract form (27, 28). The TFα we report could be quite high (up to 250 U), particularly in soft plaques but it was variable. The variable level of constitutive TFα from extract to extract may be due to testing of plaques at different stages of development. Indeed calcified plaques had lesser quantities of constitutive TFα. Additionally, the putative presence of inhibitors in some plaques may also be responsible for the observed lack of TFα in some specimens.

Importantly, we report that many plaque extracts induced generation of TFα in peripheral blood MO which could be more than 50 times higher than the respective constitutive TFα (Table 3). We propose that resident MF as well as MO attracted to the plaque may be induced to generate TFα. As MF may represent up to 60% of the cells in a plaque (29), considerable amounts of TFα could be generated.

Attempts to identify inducer of MO-TFα led us to quantify plaque proteins and lipids and correlate their concentration with TFα. Neither constitutive nor induced TFα correlated well with the concentration of cholesterol, HDL-C or triglycerides in the plaque extract. However there was a negative correlation between constitutive TFα and total protein concentration. At least three possible explanations exist for this

<table>
<thead>
<tr>
<th>Table 4</th>
<th>Constitutive and induced TFα are compared with protein, triglyceride (TG), total cholesterol (TC) and HDL-cholesterol (HDL-C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Constitutive</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>#1</td>
<td>27</td>
</tr>
<tr>
<td>#2</td>
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<tr>
<td>#3</td>
<td>14</td>
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</tr>
<tr>
<td>#5</td>
<td>6</td>
</tr>
<tr>
<td>#6</td>
<td>9</td>
</tr>
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</table>

**Fig. 2** Dose-dependent neutralization of plaque extract TFα by mab TFα

**Table 5** Platelet aggregation by plaque extract was measured on platelet-rich plasma. Aggregation was compared to constitutive and induced TFα of these extracts.

<table>
<thead>
<tr>
<th>% aggregation of platelets</th>
<th>Constitutive TFα</th>
<th>Induced TFα</th>
</tr>
</thead>
<tbody>
<tr>
<td>48</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>34</td>
<td>2</td>
<td>&lt;1</td>
</tr>
<tr>
<td>21</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>12</td>
<td>47</td>
<td>24</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>&lt;1</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>0</td>
<td>50</td>
<td>18</td>
</tr>
</tbody>
</table>
negative correlation. 1. Poor co-extraction of proteins and constitutive TFα.
2. Co-extraction of an inhibitor of TFα.
3. TFα and protein concentration may peak at different times in the evolution of an atheromatous plaque.

There is strong evidence that the high concentration of MO-MF within the plaque is a result of chemotactic factors secreted from cellular elements of the vascular wall. Indeed MO chemotactic factors including Monocyte Chemotactic Protein (MCP) and other cytokines have been demonstrated in atheromatous plaques (30). Poon et al. showed that monocyte chemotactic activity was generated and secreted by cultured rat aortic vascular smooth muscle cells in response to platelet-derived growth factor-BB. Antisense oligonucleotides and antibody to JE/MEC-1 were able to completely block monocyte migration (35). Zeliger et al. were able to demonstrate the up-regulation of MCP-1 by cultured human umbilical vein endothelial cells by inhibition of basal nitric oxide production (36).

Platelets have been shown to adhere to atheromatous lesions in vitro (37). In our experiments atheromatous plaque was able to induce platelet aggregation in 5 out of 9 cases. This may be of considerable importance in thrombogenesis since it has been reported previously that platelets powerfully enhance endotoxin-induced MO TFα, through the mediation of 12-HETE (26). Moreover, activated platelets may directly induce MO TFα generation, possibly by platelet p-selectin (32). The clinical relevance of these observations may be related to the improved outcome of some patients undergoing coronaryangioplasty who were treated with mab to IIb/IIIa platelet glycoprotein receptors (25).

We propose that plaque TFα is at least in part due to MF-MO TFα. The TFα is available on the surface of the intact MF-M0, but it also may be released (31) or may be a remnant of disrupted MF.

The following scenario is proposed: Plaque is ruptured, circulating MO are recruited by the plaque and are induced by yet unknown factors to generate TFα. Platelets attracted to the plaque enhance the generation of TFα by MO-MF. This cascade of events, which need not peak simultaneously, may be responsible for fibrin deposition over the fissure of the atheromatous plaque.

Acknowledgments

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References


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