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

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Summary

Tissue factor activity (TFa) 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 TFa when stimulated by a variety of inducers. To test the hypothesis that MO could be induced by atheromatous plaque to generate TFa, plaque extracts obtained from patients with obstructive atheromatous disease were used. These extracts were also assayed for constitutive TFa. The constitutive activity was variable from extract to extract but could be very high, up to 250 U TFa. The TFa induced in MO could be also very high, up to 200 U (i.e. 1/5 of the TFa of full strength rabbit brain thromboplastin). These findings point to a major role for MO-MF TFa 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 lipidated integral membrane-bound glycoprotein whose activity (TFa) is a powerful promoter of blood coagulation.

TFa 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. TFa 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 TFa, and have a well-established thrombogenic activity (13). Mo TFa 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 TFa 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 TFa (22). More recently, TFa has been demonstrated within atheromatous plaques obtained from coronary arteries at the time of surgery (33). Thiruvikraman 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 TFa, and that the atheromatous plaques may induce TFa generation in MO. Therefore specific inhibitors of TFa and TFa generation by MO may be an appropriate form of anticoagulation during angioplasty.

Materials and Methods

Plaque material was obtained sterily 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 tissue homogenizer with 8-10 strokes under a sterile hood. Non-dispersed material was sedimented for 5 min at $1 \times g$ 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 TFa, or studied as inducers of MN-TFa.

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 50% with phosphate buffered saline (PBS) without Ca or Mg (Grand Island Biologicals, NY), layered on Ficoll-Hypaque (Pharmacia, Piscataway, NJ) and centrifuged at $400 \times g$ 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-l% 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 l h, was added to RPMI to achieve a final concentration of l2% in the incubation mixture.

Reagents and components partaking in incubation mixtures where MO-generated TFa was assessed, were handled sterily and were endotoxin-free. MN (3×10^6/tube) were incubated with either atheromatous plaque extract and RPMI l640, or endotoxin 0.05 µg/ml (Difco, Detroit, MI) as a standard inducer of TFa, or RPMI l640 alone as negative control. After l6 h incubation at

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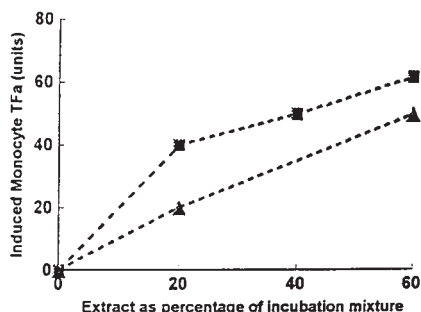
Monocyte TFa Generation Induced by Plaque Extract

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

37° C, MN were washed by suspension in 3 ml of 0.9% NaCl, and sedimented at $400 \times g$ 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 TFa. Testing for TFa 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 l000 U/ml (14). MN incubated in RPMI had a TFa, which was always below 0.5 U.

Neutralization of constitutive and induced TFa was carried out with monoclonal anti-TFa (mab TFa) kindly provided by Dr. Yale Nemerson, Mount Sinai School of Medicine (33). Specimens containing TFa 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 TFa was then measured.

Platelet aggregation was performed with the platelet Aggregation Profiler, model PAP-4 (Bio-Data Corporation, Horsham, PA). Platelet-rich plasma

Table 1 Constitutive TFa was determined on plaque extract. Plaque-induced MO TFa was determined on MNs (3×10^6) incubated for 16 h with plaque extract. Thereafter, MNs were washed, disrupted and tested for TFa

	N	Induced TFa U
Soft plaque	31	15 (0-75)
Hard plaque	7	13 (0-52)
RPMI	–	<0.5
	N	Constitutive TFa U
Soft plaque	30	22 (0-250)
Hard Plaque	5	3 (0–7)

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

	TFa (U)		
Sample	Induced (Plaque)	Induced (Plaque + FCS)	Constitutive
1	18	25	27
2	33	84	24
3	75	200	14
4	3	3	33
5	9	130	6
6	1	19	9
Endotoxin	9	5	–
RPMI	<1	<1	–

(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 TFa did not follow a normal distribution, non-parametric tests were used. The Mann-Whitney-U test was used for TFa from different samples. Paired data (i.e. TFa activity from the same samples) were evaluated with the Wilcoxon test. The relationships among induced and constitutive TFa 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 TFa in MO

To determine whether plaque extracts may induce TFa generation in MO, autologous, or blood-group-compatible MN were incubated with plaque extracts and after 16 h incubation the TFa 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 TFa by plaque extract. The generated TFa of 6 consecutive extracts were compared. Addition of 12% FCS in the MN-plaque extract incubation mixture amplified TFa generation in some, but not all of the samples. Enhancement was up to 19-fold and as much as 200 U TFa 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 TFa could be very high, in most instances severalfold higher than the TFa 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 TFa activity (Table 2).

Constitutive TFa

The constitutive TFa of 35 atheromatous plaque extracts was examined. Thirty extracts were obtained from soft plaques. The TFa 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 TFa (Table 1).

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

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

Table 3 Comparison of constitutive and induced TFa. All samples with high induced TFa (>30 U) were compared with the constitutive TFa in the corresponding extract. Similarly, all samples with high constitutive TFa (>30 U) were compared with the corresponding induced TFa

High induced with corresponding constitutive TFa		High constitutive with corresponding induced TFa	
Induced	Constitutive	Constitutive	Induced
52	1	45	3
62	31	250	3
52	2	47	24
200	14	50	18
33	1	37	14
84	24	33	3
130	6		

(over 30 U) induced TFa were compared with their corresponding constitutive TFa, 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 TFa could not be carried-over TFa.

TFa 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 TFa, constitutive or induced, on the other. However, a negative correlation was observed between total protein concentration and constitutive TFa (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 TFa, whether induced or constitutive (Table 5).

Discussion

We have demonstrated constitutive TFa 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 TFa we report could be quite high (up to 250 U), particularly in soft plaques but it was variable. The variable level of constitutive TFa from extract to extract may be due to testing of plaques at different stages of development. Indeed calcified plaques had lesser quantities of constitutive TFa. Additionally, the putative presence of inhibitors in some plaques may also be responsible for the observed lack of TFa in some specimens.

Importantly, we report that many plaque extracts induced generation of TFa in peripheral blood MO which could be more than 50 times
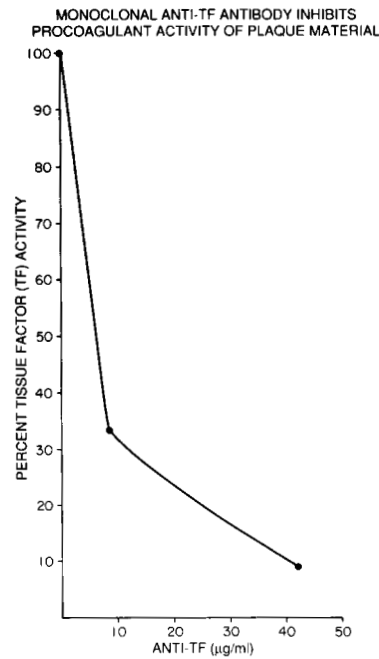

MONOCLONAL ANTI-TF ANTIBODY INHIBITS PROCOAGULANT ACTIVITY OF PLAQUE MATERIAL

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

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

% aggregation of platelets	Constitutive TFa	Induced TFa
48	10	11
34	2	<1
21	2	6
12	47	24
7	7	1
0	2	<1
0	2	1
0	7	6
0	50	18

higher than the respective constitutive TFa (Table 3). We propose that resident MF as well as MO attracted to the plaque may be induced to generate TFa. As MF may represent up to 60% of the cells in a plaque (29), considerable amounts of TFa could be generated

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

TFa Sample	Constitutive	Induced	Plaque constituents (mg/dl)				
			Protein	TG	TC	HDL-C
#1	27	18	200	4	16	6
#2	24	33	300	22	37	7
#3	14	75	200	8	6	2
#4	33	3	100	12	26	6
#5	6	9	600	35	45	9
#6	9	1	300	5	6	3

Table 4 Constitutive and induced TFa are compared with protein, triglyceride (TG), total cholesterol (TC) and HDL-cholesterol (HDL-C)

negative correlation. l. Poor co-extraction of proteins and constitutive TFa. 2. Co-extraction of an inhibitor of TFa. 3. TFa 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/MCP-1 were able to completely block monocyte migration (35). Zeiher 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 TFa, through the mediation of 12-HETE (26). Moreover, activated platelets may directly induce MO TFa generation, possibly by platelet p-selectin (32). The clinical relevance of these observations may be related to the improved outcome of some patients undergoing coronary angioplasty who were treated with mab to IIb/IIIa platelet glycoprotein receptors (25).

We propose that plaque TFa is at least in part due to MF-MO TFa. The TFa 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 TFa. Platelets attracted to the plaque enhance the generation of TFa 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.

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