Reduction of Salivary Tissue Factor (Thromboplastin) Activity by Warfarin Therapy

By Leo R. Zacharski and Robert Rosenstein

The coagulant of normal human saliva has been identified as tissue factor (thromboplastin, TF) by virtue of its ability to cause rapid coagulation in plasmas deficient in first-stage coagulation factors and to activate factor X in the presence of factor VII and by virtue of the fact that its activity is expressed only in the presence of factor VII and is inhibited by an antibody to TF. The TF is related to cells and cell fragments in saliva. Salivary TF activity has been found to be significantly reduced in patients taking warfarin. The decline in TF activity during induction of warfarin anticoagulation occurs during the warfarin-induced decline in vitamin-K-dependent clotting factor activity, as judged by the prothrombin time. The decrease in TF activity is not related to a reduction in salivary cell count or total protein content or to a direct effect of warfarin on the assay. It is hypothesized that the mechanism by which warfarin inhibits TF activity may be related to the mechanism by which it inhibits expression of the activity of the vitamin-K-dependent clotting factors. Inhibition of TF activity may be involved in the antithrombotic effect of warfarin.

Tissue factor (TF, factor III, thromboplastin) is a lipoprotein that on combining with coagulation factor VII initiates blood coagulation by the so-called extrinsic pathway. TF differs from other coagulant factors in that it does not appear to be present (at least in active form) in normal blood and it is a component of cell membranes. Because of these characteristics, its study presents unique difficulties. Although TF coagulant activity may be studied in cells cultured in vitro, this approach is cumbersome and artificial, and it provides information of uncertain physiologic significance. TF protein may be detected by an antibody to TF or by coagulation factor VII to which either a fluorescent or histochemical probe has been coupled. These substances become fixed to TF protein in tissues and therefore permit its visualization. However, the use of such methods to localize TF provides no information relative to its coagulant activity.

The desirability of having a readily available source of TF for study is evident. In this regard, attention has been directed to nonhemic body fluids as potential sources of TF activity. Although urine has been shown to contain a thromboplastin-like coagulant, it is present in low concentrations. Various pathologic effusions, amniotic fluid, bile (or other intestinal secretions), semen, sweat, or tears may be worthy of study for coagulant activity, but they are not easily obtained. Saliva, on the other hand, is especially suitable for study because of its ready availability and because saliva has long been known to possess coagulant properties. Our efforts in characterizing human salivary TF and assessing its activity in patients taking warfarin are the subject of this report.
Materials and Methods

Plasmas congenitally deficient in factors VII, VIII, IX, and XI were obtained through Dr. Peter Levine, Worcester, Massachusetts. Plasmas congenitally deficient in factors X and XII were obtained from George King Associates, Salem, New Hampshire. Normal plasma in one-tenth volume of 4% sodium citrate was prepared by centrifugation at 3200 g for 15 min. One-stage assays for factors VII, VIII, IX, XI, and XII were performed by standard methods. Assays for factors II and X were performed by methods described by others using reagents obtained from Sigma, St. Louis, Missouri. Factor VIII antigen was assayed by Dr. Leon Hoyer, Newington, Connecticut.

Saliva was collected from subjects who had had a fat-free breakfast and had abstained from coffee, tea, alcohol, and strenuous exercise since the previous evening. These included 34 normal subjects, 9 patients with cardiac valve prostheses on long-term (months to years) warfarin anticoagulation, 7 patients prior to and during induction of warfarin anticoagulation, and 1 patient receiving intravenous heparin. The normal subjects had had no medications for at least 2 wk. The patients on long-term warfarin therapy were receiving a variety of medications in addition to warfarin, as will be discussed in the section on results. Samples were obtained at intervals for up to 22 days from patients followed during induction of warfarin anticoagulation. Samples were obtained over a 4-day period in the heparinized (1000 units/hr) patient.

Samples were usually collected between the hours of 8 and 11 A.M. Salivary flow was stimulated by chewing a 1-inch length of sterile rubber tubing. Mixed oral saliva was collected into 17- X 100-mm plastic tubes (Lab-Tek Products, Miles Laboratories, Naperville, Ill.) and cleared of coarse particulate debris by centrifugation at 300 g for 15 min at room temperature.

TF was assayed by one-stage and two-stage methods. The one-stage assay, patterned after the prothrombin time assay, was performed by mixing 0.1 ml of saliva with 0.1 ml of 0.02-M CaCl₂, with the clotting reaction being started on addition of 0.1 ml of plasma. The results of all one-stage assays represent the average of duplicate determinations. In the two-stage assay the ability of salivary coagulant and rabbit brain TF to complex factor VII and subsequently to activate factor X was tested.

The incubation mixture in which partially purified factor X was activated in the presence of TF and factor VII was subsampled at intervals and mixed with normal plasma, and the clotting time was then determined. A progressive shortening of the clotting time with duration of incubation was indicative of progressive factor X activation. This test was performed as described previously, except that the control thromboplastin (rabbit brain, Ortho Pharmaceuticals, Raritan, N.J.) was appropriately diluted such that the initial clotting time approximated the clotting time obtained when an equivalent volume of saliva was tested.

Imidazole-buffered saline (IBS) was prepared according to the method of Owen et al. Lyophilized human fibrinogen was obtained from Kabi, Stockholm, Sweden, and tested in a concentration of 200 mg/dl in IBS. Known sources of TF included rabbit brain and freeze-thaw homogenates of cultured human foreskin fibroblasts prepared as described previously. Chromatographically pure lyophilized hyaluronidase (type VI from bovine testes) was obtained from Sigma, St. Louis, Missouri. Crystalline sodium warfarin was obtained from Endo Laboratories, Garden City, New York. Filtration of hyaluronidase-treated saliva was performed on filters with a pore size of 0.2 μ (Nalge, Rochester, N.Y.).

Serial twofold dilutions of hyaluronidase-treated saliva in IBS were prepared before and after filtration and before and after graded centrifugation and then assayed. A calculated line (method of least squares) was obtained for points of each dilution curve. Cell counts were performed in a hemocytometer. Smears used to identify cells were stained with Wright’s stain. In preparation for electron microscopy, hyaluronidase-treated saliva was centrifuged at 1000 g for 15 min at room temperature to remove intact cells. The supernatant was then centrifuged at 3200 g for 30 min. The button thus obtained was fixed in glutaraldehyde with cacodylate buffer, postfixed in osmium tetroxide, dehydrated in graded concentrations of alcohol, and embedded in Epon (Ladd Industries, Burlington, Vt.).

A sample of rabbit serum containing antibody to human TF was provided by Dr. Yale Nemerson, New York, New York. The effects of both this antisera and control rabbit serum on the TF activity of homogenates of cultured human fibroblasts and on salivary coagulant activity were determined as follows: First 0.3 ml of serum was mixed with 0.9 ml of IBS. Then 0.4 ml of mixed saliva or 0.4 ml of fibroblast homogenate was mixed with 0.4 ml of diluted serum and then incubated with occasional mixing for 30 min at room temperature. Then 0.1 ml of this incubation mixture was mixed with 0.1 ml of normal plasma or factor-VIII-deficient plasma and 0.1 ml of 0.02-M calcium chloride, and the clotting
Table 1. Effects of Saliva and Normal Plasma on Assay for Factor VII*

<table>
<thead>
<tr>
<th>Additive</th>
<th>Amount (ml) of Buffer, Saliva, or Normal Plasma Added to Test System (0.20 ml)</th>
<th>Clotting Times (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer control</td>
<td>0.01</td>
<td>31.2</td>
</tr>
<tr>
<td>Saliva</td>
<td>0.01</td>
<td>31.2</td>
</tr>
<tr>
<td>Saliva</td>
<td>0.02</td>
<td>31.0</td>
</tr>
<tr>
<td>Saliva</td>
<td>0.03</td>
<td>30.9</td>
</tr>
<tr>
<td>Saliva</td>
<td>0.04</td>
<td>30.6</td>
</tr>
<tr>
<td>Saliva</td>
<td>0.10</td>
<td>30.6</td>
</tr>
<tr>
<td>Normal plasma</td>
<td>0.01</td>
<td>17.3</td>
</tr>
<tr>
<td>Normal plasma</td>
<td>0.02</td>
<td>14.8</td>
</tr>
<tr>
<td>Normal plasma</td>
<td>0.03</td>
<td>13.8</td>
</tr>
<tr>
<td>Normal plasma</td>
<td>0.04</td>
<td>13.9</td>
</tr>
<tr>
<td>Normal plasma</td>
<td>0.10</td>
<td>13.9</td>
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*Average of three experiments.

time was determined (at 37°C). The factor-VIII-deficient plasma was incorporated into this experiment in order to delay coagulation by way of the intrinsic pathway (Table 2).

Data from normal subjects and from patients on long-term warfarin anticoagulation were analyzed by Student’s t-test. Slopes of clotting times for patients studied prior to and during induction of warfarin anticoagulation and for the individual receiving intravenous heparin were analyzed by a program that provided an F ratio for a linear regression with several y values for each x value. It was recognized that the change in prothrombin time was not linear (vide infra); however, the data were analyzed by linear regression analysis to demonstrate that a significant change occurred in the activity of vitamin-K-dependent factor.

RESULTS

Saliva accelerated the coagulation of normal plasma and plasmas deficient in any one of factors VIII, IX, XI, and XII. By contrast, saliva contained less than 1% of the coagulant activity of factors II, VII, and X and less than 1% of the factor VIII antigen content of normal plasma. That deficiency of factor VII blocked the coagulant activity of saliva is illustrated by the failure of incremental volumes of saliva to shorten the clotting times observed in the assay for factor VII (Table 1). Inhibition of salivary coagulant activity by an antibody to TF is illustrated in Table 2. The ability of salivary coagulant to activate factor X in the presence of factor VII (i.e., the two-stage assay for TF) was demonstrated in three experiments. The results obtained in one of these are illustrated in Fig. 1. In this particular experiment, 1:8 and 1:16 dilutions of control (rabbit brain) TF were used. Saliva clotted a solution of human fibrinogen in 2½ hr.

Table 2. Inhibition of Salivary Coagulant Activity by Anti-TF Antibody

<table>
<thead>
<tr>
<th>Incubation Mixture</th>
<th>Normal Plasma</th>
<th>Factor-VIII-deficient Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblast homogenate + control serum</td>
<td>56.4</td>
<td>57.4</td>
</tr>
<tr>
<td>Fibroblast homogenate + anti-TF serum</td>
<td>271</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Saliva + control serum</td>
<td>92.0</td>
<td>94.1</td>
</tr>
<tr>
<td>Saliva + anti-TF serum</td>
<td>191</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Saline blank</td>
<td>190</td>
<td>&gt;300</td>
</tr>
</tbody>
</table>
Centrifugation of saliva at 500 g for 15 min (in order to remove gross particulate debris in preparation for assay) had no effect on salivary coagulant activity. Attempts to use centrifugation or Micropore filtration to determine if salivary TF was particulate were frustrated by the high viscosity of saliva. This problem was overcome by treating saliva with hyaluronidase. Addition of 600 units of hyaluronidase (in 0.2 ml of IBS) to 1 ml of saliva effectively reduced the viscosity but had no effect on the coagulant activity. The cell count (average of determinations on three individuals) was 1400 cells/ml in hyaluronidase-treated saliva. Following centrifugation at 500 g for 15 min, the cell count was reduced to 366 cells/ml, and at 1000 g the cell count was reduced to zero. When assayed with normal plasma, 60% of the coagulant activity was removed by centrifugation at 500 g, and 78% was removed by centrifugation at 1000 g. Less than 0.1% of the initial coagulant activity remained following filtration on filters of 0.2 μm pore size.

Hyaluronidase treatment of saliva also permitted assessment of cell morphology on smears made with Wright's stain. Whereas cells in smears of native saliva manifested considerable distortion that prevented ready identification, cells present on smears of hyaluronidase-treated saliva were better preserved. More than 99% of such cells were buccal epithelial cells, and the remainder were neutrophils. Mononuclear leukocytes were not observed. Particulate material present in hyaluronidase-treated saliva that had been rendered cell-free by centrifugation at 1000 g for 15 min was pelleted and examined by electron microscopy. This particulate material consisted of cellular debris that was similar in appearance to fragments resulting from sonication of cells.

Salivary coagulant activity was not increased by freezing and thawing and was stable at −20°C for at least 2 wk. Suspensions of epithelial cells in IBS prepared by scraping the buccal mucosa with a spatula also manifested TF coagulant activity. Salivary TF activity was significantly less (longer clotting times) in the 9 patients on long-term warfarin anticoagulation, as compared with that in 34 normal
Fig. 2. Results of one-stage assays for TF and prothrombin time in 7 patients followed during induction of warfarin anticoagulation. These results were compared to those in normal subjects and individuals on long-term anticoagulant therapy. *Error bars in either direction represent 1 SD from the mean. † different from normals (p ≤ 0.001); black triangles indicate mean for normal subjects (n = 34) for saliva tissue factor and laboratory mean for normal prothrombin time. Open circles indicate prewarfarin mean for the 7 patients from whom the data were obtained for the regression analysis. Small black dots indicate individual data points for these patients. Larger black circle indicate mean for 9 patients on long-term warfarin therapy. Regression of clotting time (tissue indicator factor) versus days on warfarin, p ≤ 0.01; regression of prothrombin time versus days on warfarin, p ≤ 0.01.

subjects (Fig. 2). Daily warfarin doses in the patients ranged from 5 to 10 mg/day (mean 7.0 mg/day). In addition to the warfarin, 8 of the 9 patients were also taking oral iron supplements, 7 were taking folic acid, 4 were taking digoxin, 3 were taking propranolol, 2 were taking quinidine, and 2 were taking a diuretic. Four of the 9 patients were taking no medication other than iron supplement and folic acid. Of these 4 patients, 2 had salivary clotting times above the mean for the total group, and 1 had a clotting time equal to the mean.

Clotting times prior to introduction of warfarin treatment were not significantly different from normal in the 7 patients who were followed during induction of anticoagulation. However, the clotting times of this group increased significantly during warfarin treatment and became comparable to the clotting times in the 9 individuals on long-term anticoagulation (Fig. 2). As expected, the prothrombin times also increased during this period. Postwarfarin TF activity was between 15% and 20% of the prewarfarin activity.

As anticipated, the prothrombin times appeared to be curvilinear, with the greatest rate of change occurring during the first few days (Fig. 2). The scatter of the data for the salivary TF activity was such that linearity versus curvilinearity could not be determined. Therefore, for purposes of illustration, and in order to test
the significance of the change in clotting times obtained in the salivary TF assay in the prothrombin times, the data from these patients were analyzed by linear regression (log of clotting time versus number of days following initiation of warfarin therapy). Whereas data obtained over 22 days were used for construction of the regression line illustrated for salivary TF, a significant slope \( p \leq 0.05 \) was observed when data from only the first 9 days were used. A prolongation in clotting time was not noted in the individual on heparin; in fact, the slope of his curve was negative. \textit{In vitro} addition of sodium warfarin in various concentrations up to 2 mg/ml failed to alter TF activity. No differences in cell count or total protein concentration \( p \leq 0.001 \) were found when saliva from control subjects was compared with saliva from patients on long-term warfarin therapy.

**DISCUSSION**

TF is best known as a laboratory reagent used in performing the one-stage prothrombin time test. Although much has recently been learned of the biochemistry and cell biology of TF, little is known of its pathophysiologic significance. This lack of knowledge is related to inherent difficulties in readily obtaining TF-containing samples for assay. The demonstration of TF in saliva may provide a solution to this problem.

Although saliva has long been recognized as having thromboplastic properties, this activity could not heretofore be conclusively attributed to TF. We have documented the existence of TF in saliva by four criteria. First, saliva accelerated coagulation in plasma deficient in activity of one of the factors VIII, IX, XI, and XII. By contrast, saliva contained less than 1% of the coagulant activity of factors II, VII, and X and less than 1% of the level of factor VIII antigen of normal plasma. Although the clotting of a fibrinogen solution by saliva in 2½ hr may have been due to the presence of trace amounts of thrombin, enzymes other than thrombin present in saliva are more likely responsible for this finding. Second, no dose-related response was observed when incremental volumes of saliva were incorporated into the assay for factor VII, thus indicating that factor VII is necessary for the expression of salivary coagulant. Third, salivary coagulant was capable of activating factor X in the presence of factor VII. Fourth, the activity of salivary coagulant was obliterated by an antibody to human TF.

Approximately 78% of salivary TF activity is attributable to the presence of cells in saliva and the remainder to particulate cell debris. No coagulant activity is present in saliva rendered free of cells and particles by centrifugation or Micropore filtration. The question whether TF is produced by these cells or is absorbed to cell surfaces is moot at this time. Although leukocytes have been shown to possess TF activity under certain circumstances, our results suggest that buccal epithelial cells rather than leukocytes present in saliva are the source of the TF activity. The evidence for this is that more than 99% of the cells present on Wright-stained smears of salivary cell pellets are epithelial. The remainder are neutrophils. No mononuclear leukocytes are seen. Work now in progress indicates that the saliva of edentulous individuals, which is known to contain reduced numbers of leukocytes, possesses TF activity comparable to that of individuals with teeth. TF activity is also demonstrable in saline suspensions of epithelial cells prepared by scraping the buccal mucosa with a spatula.
In 1964 Doku and associates described a reduction in salivary coagulant activity that was not thought to be due to the presence of an inhibitor in patients receiving warfarin. Unfortunately, the nature of the salivary coagulant was not clearly defined at that time, and the manner in which the data were presented rendered their findings unconvincing. No further relevant observations were made until Edwards and Rickles reported reduced TF production by mononuclear leukocytes from individuals receiving warfarin. This finding could not likely be attributed to a nonspecific cytotoxic effect, because these cells retained their ability to ingest latex particles, exclude trypan blue, and undergo blastogenesis in culture. Edwards and Rickles postulated that warfarin might induce a selective lesion of cellular metabolism that renders the cells unable to synthesize TF. Since warfarin inhibits vitamin-K-dependent gamma carboxylation of glutamic acid residues of the precursor molecules for clotting factors II (prothrombin), VII, IX and X, they postulated that TF may also be a clotting factor, the activity of which is dependent on gamma carboxylation.

Our findings on buccal epithelial cells and cell fragments in human saliva confirm those of Edwards and Rickles on mononuclear leukocytes. Our results further suggest that the reduction in salivary TF from individuals on warfarin is time-dependent and is not related to a direct effect of warfarin on salivary TF or the assay used to measure TF activity. The fact that the decline in salivary TF activity accompanied the decline in clotting activity observed in the prothrombin time assay and the fact that similar percentage reductions in TF activity and vitamin-K-dependent coagulation factor activity occurred might be interpreted as indicative of a related mechanism of inhibition. However, this remains to be determined. No prolongation of salivary TF was observed in a patient receiving intravenous heparin.

The warfarin-related reduction in TF activity did not appear to be related to the disease for which the patient was being anticoagulated, nor did it appear to be related to other drugs being taken, reduced salivary cell count, or reduced protein concentration. There would obviously be an advantage to expressing TF in terms of specific activity. However, our results thus far suggest that the reduced activity observed is not related to a reduction in cell or protein concentration due to warfarin. Unfortunately it is not possible, in our opinion, to completely resolve this problem at the present time because it is impossible to quantitate TF independently of its coagulant activity, and it is not known what proportion of the total protein is represented by TF. Therefore, expressions of specific activity using current methods would not likely be very meaningful.

Besides TF, other substances of interest to the coagulationist that have been identified in human saliva include kallikrein, plasminogen activator, and plasmin. Although the functions of such substances in human saliva are unknown, there is less doubt about their roles in certain animals. In fact, coagulant or fibrinolytic activity has long been recognized to exist in the secretions of salivary glands of many species. Creatures with macabre feeding habits may depend on the coagulant activity of saliva to immobilize prey larger than themselves (e.g., certain snakes and spiders) or may depend on the anticoagulant or fibrinolytic activity to render the blood on which they feed incoagulable (e.g., leeches, vampire bats).

The pathophysiologic significance of TF in human saliva is unknown. It is
conceivable that TF may facilitate the barrier function of the buccal mucosa by securing hemostasis in the event of injury, such as during mastication. A half century ago Hunter\textsuperscript{31} advocated treating bleeding peptic ulcers by stimulating salivary flow. He postulated that bleeding would be reduced by the coagulant activity of the swallowed saliva. Ersner et al.\textsuperscript{11} applied saliva-soaked gauze to the tonsillar bed to secure hemostasis following tonsillectomy. Although current scruples might militate against such practices, medicinal properties have been attributed to saliva since antiquity, and they continue to be recognized in modern folklore.\textsuperscript{32} Animals instinctively lick an injured part, and the account of the dogs that licked Lazarus's sores is well known.\textsuperscript{33}

The advantages of using saliva to study TF include its relative (if not complete) freedom from other clotting factors that might confound the interpretation of results and the requirement for only small samples that are easily obtained by noninvasive means. Since the TF in saliva exists on cells and cell fragments, this test might be construed as a painless "biopsy."

In summary, we postulate that studies of saliva might assist in elucidating the pathophysiologic significance of TF. Our results suggest that either TF itself or perhaps an enzyme involved in TF synthesis might be dependent on the presence of vitamin K for its activity. We further suggest that the therapeutic (i.e., antithrombotic) effect of warfarin might rest, at least partially, in its ability to reduce endogenous TF activity.

ACKNOWLEDGMENT

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REFERENCES

33. Luke 16:21
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