Oral Anticoagulation Thresholds

Kathleen E. Brummel, PhD; Sara G. Paradis, BS; Richard F. Branda, MD; Kenneth G. Mann, PhD

Background—Monitoring patients on oral anticoagulation is essential to prevent hemorrhage and recurrent thrombosis. We studied tissue factor–induced whole-blood coagulation in patients on warfarin therapy with similar international normalized ratios (INRs).

Methods and Results—Contact pathway–suppressed whole-blood coagulation initiated with tissue factor was studied in 8 male subjects (group W) and in 1 individual multiple times (subject A). Coagulation profiles for group W showed that subjects with similar INRs had widely varying clot times (6.2 to 23 minutes) and thrombin–antithrombin III (TAT) profiles with rates of 25 to 40 nmol · L−1 · min−1 and maximum levels varying from 192 to 349 nmol/L. The normal control group exhibited clot times of 5.7±0.3 minutes and TAT rates of 57±13 nmol · L−1 · min−1, reaching maximum levels of 742±91 nmol/L. Subject A, who was stably anticoagulated at an INR of 2.1±0.4 for 6 months, had widely ranging profiles with clot times of 9.0 to 22.7 minutes, TAT maximums varying from 141 to 345 nmol/L, and TAT formation rates of 10 to 57 nmol · L−1 · min−1. INR did not correlate with TAT formation. Platelet activation was decreased by anticoagulants but also displayed variability. Fibrinopeptide A generation showed threshold variability independent of the INR. Factor VIII levels were increased (P=0.03) in group W (204±34.4%) compared with normal control subjects (149.4±37.4%). A significant correlation was identified between increasing factor VIII levels and years on warfarin therapy (r=0.78, P=0.01), suggesting a possible factor VIII compensatory mechanism.

Conclusions—These results suggest that control of anticoagulation in patients to a set INR therapeutic range may be less secure than anticipated. Patients with similar INRs show significant individual variability in their tissue factor coagulation response, suggesting different risks to anticoagulation when confronted with underlying vascular anomalies.

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Key Words: anticoagulants ■ hemorrhage ■ coagulation ■ thrombosis

oral anticoagulants effectively block the regeneration of reduced vitamin K, thereby depleting the amount of vitamin K–dependent (VKD) proteins available for coagulation reactions. These compounds block the glutamate-to–γ-carboxylglutamate posttranslational modification in the VKD coagulation proteins2-3 prothrombin; factors (F) VII, FIX, and FX; and proteins C, S, and Z. Glutamate–γ-carboxylglutamate modification in the NH2 termini of these proteins allows them to interact with calcium ions (Ca2+) and form membrane complexes with anionic phospholipids, membrane interactions essential for maintaining catalytic functions in hemostasis.

Tissue factor (TF)–initiated coagulation has been shown to be the mechanism for blood clotting in vivo. On exposure/ expression, TF binds to circulating plasma FVIIa. The resulting extrinsic tenase complex activates FIX and FX. FIXa, FVIIa, Ca2+, and a membrane form the intrinsic tenase complex activating the VKD zymogen FX to FXa, initiating the “propagation” phase of thrombin generation. Ultimately, prothrombin is converted to thrombin via the VKD prothrombinase complex (FXa-FVa-Ca2+-membrane). Thrombin, the key enzyme in coagulation, has many roles, including converting fibrinogen to fibrin, activating platelets, activating FV and FVIII, and combining with thrombomodulin to activate protein C. Downregulation of formation of the VKD enzymes, especially the amount of available thrombin through anticoagulant therapy, has a major influence on the coagulation cascade.
cardiac valve replacement, acute myocardial infarction, and prevention of stroke or deep vein thrombosis. Standardization of warfarin therapy with the INR is a great improvement over monitoring by PT alone. However, complications from warfarin therapy occur even with proper monitoring, with major bleeding in 1% to 5% of those treated.\textsuperscript{10,11} This value is increased (\(\approx 8\%\)) when all types of bleeds are included.\textsuperscript{12}

The PT assay, performed by adding excess TF and Ca\textsuperscript{2+} (thromboplastin reagent), is sensitive to FVII, FX, FV, prothrombin, and fibrinogen. The PT ignores the contributions of FVIII, FIX, and FXI (the defects in hemophilia A, B, and C), as well as blood cells. The method we have used is sensitive to all these agents and provides in-depth insight into coagulation monitoring.

The present study was undertaken to evaluate TF-initiated whole blood collected from individuals stably anticoagulated to determine whether the INR predicts coagulation when sensitive, inclusive tests of the hemostatic competence are used. The whole-blood model\textsuperscript{13–15} permits evaluation of coagulation, which integrates all blood component contributions to the hemostatic process at nearly physiological blood conditions, with a TF stimulus that is at biologically reasonable levels.

**Methods**

**Materials**

HEPES, Tris-HCl, EDTA, TFA, L-\(\alpha\)-phosphatidyl serine (PS), and L-\(\alpha\)-phosphatidyl choline (PC) were purchased from Sigma Chemical Co. High-performance liquid chromatography--grade H\textsubscript{2}O and CH\textsubscript{3}CN were purchased from Fischer Scientific. Benzamidine-HCl was purchased from Aldrich, Inc. Recombinant TF was a gift from Drs Roger Lundblad and Shu-Len Liu (Baxter Healthcare Corp) and was relipidated in 25% PS/75% PC (PCPS) vesicles by a previously described protocol.\textsuperscript{16,17} Corn trypsin inhibitor was prepared as described.\textsuperscript{14} D-Phenylalanyl-l-prolyl-l-arginine chloromethyl ketone (FPRck) and biotinylated FPRck were gifts from Dr Richard Jenny (Hematologic Technologies). ELISA kits were used to estimate thrombin-antithrombin-III (TAT; Behring) and platelet osteonectin (a gift from Dr Richard Jenny). Murine monoclonal \(\alpha\)-Fbg3A, which recognizes the \(\alpha\)-chain of fibrinogen,\textsuperscript{15} was provided by the Monoclonal Facility (University of Vermont). Goat \(\alpha\)-mouse IgG horseradish peroxidase was purchased from Southern Biotechnology Associates.

**Subjects**

Nine male subjects (average age, 68 ± 12 years) stably anticoagulated with warfarin with no prior history of bleeding were studied. Warfarin therapy ranged from 0.16 to 10 years. Eight subjects with warfarin with no prior history of bleeding were studied. Nine male subjects (average age, 68 ± 12 years) stably anticoagulated with warfarin therapy occur even with proper monitoring, with major bleeding in 1% to 5% of those treated.\textsuperscript{10,11} This value is increased (\(\approx 8\%\)) when all types of bleeds are included.\textsuperscript{12}

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**Whole-Blood Coagulation**

Blood was collected by venipuncture, and 1-mL aliquots were distributed into tubes containing corn trypsin inhibitor (100 \(\mu\)g/mL), TF, and PCPS (12.5 pmol/L and 25 nmol/L) as previously described.\textsuperscript{13–15} Samples were quenched at intervals over 1 hour with an inhibitor cocktail: 50 mmol/L EDTA and 20 mmol/L benzamidine-HCl in HEPES-buffered saline, pH 7.4, plus 10 \(\mu\)L of either 10 mmol/L FPRck or biotinylated FPRck in 10 mmol/L HCl. Clot time (CT) was determined visually (2 observers). After quenching, samples were centrifuged (15 minutes at 2000 rpm); clot material was separated from the solution phase, divided into aliquots, and stored at \(-80^\circ\)C.

**Immunoassays and Calculated CTs**

Commercial ELISAs for TAT complex formation and platelet \(\alpha\)-granule osteonectin release were performed as previously described.\textsuperscript{14} From TAT ELISA data, regression analysis was performed on the rising part of the curve to generate a linear equation. With an estimated 10 nmol/L TAT formation\textsuperscript{13} used as the point of CT, the equation was solved to generate objective initiation phase CTs (CT\textsubscript{I}). Rates were determined from the slope of the curves.

**Fibrinopeptide and Fibrinopeptide Detection**

Fibrinopeptide A (FPA) was isolated by high-performance liquid chromatography.\textsuperscript{13} Soluble fibrinogen was analyzed by nonreduced SDS-PAGE according to a modified Laemmli procedure.\textsuperscript{15,18} The proteins were transferred by semidry methods to nitrocellulose membranes (Bio-Rad),\textsuperscript{19} blotted with \(\alpha\)-mouse IgG horseradish peroxidase (1:5000), developed, and quantified.\textsuperscript{13}

**Statistical Analysis**

Values are expressed as mean ± SEM. ANOVA and correlation analysis were performed with SAS software. Significance was defined as \(P<0.05\).

**Results**

**TAT Complex Formation**

TAT profiles from blood obtained from group W individuals (5 individuals illustrated) showed substantial variations not predicted by the INR (Figure 1A). Subjects with similar INRs had TAT profiles that differed in the duration of the “initiation” phase of TAT formation, rate of activation phase, and maximum TAT level achieved. The control curve (●) generated from 5 normal control subjects showed a CT of 5.7 ± 0.3 minutes with maximum TAT levels of 742 ± 91 nmol/L produced at an average rate of 57 ± 13 nmol \cdot L\textsuperscript{-1} \cdot min\textsuperscript{-1}. Subject 3 (▲) had an INR of 1.7, a CT of 6.2 minutes, and a maximum TAT of 340 nmol/L produced at a rate of 40 nmol \cdot L\textsuperscript{-1} \cdot min\textsuperscript{-1}. In comparison, subject 1 (∗), with an INR of 1.9, had a CT of 11.5 minutes with a maximum TAT value of 192 nmol/L produced at a rate of 25 nmol \cdot L\textsuperscript{-1} \cdot min\textsuperscript{-1}. The maximum TAT levels achieved by group W, including the control subjects, showed a strong correlation with prothrombin concentration (\(r = 0.96\)). Without the control subjects, the correlation was not significant (\(P = 0.51\)). INR did not correlate with TAT for group W (\(P = 0.32\)).

An individual studied repeatedly, subject A (5 experiments illustrated), also exhibited significant variability in the initiation and propagation phases of TF–induced thrombin generation although similar INR values (Figure 1B) were obtained. With an INR of 2.5, CT varied from 10 (∗) to 22 minutes (∗∗). TAT profiles varied from 210 nmol/L TAT
formation at 30 minutes (>) at a rate of 26 nmol · L⁻¹ · min⁻¹ to ≈100 nmol/L. TAT formation at a rate of 10 nmol · L⁻¹ · min⁻¹ (♀). INR was not strongly correlated with maximum TAT levels for subject A (P=0.09).

Figure 2 (♀) illustrates the variability between INR and CT, from all experiments, including the control group (♀). From the Table, a comparison of individuals from group W shows that stably anticoagulated individual(s) exhibited predicted visual CTs from 8.5 to 23 minutes and calculated CT, of 8.7 to 20.9 minutes. Subject A showed significant variability, with visual CT ranging from 8.2 to 22.7 minutes and CT, ranging from 10.3 to 23 minutes. After warfarin therapy (the Table, A11), the CTs for subject A normalized (~6 minutes), as did TAT maximum (789 nmol/L) and rate of TAT formation (55 nmol · L⁻¹ · min⁻¹). The CT profiles for all subjects were similar to the CT profiles with discrepancies only at the longer (visual) CTs. A significant correlation was seen between CT and INR (P=0.02) for group W. Subject A exhibited no correlation between CT and INR.

Table: Comparison of Coagulation Profiles From Stable, Orally Anticoagulated Individuals

<table>
<thead>
<tr>
<th>Subjects</th>
<th>INR</th>
<th>Visual Clot Time, min</th>
<th>Calculated Clot Time, min</th>
<th>TAT, nmol/L</th>
<th>TAT Rate, nmol/min</th>
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<tr>
<td>Group W</td>
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</tr>
<tr>
<td>1</td>
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</tr>
<tr>
<td>7</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>8</td>
<td>2.4</td>
<td>11.0</td>
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<td>312</td>
<td>34</td>
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<tr>
<td>Control group (n=5)</td>
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<tr>
<td></td>
<td>1.0</td>
<td>5.7±0.3</td>
<td>5.8±0.6</td>
<td>742±91</td>
<td>57±13</td>
</tr>
</tbody>
</table>

| Subject A |     |                       |                           |             |                   |
| A1        | 2.6 | 11.3                  | 10.3                      | 141         | 17                |
| A2        | 1.4 | 9.0                   | 10.5                      | 335         | 33                |
| A3        | 1.3 | 8.2                   | 10.7                      | 345         | 57                |
| A4        | 1.9 | 10.7                  | 12.9                      | 224         | 28                |
| A5        | 2.1 | 19.5                  | 16.0                      | 147         | ND                |
| A6        | 2.1 | 22.7                  | 19.3                      | 216         | 13                |
| A7        | 2.4 | 22.5                  | 18.8                      | 196         | 17                |
| A8        | 2.5 | 22.0                  | 22.9                      | 210         | 10                |
| A9        | 2.3 | 14.3                  | 16.1                      | 299         | ND                |
| A10       | 2.5 | 10.0                  | 10.6                      | 249         | 26                |
| A11       | 1.0 | 5.8                   | 6.0                       | 789         | 55                |

ND indicates not determined.

Platelet Activation

Representative data for platelet activation as measured by α-granule osteonectin release are shown in Figure 3. In the control group (♀), ~80% osteonectin was released by CT (arrow, Figure 3A) and reached maximum levels of ~60 nmol/L. In group W, the threshold for platelet activation was delayed compared with normal blood, as was the amount of

Figure 1. Thrombin generation determined from TAT complex formation. A, Selected TAT (nmol/L) vs time (min) profiles for group W. Control curve (♀) was generated from 5 age-related men. Visual CT (arrow ♀) was 5.7±0.3 minutes. Five individuals are depicted: subjects 1 (♀), 2 (♀♀), 3 (♀♀), 5 (♀♀), and 6 (♀♀). B, Five draws of subject A are depicted with date (month/day) of blood draw shown: A(3) (♀♀), A(7) (♀♀), A(8) (♀♀), A(9) (♀♀), and A(10) (♀♀). Visual CTs (arrow) and INR are depicted under each graph with corresponding symbols. *Aspirin use.

Figure 2. Comparison of CT, with INR. All blood draws (♀) are depicted with control group (♀). Control group CT was 5.8±0.34 minutes.

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Osteonectin detected before CT. For subject 3 (△), ~31% osteonectin was released by CT; for subject 6 (●), 36%; for subject 1 (○), 55%; and for subject 2 (□), 6%. Also, maximum levels of osteonectin released by 30 minute appeared slightly lower (~40 nmol/L) in several subjects although platelet levels did not vary. Platelet levels were 2.37±0.37×10^8 platelets/mL for the control subjects and 1.82±0.18×10^8 platelets/mL for group W subjects.

In subject A (Figure 3B), the thresholds for platelet activation varied substantially. The amount of osteonectin released before CT varied from 55% (○) to 68% (●) to 27% (□). The maximum levels of osteonectin released appeared always to reach those of the control group. Platelet levels for subject A were 2.49±0.13×10^8 platelets/mL.

**FPA Generation**

FPA release data are illustrated in Figure 4. Control levels (●) are shown, with ~50% FPA released before CT (arrow), reaching maximum levels of ~12 μmol/L. Similar results are seen in group W (Figure 4A) and in subject A (Figure 4B). Once the threshold is reached, maximum FPA release occurs (12 to 14 μmol/L), which is similar to the control group.

**Fibrin Formation**

The solution phase of whole blood was analyzed for fibrinogen to determine when insoluble fibrin formation occurs. In Figure 5 (top), soluble fibrinogen products from a control individual are illustrated with both CT and CTi of 6 minutes. Fibrinogen disappears from solution immediately after CT. In subject A’s draw A7 with an INR of 2.4, CT occurred by 22 minutes, and 90% of fibrinogen was removed from solution at that time. The CTi was calculated to be ~19 minutes. Subject 6 with a similar INR of 2.5 had 70% of fibrinogen removed from solution by visual CT (10.5 minutes), which coincided with CTi (11.0 minutes).
Coagulation Profile

The coagulation profiles for TAT and FPA data for the individuals (INR 2.4 and 2.5) presented in Figure 5 are overlaid in Figure 6. The data are illustrated as relative percent reaction versus time. The TAT (□) propagation phase for the individual with an INR of 2.5 (CTi of 11 minutes) begins at ≈10.5 minutes, at which time ≈50% FPA (●) had already been released from fibrinogen. The profile for the other individual with an INR of 2.4 shows significantly displaced thresholds. The initiation phase of TAT (○) generation is extended to 18 to 19 minutes and FPA (○) release threshold to 11 to 12 minutes.

Factor Levels

FVIII levels were increased ($P=0.03$) in group W ($204±34.4\%$) compared with normal control subjects ($149±37.4\%$). FIX levels were $48.0±12.8\%$ for group W and $42.2±14\%$ for subject A ($107.8±25.8\%$ for control group). A strong positive correlation for all subjects was detected between years on warfarin therapy and FVIII levels ($P=0.01$, $r=0.78$; Figure 7). The longer an individual was on warfarin therapy, the higher his FVIII level was, suggestive of a compensatory mechanism. Interestingly, von Willebrand factor showed no corresponding correlation ($P=0.70$, $r=−0.18$). Prothrombin, FV, FVII, FIX, FX, antithrombin III, fibrinogen, and platelets also did not show a correlation at this sample size.

Discussion

Our data indicate that standardization for control of warfarin anticoagulant therapy may be less secure than anticipated. Individuals with similar INR values have very different TF threshold responses to coagulation. Even a single individual studied over the course of anticoagulant therapy is highly variable. The data show the wide range of anticoagulation that can be achieved without clinical complications, suggesting that bleeding risks still present are due to preexisting vascular anomalies.

The TF concentration chosen to evaluate the PT was selected to produce a CT of 11 to 15 seconds. The high concentration of thromboplastin reagent used was chosen to...
avoid competition from the contact activation pathway. However, the in vitro concentrations of thromboplastin reagents used in these assays are massive compared with the concentrations likely encountered during pathological vascular challenge. It should be recalled that PT is also not influenced by platelets or blood cells, FVIII, FIX, and FXI, which are important indicators of coagulation pathologies. The whole-blood coagulation model used in the present study, initiated with very low levels of TF (12.5 pmol/L), is able to study the interactions of all these agents. This allows evaluation of coagulation under TF initiation conditions similar to those encountered during a “Simplate” bleeding time. In previous studies, we have shown that thrombin generation occurs with an initiation phase of thrombin formation that is a consequence of the FXa generated from the extrinsic tenase complex, followed by a propagation phase that is due largely to the FXa generated from the intrinsic tenase complex. Once \( \approx 10 \text{ nmol/L} \) thrombin is generated, clotting occurs and the propagation phase begins, which is the phase when most thrombin is generated. The quality of the propagation phase is significantly influenced by congenital, acquired, and pharmacologically induced alterations in coagulation.

In the present study, during anticoagulant therapy, lower levels of prothrombin are produced (26±8.4%, group W), as well as other VKD proteins required for the generation of thrombin. This is accompanied by an increase in the duration of the initiation phase and a decreased propagation phase rate in thrombin formation (Figure 1). This result is similar to that seen in individuals who are FV, FIX, and FVIII deficient, those treated with antiplaatelet agents, or patients who are thrombocytopenic. Platelet activation (Figure 3), FPA formation (Figure 4), and fibrinogen depletion from solution (Figure 5) are consistent indicators of the CT and illustrate the individual variability. The visual and calculated CTs (CT and CTi) observed in group W and subject A (the Table) show that similar INR values do not predict clotting thresholds produced when blood is subjected to biologically relevant levels of TF. This variable initiation phase is indicative of altered sensitivity to the TF-induced thresholds of thrombin formation.

Our results suggest that great plasticity exists in the hemostatic control system and a relatively large latitude in the safe manipulation of blood by anticoagulants. The risks associated with anticoagulation are probably conditioned by the continuity of the vascular tree and the preexistence of vascular pathology. There is an extraordinary window for anticoagulation that will only manifest difficulty with vascular imperfection. Thus, the 1% to 5% of the population on warfarin under good INR control who suffer from major bleeds probably couple anticoagulation with underlying vascular pathology that is invisible to blood analyses. The 9 individuals in our study exhibited no significant bleeding or thrombotic complications. At times, subject A’s threshold to TF response extended over the course of 20 minutes, a profile similar to that for hemophilia, yet he exhibited no underlying problems from day to day, and his INR was stable.

The general consensus is that evaluating anticoagulation in terms of an INR value is superior to the alternative of PT prolongation. Since the introduction of the INR, bleeding episodes on anticoagulation have been reduced; however, bleeding still remains a major problem. In our study, INR was correlated with prothrombin levels for group W \( (P=0.0005, n=8) \) and subject A \( (P=0.04, n=11) \). This observation agrees with the concept that the antithrombotic effect of warfarin reflects its ability to lower prothrombin levels, which is considered a risk factor for thrombosis.

FVIII levels have been shown to be associated with venous thrombosis. Increases in plasma levels of FVIII (182±66% versus 157±54% for control subjects; \( P=0.009 \)) were found in individuals with recurrent venous thromboembolism. Individuals in our study had FVIII levels ranging from 172% to 276% for group W and 114% to 150% during the course of treatment and 168% after discontinuation of warfarin therapy for subject A. The increase in plasma levels of FVIII was correlated with the length of time an individual was on warfarin therapy \( (P=0.01; \text{Figure 7}) \). No correlation was detected between the circulating carrier of FVIII, von Willebrand factor, and years on warfarin therapy. This increase in FVIII levels over the course of warfarin therapy suggests the possibility that a compensatory pathway partially corrects for the vitamin K–deficient state that exists in these subjects.

Our study was performed to gain insight into the underlying coagulation response of individuals on warfarin, not to suggest new methods of treatment or evaluation. The data show that the level of anticoagulation one can achieve without hemorrhagic complications suggests that the range of intervention can be larger than anticipated in the past. We conclude that individuals characterized by identical INRs may have extraordinarily different thresholds to TF–induced coagulation that may affect hemorrhagic risk in the background of vascular anomalies. These observations apply both to a group and to a single individual sampled at multiple intervals during anticoagulant therapy. The whole-blood response of an individual to dilute TF is not related to the INR. Thrombin generation, platelet activation, FPA release, and fibrin formation thresholds in response to TF are not predicted by the INR.

Acknowledgments

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