Dose-Dependent Antithrombotic Effect of Warfarin in Rabbits

By Sanford N. Gitel and Stanford Wessler

One-hundred and fifty-one rabbits, divided into controls and animals treated with varying daily doses of warfarin, were subjected to the stasis assay, and the amount of thrombosis quantitated after intravascular coagulation was initiated either by activated factor X or tissue thromboplastin. Following 8–10 days of warfarin administration, there was a significant dose-dependent decrease in the vitamin-K-dependent coagulation factors paralleled by an increase in the prothrombin time ratio. Whether thrombosis was initiated by activated factor X or tissue thromboplastin, there was, with increasing drug dose, a progressive increase in the inhibition of stasis thrombosis. This significant antithrombotic effect occurred even when the vitamin-K-dependent coagulation activities were at a mean value of 50%.

Warfarin dosage is currently determined in the US by an empirically derived range of prothrombin time ratios for all patients (1.5–2.5) and, particularly at the upper portion of that range, bleeding has been a major risk factor. Although in the 1960s 2 controlled trials of low-dose coumarin prophylaxis in patients with coronary artery disease gave conflicting results, it has been recently suggested that the intensity of coumarin therapy might be determined by the clinical condition for which the drug is indicated, and preliminary trials of this type have been undertaken abroad.

Prior to recommending a low-dose warfarin regimen in man, it would be prudent to demonstrate an antithrombotic effect in animals over a range of prothrombin time ratios. Since a model for the production of stasis thrombosis has provided quantitative support for the efficacy of low-dose heparin, a similar approach was adopted for warfarin. In the present study the antithrombotic power of the oral anticoagulant was determined over a range of prothrombin time ratios when rabbits were challenged with tissue thromboplastin or activated factor X (Xa)—two thrombogenic agents that initiate thrombosis at the two steps in the coagulation sequence where warfarin has so far been shown to exert an antithrombotic action.

MATERIALS AND METHODS

Bovine serum albumin (BSA), bovine factor-II-VII-deficient plasma, bovine factor-VII-deficient plasma, and bovine factor-VII-X-deficient plasma were obtained from Sigma Chemical Co., St. Louis, Mo. Crystalline sodium warfarin (lot 80-205) was generously provided by Endo Laboratories, Wilmington, Del. Rabbit tissue thromboplastin (Simplastin) was a product of General Diagnostics, Morris Plains, N.J. Factor Xa was isolated as described previously.

Male New Zealand white rabbits, average weight 2 kg, were obtained from Camm Research Institute, Wayne, N.J. Rabbit plasma samples were collected as described previously. Normal rabbit plasma was a pool of plasma from 30 untreated male New Zealand white rabbits.

The prothrombin time, prothrombin activity, factor X activity, factor VII activity, and the rate at which plasma inhibits Xa (Xa inhibitory activity) were determined by published procedures.

Stock procoagulant solutions for infusions into the thrombosis animal model were prepared by diluting tissue thromboplastin or Xa in physiologic saline containing 5 mg BSA/ml, such that injection of 0.5 ml into a control rabbit resulted in a grade 3 or 4 thrombus (see below). Three-milliliter aliquots of the stock solutions were stored at 20°C and thawed at 37°C immediately before use.

The stasis assay was utilized to measure the extent of thrombosis produced in rabbit jugular veins. This assay exploits the capacity of retarded blood flow to markedly potentiate intravascular coagulation and has been employed to measure minute quantities of thrombogenic compounds as well as to determine the antithrombotic effects of various drugs. Thrombosis was initiated in each rabbit by the injection into a marginal ear vein of 0.5 ml of a stock solution containing either 5 μg of Xa or 60 μg of tissue thromboplastin. Thrombi are graded on a scale of 0–4, with 0 representing no thrombus and 4 reflecting a complete thrombotic cast of the vein lumen. An antithrombotic response to a drug is indicated by a decrease in the extent of thrombosis elicited by a standard dose of inspire, for example, from a score 3 in control rabbits to a score 0, 1, or 2 in treated animals.

A series of 6 experiments were performed using 151 rabbits. In a single experiment, 24–28 animals were divided into 3 or 4 groups of 6–8 each. One group, the controls, was injected with 1 ml of normal saline per day for 8–10 days. The other groups received daily intramuscular injections of normal saline containing warfarin at the doses indicated in the Results section for 8–10 days. For each animal, pre- and posttreatment plasma samples were obtained: the latter immediately before the stasis assay was performed. No attempt was made to regulate the prothrombin times in warfarin-treated rabbits, since preliminary experiments indicated that such an approach was not technically feasible.

Differences in coagulation parameters were analyzed for statistical significance using the Student’s t test and the stasis assay data by the Wilcoxon rank sum test. Correlations between prothrombin time ratios and factor VII or Xa inhibitory activity levels were determined using a standard exponential regression program.

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Table 1. Coagulation Parameters in Rabbits Before and After Warfarin Treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/day)</th>
<th>PT Ratio</th>
<th>Percent Normal Rabbit Plasma</th>
<th>Stasis Scores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>II</td>
<td>VII</td>
</tr>
<tr>
<td>Pretreatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A (7)</td>
<td>1.02 ± 0.03*</td>
<td>101 ± 3</td>
<td>98 ± 5</td>
<td>102 ± 5</td>
</tr>
<tr>
<td>B (7)</td>
<td>0.98 ± 0.02</td>
<td>105 ± 2</td>
<td>98 ± 5</td>
<td>89 ± 6</td>
</tr>
<tr>
<td>C (7)</td>
<td>1.00 ± 0.03</td>
<td>98 ± 4</td>
<td>101 ± 3</td>
<td>99 ± 4</td>
</tr>
<tr>
<td>D (7)</td>
<td>1.03 ± 0.02</td>
<td>103 ± 3</td>
<td>92 ± 5</td>
<td>89 ± 5</td>
</tr>
<tr>
<td>Posttreatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A (7)</td>
<td>1.03 ± 0.03</td>
<td>103 ± 2</td>
<td>99 ± 3</td>
<td>97 ± 6</td>
</tr>
<tr>
<td>B (7)</td>
<td>0.3 ± 0.05</td>
<td>79 ± 9</td>
<td>67 ± 10</td>
<td>72 ± 9</td>
</tr>
<tr>
<td>C (7)</td>
<td>0.5 ± 0.07 $</td>
<td>48 ± 7</td>
<td>50 ± 9</td>
<td>72 ± 6</td>
</tr>
<tr>
<td>D (7)</td>
<td>0.7 ± 0.15 $</td>
<td>25 ± 6</td>
<td>26 ± 8</td>
<td>55 ± 8</td>
</tr>
</tbody>
</table>

*Animals in groups A-D remained in that group throughout the experiment.
†Daily dose of warfarin.
§Xa inhibitory activity.
IlThrombosis induced by Xa.
Number of rabbits in each group.
**Mean ± standard error.
††Differs significantly from control value, p < 0.05.

RESULTS

Effect of Warfarin Dose on Coagulation Parameters

Previous experiments had demonstrated that two antithrombotic actions of warfarin—early depression of factor VII and the delayed but additive increase in Xa inhibitory activity—occur by the eighth day of drug administration. That such an effect had been obtained in the present investigation is shown in Tables 1 and 2.

Table 2 provides an example, from one experiment, of the type of data collected for all 6 experiments that are summarized in Table 2. There were no significant differences in coagulation parameters among the treated and control groups prior to warfarin administration. After 8–10 days of treatment, there was a significant dose-dependent increase in the prothrombin time ratio, paralleled by an increase in Xa inhibitory activity ($r^2 = 0.85$) and a decrease in the vitamin-K-dependent clotting factors ($r^2 = 0.91$) for factor VII.

Effect of Warfarin Dose on Thrombotic Response

The concentrations of Xa or tissue thromboplastin for all experiments were selected so as to provide either a score 3 or 4 thrombus in control animals. Table 1 presents a typical example of the thrombotic scores obtained following infusions of Xa. These scores were used to determine the statistical significance of the warfarin-induced depression of stasis thrombosis.

Whether thrombosis was initiated by Xa or tissue thromboplastin, there was, with increasing drug dose, a progressive increase in the inhibition of stasis thrombosis. This increase was observed even at the lowest dose of warfarin, although in such instances, the inhibition of thrombosis did not reach statistical significance. Once the prothrombin time ratio was at or above 1.5, however, a significant inhibition of thrombosis was obtained. This effect occurred even when the vitamin-K-dependent activities were in the 45%–55% range, representing a mean value of 50% (the average of the 3 vitamin-K-dependent clotting factors measured).

DISCUSSION

Animal models never provide final answers but offer only approximations; however, for a model to be a good one it must provide a new insight, have relevance to a particular problem, and respond predictably.$^{20}$ The results obtained in this study meet these criteria in offering evidence that warfarin, like heparin, provides
antithrombotic spectrum over a range of prothrombin time ratios.

Previous experiments had demonstrated at least 2 actions of warfarin—depression of factor VII activity and increased Xa inhibitory activity—that contribute to the antithrombotic effect of this drug in rabbits. Both actions have also been observed in man. Because no pretreatment values were available in man and only a single warfarin dose was employed in rabbits, no correlation between prothrombin time and either factor VII or Xa inhibitory activity had been obtained. The data in this report demonstrate a definite correlation between prothrombin time and both factor VII and Xa inhibitory activity in rabbits, suggesting that such a correlation occurs in man.

The antithrombotic effect also increased with increased prothrombin times and became statistically significant when the mean value of the vitamin-K-dependent coagulation activities was in the range of 40%-50% (Table 2). There was also an antithrombotic effect at mean activities above 50%, but with the size of the rabbit pool used, these data did not reach statistical significance, although the extent of inhibition did correlate with the prothrombin time. The experimental design was not aimed at the ablation of thrombosis, but rather at the recognition of a statistically significant impairment in the extent of thrombosis. The inhibition obtained in the present study, when the coagulation factor activities were in the range of 40%-50% (prothrombin time 1.5), is comparable to that found using low-dose heparin in the stasis assay—an effect that translated into a clinically desirable result in man.

Although prothrombin times in rabbits treated with warfarin cannot be compared directly to prothrombin times among patients receiving oral anticoagulants, depression of vitamin-K-dependent coagulation factors may be utilized to compare the intensity of therapy between the two species. One of the common methods of expressing the intensity of anticoagulant therapy is to determine the equivalent saline dilution of normal human plasma expressed as percent dilution. Utilizing rabbit brain thromboplastin and saline dilution curves, the recommended anticoagulant level in the US is within a dilution range of 10%-30%. Because of the sensitivity of the thromboplastin reagents to abnormal coagulation factors (PIVKA s) present in the plasma of patients treated with coumarin drugs, direct comparisons of such plasmas with diluted normal plasma cannot be accomplished. In contrast to man, the only PIVKA so far measured in the rabbit, prothrombin, is not released into the circulation. This finding suggests that a warfarin-induced depression of the vitamin-K-dependent coagulation factors should correspond closely to normal human diluted plasma, allowing comparisons of the concentrations of coagulation factors between dilutions of normal plasma and the undiluted plasma of rabbits given warfarin. In this investigation, a warfarin dose of 0.5 mg/day produced a significant antithrombotic response when the mean value of the vitamin-K-dependent coagulation factors was in the range of 40%-50% (Table 2). These values are higher than the maximum dilution, 30%, recommended for therapeutic efficacy in man.

The observation of an antithrombotic effect in rabbits at vitamin-K-dependent coagulation factor levels of 40%-50% translates in man to a prothrombin time ratio, using Simplastin, that is below the lower level (1.5) of the so-called therapeutic range. This finding, coupled with the preliminary trials of low-dose coumarin prophylaxis abroad, strongly suggests the feasibility of clinical trials of low-dose warfarin in man.

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