Radioiodinated Soluble Canine Fibrin

PREPARATION AND EVALUATION AS A THROMBUS LOCALIZING AGENT IN THE DOG

By R. Edward Coleman, Sylvia S. L. Harwig, John F. Harwig, Laurence A. Sherman, and Michael J. Welch

ABSTRACT

To develop a thrombus localizing tracer which has characteristics superior to labeled fibrinogen for external detection, we evaluated radioiodinated soluble fibrin. Labeled soluble fibrin was prepared by clotting and dissolving radioiodinated (131I) canine fibrinogen under specified conditions. Biological clearance studies revealed rapid clearance of the labeled soluble fibrin from the blood with a half-life of 5 hours. The accumulation of labeled soluble fibrin and fibrinogen in induced venous thrombi, coronary artery thrombi, and the myocardium was compared in dogs. In venous thrombi, soluble fibrin and fibrinogen exhibited maximum thrombus-blood ratios when they were injected 4 hours after thrombus induction; the thrombus-blood ratio was greater for soluble fibrin than it was for fibrinogen when these agents were injected 4, 8, or 24 hours after thrombosis induction. In induced coronary artery thrombi, soluble fibrin and fibrinogen accumulated to the same extent. Since the blood clearance of soluble fibrin is faster than that of fibrinogen, a higher thrombus-blood ratio was obtained with soluble fibrin in coronary artery thrombi. The thrombus-infarcted myocardium, thrombus-normal myocardium, and infarcted myocardium-normal myocardium ratios obtained with soluble fibrin were slightly higher than those obtained with fibrinogen. Thus, soluble fibrin offers some advantages when it is compared with fibrinogen as a thrombus detecting agent.

Methods

PREPARATION OF IODINATED CANINE FIBRINOGEN AND SOLUBLE FIBRIN

The 129I-fibrinogen uptake test consists of external probe counts over the deep venous system of both lower extremities and is a sensitive method of detecting thrombi forming in the deep veins of the legs (1-3). A disadvantage of this procedure is that it cannot detect thrombi forming in the pelvis or the upper thighs due to photon attenuation by overlying tissues and background activity in the bladder and the large vessels (1). To detect thrombi in the pelvis and the thighs scintigraphically, fibrinogen labeled with 131I and 123I has been used (4, 5). However, since human fibrinogen has a biological half-life in the blood of approximately 120 hours (6), a large amount of background vascular activity is still present if thrombus detection is attempted during the first few days after injection of the labeled fibrinogen.

Recently, fibrinogen prepared at high levels of iodination has been investigated as a thrombus localizing agent (7). Highly iodinated fibrinogen may be a better thrombus localizing agent than the standard fibrinogen preparation labeled with 0.5 iodine atoms per molecule of fibrinogen, since its relatively unchanged thrombin coagulability and its more rapid blood clearance result in a higher thrombus-blood ratio (7). In the present investigation, we studied another fibrinogen-derived species, soluble fibrin, as a thrombus localizing agent. Previous work in this laboratory has indicated a biological half-disappearance time (t1/2) of less than 10 hours for soluble fibrin (8). Therefore, the thrombus-blood ratio with soluble fibrin should be higher than that with fibrinogen if both are incorporated into thrombi to the same extent. This report describes our method of preparing labeled soluble fibrin and compares the accumulation of radioiodinated fibrinogen and soluble fibrin in arterial and venous thrombi.

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radioiodine was added followed by 5-10 \( \mu \text{g} \) (10-20 \( \mu \text{l} \)) of crude enzyme (Sigma Chemical). The iodination was then initiated by the addition of 10 \( \text{nmol} \) of \( \text{H}_2\text{O}_2 \) and allowed to proceed for 30 minutes at 37\(^\circ\)C with oscillating mixing. The reaction was quenched by the addition of cysteine solution (0.05M, 0.1 ml). The iodinated fibrinogen was separated by the ammonium sulfate precipitation procedure at 30% saturation of the ammonium sulfate (12). Clotting was accomplished in a 0.05% fibrinogen solution containing 0.09M KCl, 0.05M NaBr, 0.015M NaHPO\(_4\), 0.03M KH\(_2\)PO\(_4\), (14), and 4 \( \times \) \( 10^{-3} \)M iodoacetamide at pH 6.29 and a bovine thrombin (25 NIH units/ml) concentration of 1 unit/ml fibrinogen. The thrombin was purified by ion-exchange chromatography. After 10-20 minutes of incubation, the clot was carefully removed and washed with a 0.135M ammonium acetate-0.0165M tosyl arginyl methyl ester solution, then dissolved in 0.3M Tris-HCl at pH 5.3 (15). The dissolution process was complete within 10 minutes with a final protein concentration of 0.4-0.5 mg/ml. The isotopic coagulability (12) of the soluble fibrin solution was always greater than 80%; the solution retained this value after freezing and thawing.

**Biological Clearance Studies of Iodinated Soluble Fibrin**

Three mongrel dogs were anesthetized with sodium pentobarbital (30 mg/kg, iv). After the intravenous injection of 250 \( \mu \text{g} \) of \( ^{131} \text{I} \)-labeled soluble fibrin, serial 3-ml blood samples were withdrawn into tubes containing 0.03 ml of a 17.6% sodium citrate-8.4% citric acid solution at 5, 30, 60, 120, 210, and 300 minutes after injection. Two blood samples were taken on the second day. An aliquot of each sample was clotted, and the clot, the clot supernatant fluid, and the plasma were counted in a well-type scintillation counter. A total plasma clearance curve was constructed by plotting the plasma activity at the various sampling times as a percent of the activity in the plasma sample. Another clearance curve was constructed by plotting the activity in the coagulable protein at the various sampling times as a percent of that in the first sample.

**Thrombus Uptake**

Thrombi were induced in the femoral veins of ten dogs and the coronary arteries of six dogs. The thrombi were induced by intimal injury with an electric current (16, 17) while the dog was under sodium pentobarbital anesthesia (30 mg/kg, iv). Venous thrombosis was induced by inserting a catheter containing a stainless steel guide wire, which also served as an electrode, into an external jugular vein and passing the catheter into a femoral vein under fluoroscopic control. The wire was connected to the anode of a variable power supply. The cathode was connected through an ammeter to a skin clamp on the leg with the femoral catheter. A direct current of 5 ma and approximately 2 v was applied for 1 hour. The guide wire and the catheter were then removed.

Coronary artery thrombosis was induced by the method of Salazar (18). A catheter was passed from the femoral artery into the left main coronary artery. A Teflon-coated stainless steel electrode with the tip exposed for approximately 3 mm was then advanced through the catheter under fluoroscopic control into the left anterior descending coronary artery. The electrode in the coronary artery was connected to the anode of the variable power supply, and the cathode was connected through an ammeter to the chest wall. The electrocardiogram (ECG) was continuously monitored. A current of 500 \( \mu \text{a} \) was applied until definite ECG evidence of myocardial ischemia was noted; usually about 30-45 minutes was required to induce this response. The catheter was then removed and the incision closed. Lidocaine (20 mg/ml) was administered intravenously as a 50-ml bolus when ventricular premature contractions were detected.

Labeled fibrinogen (approximately 400 \( \mu \text{g} \)) and soluble fibrin (approximately 200 \( \mu \text{g} \)) were administered intravenously into the same dog 2, 4, 8, or 24 hours after venous thrombosis had been induced. In the dogs with coronary artery thrombosis, the fibrinogen and soluble fibrin were injected 4 hours after thrombosis induction. In three of the dogs with coronary artery thrombosis, standards of the injected dose were retained for quantification of tissue distribution studies.

The dogs were reanesthetized 24 hours after injection of the labeled tracers. In the dogs with venous thrombi, a venogram was performed with 10 ml of meglumine diatrizoate to demonstrate the size and the location of the thrombus. The thrombosed vein was then exposed, ligated, and resected. The thrombus, which was adherent to the vascular endothelium in every dog, was then removed from the vein and washed with 0.9% saline solution. A blood sample was obtained at the time of thrombus removal. The blood and the thrombus were weighed and counted in a well-type scintillation counter. The thrombus-blood ratio for soluble fibrin and that for fibrinogen were calculated as counts/min g\(^{-1}\) thrombus divided by counts/min g\(^{-1}\) blood.

In the dogs with coronary artery thrombosis, a blood sample was obtained prior to death and counted. At the time the dogs were killed, the left anterior descending coronary artery and the area of infarct were identified. The coronary artery thrombus was removed, washed, weighed, and counted. Tissue samples from infarcted and normal myocardium were taken, washed, weighed, and counted. The percents of the injected dose per gram of thrombus, normal myocardium, and infarcted myocardium were determined in three dogs. In all six dogs with coronary artery thrombosis, the thrombus-blood, thrombus-infarcted myocardium, thrombus-normal myocardium, and infarcted myocardium-normal myocardium ratios were determined.

The results of these studies were analyzed statistically by Student's t-test for paired observations (19).

**Results**

The plasma and coagulable protein clearance curves of soluble fibrin were similar, and both curves demonstrated rapid tracer clearance from the blood; only 28% of the coagulable protein remained in the blood after 5 hours, and 5% remained after 20 hours. When the coagulable protein clearance curve was analyzed by a conventional curve-stripping technique (20), a biological
Labeled Soluble Fibrin

$t_w$ of 5 hours was obtained from the longest component.

Deep vein thrombi were readily detectable by venography in all dogs. Soluble fibrin and fibrinogen thrombus-blood ratios were greatest when both preparations were injected 4 hours after thrombosis induction; the ratio for soluble fibrin was approximately three times that for fibrinogen (Table 1). When the tracers were injected 2 hours after thrombosis induction, the thrombus-blood ratio was higher with fibrinogen than it was with soluble fibrin. When the tracers were injected at 8 and 24 hours after thrombosis induction, the soluble fibrin thrombus-blood ratios were higher than the fibrinogen ratios, but the difference was not as great as that seen at 4 hours. The only time period in which results with the two tracers were significantly different was 4 hours after thrombus induction.

The uptakes (% injected dose/g) of soluble fibrin and fibrinogen in coronary artery thrombi were nearly identical (Table 2). Slightly less of the injected dose of soluble fibrin accumulated in both the infarcted and the normal myocardium in comparison with fibrinogen accumulation.

### Table 1

<table>
<thead>
<tr>
<th>Time of injection after thrombus induction (hours)</th>
<th>N</th>
<th>Thrombus-blood ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Soluble fibrin</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>7.2 ± 2.8</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>43.3 ± 9.1</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>19.4 ± 14.8</td>
</tr>
<tr>
<td>24</td>
<td>2</td>
<td>6.1 ± 0.7</td>
</tr>
</tbody>
</table>

The thrombus-blood ratios are means ± sd expressed as counts/min g⁻¹ thrombus divided by counts/min g⁻¹ blood. * Statistically significant ($P < 0.05$) compared with the soluble fibrin group.

### Table 2

<table>
<thead>
<tr>
<th>Dog</th>
<th>Soluble fibrin</th>
<th>Fibrinogen</th>
<th>Soluble fibrin</th>
<th>Fibrinogen</th>
<th>Soluble fibrin</th>
<th>Fibrinogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.33</td>
<td>0.39</td>
<td>0.0029</td>
<td>0.0043</td>
<td>0.016</td>
<td>0.023</td>
</tr>
<tr>
<td>2</td>
<td>0.40</td>
<td>0.45</td>
<td>0.0016</td>
<td>0.0030</td>
<td>0.0091</td>
<td>0.019</td>
</tr>
<tr>
<td>3</td>
<td>0.38</td>
<td>0.25</td>
<td>0.0036</td>
<td>0.0029</td>
<td>0.025</td>
<td>0.037</td>
</tr>
<tr>
<td>MEAN ± SD</td>
<td>0.37 ± 0.04</td>
<td>0.36 ± 0.10</td>
<td>0.0027 ± 0.0010</td>
<td>0.0034 ± 0.0008</td>
<td>0.017 ± 0.008</td>
<td>0.026 ± 0.009*</td>
</tr>
</tbody>
</table>

Labeled fibrin and fibrinogen were injected 4 hours after thrombus induction, and tissue was removed 24 hours after injection. Results are expressed as % injected dose/g.

* Statistically significant ($P < 0.025$) compared with the soluble fibrin group.

The thrombus-blood, the thrombus-infarcted myocardium, and the thrombus-normal myocardium ratios for soluble fibrin injected 4 hours after coronary artery thrombosis induction were significantly greater than the corresponding ratios for fibrinogen (Table 3). There was no significant difference in the infarcted myocardium-normal myocardium ratio with the two tracers.

### Discussion

Since Hobbs and Davies (21) first reported that labeled fibrinogen was localized in experimental venous thrombi and could be detected externally, considerable effort has been devoted to the development of a safe and accurate method of detecting thrombi. Various labeled tracers (1-5, 22-32) have been investigated as thrombus localizing agents suitable for detecting venous thrombi and thromboemboli. Many of these tracers have also been evaluated as imaging agents.

The use of a labeled thrombolytic agent for thrombus detection was first reported in 1962 by Ouchi and Warren (26). They were able to detect venous thrombi with $^{131}$I-plasmin, but the tracer was cleared slowly from the blood. Subsequently, the plasminogen activators, streptokinase and urokinase, were also evaluated as thrombus imaging agents (22-25). Preliminary studies with streptokinase and urokinase labeled with $^{131}$I and $^{99m}$Tc were encouraging (22, 24, 25). Later studies were unable to demonstrate satisfactory imaging of emboli (23, 33), but imaging of lower extremity venous thrombi has been reported (34).

Although labeled rabbit antibody to human fibrinogen has been demonstrated to accumulate in areas of thrombosis both in deep veins (27) and the left atrium (28), this agent has several disadvantages as an imaging agent. The half-life of this tracer in the circulation is similar to that of labeled fibrinogen, i.e., approximately 5-7 days; thus,
there is high vascular background activity for the first few days after injection. Furthermore, immune reaction to the labeled rabbit antibody can occur.

Macroaggregated albumin labeled with $^{99m}$Tc has been demonstrated to localize in areas of deep venous thrombosis when the radiotracer is injected into a superficial foot vein (29). However, Rosenthal and Greyson (30) have found that this test is nonspecific, since abnormal images occur in patients with venous obstruction, venous stasis, and endothelia damage as well as in those with venous thrombosis.

Kwaan and Grumet (31), using autologous leukocytes labeled with $^{51}$Cr and external scintillation probe counting, were able to detect areas of deep venous thrombosis; their results were well correlated with venographic findings. Attempts to use labeled platelets for thrombus localization have not been successful (32).

The fibrinogen uptake test using external probe counting has been demonstrated to be a safe, accurate procedure for the diagnosis of forming thrombi in the deep venous system of the lower thigh and calf (1-3). This test is particularly useful in following the extent of thrombosis over a period of time (1). The test cannot detect thrombi forming in the upper thigh or pelvis, since there is a large amount of background activity in the bladder and the large vessels (1). Charkes et al. (4), using $^{131}$I-fibrinogen, and DeNardo et al. (5), using $^{123}$I-fibrinogen, have suggested that these agents might be useful in imaging thrombi in the pelvis and the upper thigh. However, since fibrinogen has a $t_{1/2}$ in the blood of approximately 120 hours (6), the blood pool background activity is still a problem if imaging is attempted within the first few days after injection.

Since McFarlane (35) had reported that iodination of fibrinogen with more than 0.5 iodine atoms per molecule altered the clearance of fibrinogen, we recently investigated a more highly iodinated fibrinogen as a thrombus localizing agent. Fibrinogen labeled with 25 iodine atoms per molecule is cleared more rapidly than fibrinogen labeled with 0.5 iodine atoms per molecule but has approximately the same coagulability (7). The thrombus-blood ratio obtained when animals were injected with the more heavily iodinated fibrinogen was higher than that obtained when fibrinogen labeled with 0.5 iodine atoms per molecule was used.

Soluble fibrin is cleared from the circulation more rapidly than fibrinogen labeled with either 0.5 or 25 iodine atoms per molecule (7, 8). The percent of the injected dose of soluble fibrin or fibrinogen incorporated in experimental coronary artery thrombi 4 hours after induction is approximately equal. Consequently, the thrombus-blood ratio is higher for soluble fibrin than it is for fibrinogen.

We have demonstrated high thrombus-blood and thrombus-myocardium ratios with soluble fibrin. A previous study using the same method of inducing coronary thrombosis demonstrated that coronary artery thrombi can be detected by imaging when the thrombus-blood ratio with $^{131}$I-fibrinogen is greater than 3 (36). Since the ratio with soluble fibrin is much greater than 3, external detection of coronary artery thrombi should be possible. It may also be possible to use labeled soluble fibrin to evaluate the relationship of coronary artery thrombosis to myocardial infarction in patients with early infarction. Whether the relationship between coronary thrombosis and myocardial infarction in man is causal is still being debated (37), and resolution of this question would be of considerable interest. Some investigators have found coronary artery thrombi in most patients dying of myocardial infarction, whereas others have found a low incidence of coronary artery thrombosis. Hackel et al. (38) have demonstrated a significant difference in the incidence of coronary artery occlusion at autopsy in patients dying of acute myocardial infarction with and without "power failure."
patients with power failure at the time of death had a greater incidence of coronary artery occlusion, and the occlusion was usually related to thrombosis. Erhardt et al. (39) found radioactivity in all portions of the coronary thrombi in six of seven patients who died of myocardial infarction after having received labeled fibrinogen. They suggested that thrombosis was a secondary event in myocardial infarction.

The \(^{125}\text{I}\)-fibrinogen uptake test will continue to be the procedure for screening large patient populations. Since this procedure does not detect iliac vein thrombi, imaging is necessary to detect them. Soluble fibrin seems to have some advantages compared with fibrinogen as an agent to image thrombi. Since soluble fibrin is cleared so rapidly, labeling with \(^{123}\text{I}\) or \(^{99m}\text{Tc}\) is desirable, since these radioisotopes have superior decay characteristics for imaging compared with \(^{131}\text{I}\) or \(^{131}\text{I}\).

References


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