Continued Incorporation of Circulating Radiolabeled Fibrinogen into Preformed Coronary Artery Thrombi

Ali Salimi, M.D., G. Charles Oliver, Jr., M.D., Joan Lee, M.S., and Laurence A. Sherman, M.D.

SUMMARY This study measured the growth over time of experimental coronary artery (CA) thrombi by radiolabeled fibrinogen uptake. $^{125}$I-fibrinogen was injected into dogs and 24 hours later CA thrombi were induced by electrical current. Twenty-four hours after CA thrombus induction, $^{125}$I-albumin (group A) or $^{125}$I-fibrinogen (group B) was injected. At 48 hours after thrombi induction, the thrombi were removed, divided into segments, and their radioactivity measured. The $^{125}$I-fibrinogen in the group B thrombi was significantly greater than the $^{125}$I-albumin in group A thrombi ($P < 0.001$). In further studies, the $^{125}$I-fibrinogen was given 48 and 72 hours post CA thrombus formation. Significant $^{125}$I was also found in these CA thrombi. $^{125}$I-fibrinogen and $^{125}$I-fibrinogen incorporation occurred in virtually all segments of the thrombi. The results suggest 1) CA thrombi growth continues for at least 72 hours after initial formation; 2) the presence of $^{125}$I in the thrombi was due to gradual fibrin deposition throughout the thrombus.

THE FORMATION AND GROWTH OF CORONARY ARTERY THROMBI clearly plays a role in the process of myocardial infarction. At this time insufficient information exists about such factors as the time period over which a thrombus grows, its rate of growth, and its pattern of growth to determine whether the development of coronary thrombi is a primary or secondary process. The evidence from prior investigations is inconclusive and at times conflicting.

Recent autopsy studies of patients with documented myocardial infarction concluded that the thrombi were formed after myocardial infarction. Before death, patients in these studies had been given $^{125}$I-fibrinogen to detect peripheral venous thrombi; the radioactive protein was taken up by all segments of coronary artery thrombi examined at postmortem. In contrast, other studies in patients who had undergone $^{125}$I-fibrinogen scanning for venous thrombi showed that radioactivity in the venous thrombi increased for several days, a finding that suggests active continued growth of thrombi can occur. $^{125}$I appeared in venous thrombi of dogs when $^{125}$I-fibrinogen was injected 30 min after clot formation. No thrombus radioactivity occurred in dogs injected with radiolabeled albumin. This finding adds evidence to the postulate that increasing thrombus radioactivity indicates fibrin deposition rather than accumulation of inflammatory fluid.

In this study, we collected data to evaluate the time course of coronary thrombus development. We used the electric current thrombus model in dogs.

Methods

Oxalated dog plasma was adsorbed with BaSO$_4$. We modified Regocczi's method$^6$ to purify fibrinogen from the adsorbed plasma. A saturated solution of NH$_4$SO$_4$ was slowly added to the plasma with stirring, until the mixture was 12% v/v NH$_4$SO$_4$, at 4°C for 15 min. The precipitate was centrifuged at 3000 g x 30 min, and discarded. The NH$_4$SO$_4$ concentration in the supernatant plasma was brought up to 23% and centrifuged. We dissolved the precipitated fibrinogen in 0.15 M NaCl, 0.01 M Na citrate buffer, pH 7.4 (½ the original volume), and reprecipitated with 23% NH$_4$SO$_4$.

The purified fibrinogen redissolved in the saline-citrate buffer. The clottability was ≥ 94%. It was stored frozen in aliquots. Labeling with $^{125}$I or $^{125}$I was by the ICI method of McFarlane,$^7$ with ≤ 0.3 atoms iodine/molecule fibrinogen. For control experiments, we labeled albumin with $^{125}$I, also by the ICI method.

Seventeen mongrel dogs had coronary artery thrombus produced by a closed chest technique. A modified electrical current technique$^{6-9}$ induced thrombi. Following general anesthesia with sodium pentobarbital, the left coronary artery was catheterized with an 8F Sones catheter. A Teflon coated guidewire was stripped of Teflon at the tip for 4–5 mm. We passed the guide wire through the catheter into the left anterior descending or circumflex coronary artery. The positive current from a 6 volt battery was connected through a potentiometer to the guidewire and the negative terminal was attached to the chest wall. The current ranged from 300–600 μ amp. ECGs were obtained at baseline and at 10 min intervals, following initiation of electrical current. The ECG leads included an intracorony, one precordial (approximating V$_a$), I, II, III, aV$_R$, aV$_L$, and aV$_F$. When changes compatible with acute myocardial infarction appeared on the ECG, we discontinued the current. After approximately 30 min the guidewire and catheter were removed. The carotid artery was tied following application of penicillin and streptomycin.

In preliminary studies, we induced CA thrombi and followed the animals without injecting radiolabeled proteins. Pre- and post-CA thrombi platelet counts, prothrombin times, activated partial thromboplastin times, fibrinogen levels, serum fibrinogen related antigen levels, protamine sulfate precipitation, and ethanol gelation tests were performed. Inconstant changes occurred, except for increased fibrinogen levels consistent with a response to injury. Because of these

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negative findings, the tests were not included in the studies reported here.

Initially, the animals were divided into two groups, A and B (Table 1). Both groups received $^{131}$I-fibrinogen injections 24 hours before induction of a coronary artery thrombus. The $^{131}$I-fibrinogen served as a measure of fibrin deposition in the initially formed thrombus. Twenty-four hours following formation of the thrombus, control group A (six dogs) received $^{131}$I-albumin injections. This measured diffusion into, and resultant contamination of thrombi by soluble proteins. Experimental group B (six dogs) followed the same protocol except that at 24 hours post thrombus formation, $^{131}$I-fibrinogen was injected instead of $^{131}$I-albumin. Preliminary results showed significantly greater uptake of the post thrombus $^{131}$I-fibrinogen in group B as compared with the $^{131}$I-albumin in group A. Accordingly, in two subsequent groups, we delayed the post thrombus $^{131}$I-fibrinogen injections. Group C (three dogs) received $^{131}$I-fibrinogen at 48 hours post thrombus and group D (two dogs) received $^{131}$I-fibrinogen at 72 hours (Table 1).

All animals were sacrificed 24 hours after injection of the second radiolabeled protein. The hearts were immediately dissected. After noting the anatomic relations, the coronary arteries containing the thrombi were removed and washed with a saline flush for one min. Then the thrombi were detached from the arteries. Preliminary experiments established that the saline rinses would not adequately remove soluble $^{131}$I-albumin. We placed the thrombi in a nylon net in an agitator and extensively washed them with a heparinized 0.15 M NaCl, 0.01 M EACA, and 0.01 M EDTA solution for 3 hours. Shorter periods of washing resulted in greater retention of $^{131}$I-albumin.

The washed thrombi were divided into 4–7 sections measuring 3–4 mm length, and numbered, with section 1 (proximal) being closest to the coronary ostium. The sections were placed in pre-weighted test tubes and dried in a 100°F oven overnight. The tubes were reweighed and the weight of the thrombus sections calculated. The radioactivity of the dried sections was measured in a dual-channel Packard gamma counter with correction for crossover of $^{131}$I activity into the $^{131}$I-channel. The specific radioactivity (cpm/mg) of each section was calculated.

At the time of sacrifice, a blood sample was also taken and the specific radioactivity of the dried blood determined. We expressed the thrombus uptake as the “thrombus/blood ratio” (TBR). The TBR is the specific radioactivity of the thrombus divided by the specific radioactivity of the blood. This permits quantitation of the relative uptake of each isotope, by dividing the TBR of $^{131}$I by that for $^{181}$I (TBR-$^{131}$I/TBR-$^{181}$I). A high value indicates greater $^{131}$I uptake and thus an older thrombus segment. A low value reflects $^{181}$I uptake and a more recently formed segment.

**Results**

In each heart, we found a thrombus in the expected location totally occluding the involved coronary artery and an area of necrosis corresponding to the zone supplied by the

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**Table 1. Outline of Experimental Design**

<table>
<thead>
<tr>
<th>Prethrombosis (24 hr)</th>
<th>Thrombosis Post Thrombosis</th>
<th>34 hr</th>
<th>48 hr</th>
<th>72 hr</th>
<th>96 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. $^{131}$I-F</td>
<td>$^{131}$I-F</td>
<td>S</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. $^{131}$I-F</td>
<td>$^{131}$I-F</td>
<td>S</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. $^{131}$I-F</td>
<td>$^{131}$I-F</td>
<td></td>
<td>S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. $^{131}$I-F</td>
<td>$^{131}$I-F</td>
<td></td>
<td></td>
<td>S</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: $^{131}$I-F = $^{131}$I-fibrinogen injection; $^{131}$I-F = $^{131}$I-fibrinogen injection; $^{131}$I-A = $^{131}$I albumin injection; S = Sacrifice.

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**Table 2. Thrombus/Blood Ratios of $^{131}$I-fibrinogen and $^{131}$I-albumin**

<table>
<thead>
<tr>
<th>Group</th>
<th># animals</th>
<th># segments</th>
<th>1st Isotope ($^{131}$I-fibrinogen) (mean ± SD)</th>
<th>2nd Isotope ($^{131}$I-albumin) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6</td>
<td>28</td>
<td>24.2 ± 3.7</td>
<td>0.27 ± 0.04</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>31</td>
<td>26.2 ± 2.90</td>
<td>4.02 ± 0.74</td>
</tr>
</tbody>
</table>

$^{131}$I-albumin vs $^{131}$I-fibrinogen: P < 0.001.

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**Figure 1. The thrombus/blood ratio of the specific radioactivity of the segments of two representative coronary artery thrombi. The thrombus in the left panel is from the control group A, and that in the right panel is from group B.**
vessel. The other coronary arteries did not contain thrombi. We attributed this absence of postmortem clots in the coronary arteries to the rapid method of sacrifice and extraction of the heart. The thrombi adhered to the walls of the coronary arteries, but detached without injury. In almost all thrombi, the earliest portion of thrombus to be formed was identified by a brownish discoloration corresponding to the position of the guidewire tip (according to preliminary experiments). The segment containing the discoloration was isolated in five thrombi, and designated as the "thrombus nidus."

We found substantial $^{121}$I fibrinogen radioactivity in all segments of every thrombus in the control group of six dogs (group A) which also received $^{121}$I-albumin. In contrast we found only negligible amounts of $^{121}$I-albumin radioactivity in these segments (table 2). Substantial uptake of $^{121}$I-fibrinogen occurred in segments from group B. Comparison of the TBR for group A $^{121}$I-albumin vs the $^{121}$I-fibrinogen in group B yielded a value of $P < 0.001$ (table 2). Group A and B protocols were identical, except for the nature of the second labeled protein injection ($^{181}$I-albumin in A, $^{121}$I-fibrinogen in B). These results indicate that the $^{181}$I-fibrinogen in the thrombi of the experimental groups was not merely the result of diffusion of soluble plasma protein, but rather represents incorporation of fibrinogen into the thrombus as fibrin. Incorporation of $^{121}$I-fibrinogen also occurred in all CA thrombi segments of groups C and D (table 3). They received $^{121}$I-fibrinogen at 48 and 72 hours post thrombus formation. Although the injection times in these groups are not strictly comparable to groups A and B, their $^{121}$I incorporation was similar to group B and much greater than control group A. This indicates that incorporation continued past 24 hours and up to 72 hours.

The TBR of $^{121}$I and $^{131}$I in the proximal, middle, and distal segments of each thrombus was compared (table 3). This is a measure of early ($^{121}$I) versus late ($^{131}$I) growth in different segments of the thrombi. All segments contained significant $^{131}$I; all except one contained $^{121}$I. The amounts of both isotopes varied considerably between corresponding segments of different thrombi as well as between different segments of an individual thrombus. Representative samples from groups A and B are in figure 1. The ratio of the relative values of $^{121}$I/$^{131}$I also varied in different segments. This fluctuation probably relates to changes in the activity of the thrombus formation process and the temporal relationship of formation of different segments wherein the older segments show a greater $^{121}$I/$^{131}$I ratio. Additionally, the segments probably differed in the portion of fibrin to nonfibrin solid material such as red cells and platelets.

Significant $^{131}$I radioactivity was found in the "thrombus nidus" in four of five thrombi. In three of these the ratio of relative values of $^{121}$I/$^{131}$I was very low, indicating substantial recent ($^{121}$I) thrombus formation. Only one "thrombus nidus" had insignificant $^{131}$I radioactivity. In three dogs the $^{131}$I/$^{121}$I ratios in the distal segments of the thrombi were strikingly lower than those of the proximal segments, probably reflecting greater recent activity in the distal end. This was not true for the proximal segments of any thrombus.

<table>
<thead>
<tr>
<th>Table 3. Relative Thrombus Blood Radioactivity of $^{121}$I and $^{131}$I-fibrinogen</th>
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<tbody>
<tr>
<td>Series</td>
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<tr>
<td>--------</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>D</td>
</tr>
</tbody>
</table>

Abbreviations: $^{121}$I-Thrombin = thrombin; $^{121}$I-TBR = thrombus to blood ratio.
The TBR obtained for 121I-fibrinogen radioactivity was comparable in the three groups, except for one dog that received the second isotope 72 hours after thrombus production. This thrombus had the aforenoted very low 121I-radioactivity in the "thrombus nidus."

Discussion

Coronary artery thrombosis was once considered a sine qua non of myocardial infarction. Now this assumption is being challenged. Some authors believe coronary artery thrombi are a primary event leading to myocardial infarction. Others maintain that the thrombi are secondary, the effect rather than cause of infarction. Advocates of both theories base their arguments largely on the anatomic appearance of postmortem material. Such examinations cannot delineate the dynamic activity of coronary artery thrombus formation.

Recent studies by Erhardt et al. attempted to provide badly needed information on the time at which coronary artery thrombi formed. Their studies provided seemingly strong support for the "secondary" theory alluded to above. After injection of 121I-fibrinogen for detection of venous thrombi, Erhardt et al. found 121I in segments of coronary artery thrombi removed postmortem. They concluded that the coronary artery thrombi developed after the infarction, since the 121I-fibrinogen was given post infarction. Their reports considered but rejected the possibility of a mixture of pre and post infarction fibrin in the thrombus. They cited Hackel et al. who found that labeled fibrinogen was not incorporated into already formed venous thrombi. Of note, in the latter study the isotope was given at least ten days after induction of a venous thrombus, making the negative results unnecesary to the early, acute phase of either venous or arterial thrombus formation.

Other studies demonstrated that previously formed venous thrombi are capable of incorporating radioactivity. Mavor et al. found that 13 out of 50 patients with ileo-femoral venous occlusion had positive peripheral venous 121I-fibrinogen scans, when the isotope was given at least 48 hours after the first clinical evidence of thrombosis. However the presence of fibrinogen radioactivity in thrombi is not unequivocal evidence of fibrin formation. Chapman pointed out Erhardt et al. could not exclude the possibility of soluble fibrinogen finding access through vasa vasorum to the thrombus and contaminating it. Such diffusion has been described in vitro and in vivo with 121I-fibrinogen.

The present investigation considered the possibility of growth of recently formed coronary thrombi. Our use of two isotopes permitted comparison of late thrombus growth with the initial stage of thrombus formation. In all but one thrombus segment, we could detect the radioactivity of 121I-fibrinogen administered after induction of the thrombus. The levels were significantly higher than those achieved in a control group similarly given 121I-albumin. The higher levels of 121I-fibrinogen versus 121I-albumin indicates that 121I-fibrinogen was actually incorporated in the thrombus by an active process and was not merely due to diffusion of soluble plasma fibrinogen from the vasa vasorum or vessel lumen.

In four of five dogs, the "thrombus nidus" evinced substantial, recent (121I-fibrinogen) radioactivity. Thus even in the initial nidus, fibrin deposition occurs long after the inception of the thrombus. The 121I/13I ratios varied in different segments of the same thrombus. We attributed this to differing rates of fibrin formation and dissolutions in different segments of the same thrombus.

Our demonstration of active incorporation of fibrinogen into previously formed coronary artery thrombi makes it necessary to re-evaluate the conclusions Erhardt et al. reached. It appears that their conclusion that no coronary artery thrombus existed prior to the myocardial infarction is not justified. The exact manner in which fibrin is laid down in an existing thrombus remains to be elucidated. One possibility is a dissolution and reformation of fibrin with no net increase in size. Another is deposition of new fibrin in layers between the already formed strands, resulting in a denser thrombus. Lengthwise growth, however, remains a possibility, with a net increase in the length and mass of the thrombus. Our data do not allow us to choose between these alternatives.

In conclusion, we have demonstrated that experimental coronary artery thrombi once formed may have growth activity for at least 72 hours. This activity varies at different times and in different parts of the thrombus. While the continued incorporation of fibrinogen may represent a balance between dissolution and reformation of the thrombus, the potential for a net increase in size exists. Because coronary artery thrombi may continue to grow for at least several days, it is hazardous to categorize clinical thrombi as simply primary or secondary. They may be either or both. Available techniques do not permit these distinctions.

The data presented have implications for anticoagulant and thrombolytic therapy. Such drugs may have a role in limiting coronary artery thrombus growth and potential extension of an infarct in the early post infarction period.

Addendum

Since submission of this manuscript, Moschos et al. published data showing uptake of 121I-fibrinogen administered to dogs after induction of experimental coronary thrombi. Their conclusions were similar to ours but methodological differences, including failure to do exhaustive washing of thrombi and the lack of radiolabeled protein controls, may have influenced their results.

Acknowledgment

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References


The Effect of Acute Coronary Artery Occlusion on Subepicardial Transmembrane Potentials in the Intact Porcine Heart

EUGENE DOWNAR, M.D., MICHEL J. JANSE, M.D., AND DIRK DURRER, M.D.

SUMMARY Subepicardial transmembrane potentials were recorded from intact pig hearts to observe the changes induced by acute ischemia. Ischemia shortened action potential duration, and decreased its amplitude, upstroke velocity, and resting potential. The cells were unresponsive after 12 to 15 minutes of coronary artery occlusion, yet near normal action potentials could be restored by flushing the occluded artery with saline as late as 40 minutes after occlusion. The unipolar extracellular electrogram reflected unresponsiveness by a monophasic potential. Local refractory periods initially shortened by up to 100 msec. Later, postpolarization refractoriness occurred and refractory periods lengthened often in excess of basic cycle length, thus resulting in 2:1 responses. The onset of early ventricular arrhythmias often coincided with a period of alternation and 2:1 responses, especially when these got out of phase in different regions. Reperfusion frequently led to ventricular fibrillation, and was associated with marked inhomogeneity in cellular responses. Re-entry within ischemic myocardium was the most likely mechanism for arrhythmias.

TO UNDERSTAND the mechanism of early ischemic arrhythmias, it is vital to know of the electrophysiological alterations which occur during the initial stages of myocardial ischemia. Several studies have been conducted in which recordings were made with microelectrodes from intact hearts; only relatively minor changes were reported following coronary artery occlusion, such as slight shortening of action potential duration and loss of resting membrane potential.\(^1\)-\(^8\) These changes fail to explain the marked delay and fragmentation of activation that occurs in the ischemic area,\(^4\)-\(^7\) and the arrhythmias which develop in the early stages following coronary artery occlusion.\(^8\)-\(^9\)

Marked changes in transmembrane potential have been reported from in vitro studies in which injured parts of the heart were excised and maintained by superfusion in a tissue bath after varying periods of ischemia.\(^10\)-\(^12\) Lazzara et al.\(^13\) used this technique to study the effect of a 30 minute period of ischemia, but the study was limited by a delay of 15 minutes before making intracellular recordings. Thus, little is known about the changes in transmembrane potential during the very early stages of myocardial ischemia, cor-

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