Accelerated Thrombolysis and Reperfusion in a Canine Model of Myocardial Infarction by Liposomal Encapsulation of Streptokinase

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The aim of thrombolytic therapy for acute myocardial infarction with plasminogen activators such as streptokinase is to lyse the coronary thrombus and reestablish blood flow as quickly as possible so that heart tissue loss is minimized and mortality rates are improved. Streptokinase has been encapsulated in large unilamellar phospholipid vesicles and tested in an animal model of acute myocardial infarction. The time required to restore vessel patency has been reduced more than 50% when compared with findings for free streptokinase. The total dosage of streptokinase required was lower, and smaller remnant thrombi were observed with the encapsulated agent. Results from this initial unoptimized study may have significant implications for further reduction in mortality from heart attacks by therapy with plasminogen activators. (Circulation Research 1990;66:875–878)

The formation of an occlusive thrombus within an atherosclerotic coronary artery is a principal event initiating acute myocardial infarction.1–3 Recently developed therapeutic approaches useful for the rapid restoration of vessel patency in patients, along with a decreased mortality and improved ventricular function, have included the use of fibrinolytic agents, or, more precisely, plasminogen activators. By effecting the conversion of plasminogen to plasmin, the fibrinolytic agents act to stimulate the body’s own system for dissolving clots and thereby, ideally, reestablish coronary artery blood flow. Plasminogen activators such as streptokinase (SK), urokinase, tissue plasminogen activator, and genetically engineered one- and two-chain versions of tissue plasminogen activator and urokinase have been administered effectively by intravenous infusion over a wide range of dosages. All of these agents show similar incidences and rates of reperfusion and problems with bleeding complications.4,5

We now report a substantial reduction in the time required for reperfusion in a canine model of acute myocardial infarction. This was accomplished by liposomal encapsulation of the plasminogen activator SK. It has been known that enclosure in phospholipid microspheres can increase selectivity and potency of certain medications, and at least one prediction suggests that 15 such therapies may appear over the next decade.6–8 In particular, it was reported more than 10 years ago that liposomes tend to accumulate in regions of experimental myocardial infarction in the dog with some apparent dependence on surface charge.9 However, subsequent pertinent studies have indicated that the early circulatory distribution of liposomes is, among other things, related to blood flow, independent of charge, and may be limited to vesicles of certain size.10,11 Regardless of the exact mechanism(s) involved, liposomes have been demonstrated to facilitate platelet disaggregation with prostaglandin E1,12 to increase the antithrombotic activity of isocarbacyclin,13 and to reduce reperfusion injury by the delivery of catalase and superoxide dismutase to myocardial tissues.14

Because we anticipated that encapsulation of plasminogen activators might increase efficacy, improve selectivity, possibly reduce or eliminate antigenic complications, and retard systemic deactivation of the agent and its descendant species, we undertook comparative thrombolysis studies with SK in both free and liposomal-enclosed forms. The results from prior in vitro experiments in our laboratories demonstrated that liposomal-encapsulated streptokinase (LESK) retained its ability to lyse white thrombi and, furthermore, that encapsulation significantly pro-

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ected the SK from deactivation during 15- and 30-minute incubations in platelet-poor plasma. In this paper, findings from reperfusion tests on an animal model of acute myocardial infarction are compared for SK and LESK.

Materials and Methods

Preparation of Liposomes

Large unilamellar phospholipid vesicles were prepared through the detergent removal method. Briefly, a solution of 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC; 50 mg) in 2.5 ml chloroform/methanol (1:1 vol/vol) in a 10-ml Pyrex test tube was evaporated with a stream of nitrogen and lyophilized overnight. The thin film of dried PC was then suspended in 1 ml of 3.29 M n-octyl-β-D-glucopyranoside (OG) in Tris-buffered saline (TBS [20 mM Tris-HCl at pH 7.5 plus 100 mM NaCl]) by warming at 37°C for 2 hours with frequent stirring. After the PC had completely dissolved, 1.5 µg of [14C]PC (0.1 µCi) was added to monitor the amounts and recovery of liposomes. This solution of lipid and detergent was denoted as the PC-OG solution. Pharmaceutical-grade SK in a vial of 750,000 units was dissolved in 1 ml TBS. This SK solution was then added to 4 ml PC-OG. This mixture was dialyzed (molecular weight cutoff, 12,000–14,000) against 21 TBS for a total of 48 hours, with dialysate changes after 8 and 24 hours. The dialyzed sample was gel-filtered at 1 ml/min over Sepharose CL-6B (35 cm × 1.5 cm i.d., prereliquilibrated in TBS) to separate LESK from nonencapsulated SK. The observed encapsulation efficiency of SK in liposomes, as determined by measurement of SK activity and total protein, was about 30%. Over 92% of the SK in the final LESK suspension resided within the vesicles. SK activity and protein concentration were assayed with commercial kits according to the manufacturers’ instructions (Helena Laboratories, Beaumont, Texas; Biorad, Richmond, California, respectively). For the liposomes, it was necessary to dissolve vesicles by the addition of detergent to a final concentration of 40 mM before measuring activity or concentration. This concentration of octylglucoside did not affect the measurement of either SK activity or concentration. Additional details on the preparation and characterization of the liposomes have been described elsewhere.

Animal Tests

The present study examined the performance of the liposome suspensions in vivo, using a minor modification of an established canine model of myocardial infarction based on a thrombus obstruction. Animals were treated under procedures that were examined and approved by an institutional review panel of The University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma. Mongrel dogs of either sex were anesthetized with intravenous sodium pentobarbital (30 mg/kg). An endotracheal tube was inserted, and the animals were ventilated with room air by use of a constant volume respirator. A left thoracotomy was performed in the fourth intercostal space, and the heart was exposed. The left circumflex coronary artery was isolated proximal to the first obtuse marginal branch, and left circumflex coronary artery flow was measured with a 20-MHz pulsed-Doppler or electromagnetic flow probe. Arterial thrombosis was initiated by the injection of 100 units thrombin (Sigma Chemical, St. Louis, Missouri) and 0.1 ml whole blood into a 5–10-mm long segment of proximally and distally left circumflex coronary artery. After 10 ensnared minutes, the proximal ligature was released; after an additional 5 minutes, the distal ligature also was released. In some experiments, as many as three injections were necessary to form an occlusive thrombus (Table 1). The thrombus was allowed to mature for 30 minutes before administration of SK.

The relative dosages of the two thrombolytic preparations were identical. An initial bolus of 20,000 units preceded an intravenous infusion of 2,000 units/min (0.1 ml/min) until recanalization was observed. Reperfusion was documented by 1) a resolution of electrocardiographic changes induced by ischemia, 2) recovery of left circumflex arterial flow, and 3) the loss of cyanosis within the left circumflex coronary artery distribution. At the conclusion of the experiment, the artery was dissected, and thrombus mass was determined gravimetrically.

Results

The thrombolytic activity of LESK was directly compared with free streptokinase. Similar to reported findings for the left anterior descending coronary artery, the average time required for reperfusion with SK alone was about 78±43 minutes (mean±SD, see Table 1). In stark contrast to the values from free SK, the LESK greatly reduced thrombolysis times to an average of 32±28 minutes, with some observed reperusions occurring as quickly as...

| Table 1. In Vivo Thrombolysis Experiments With Free and Encapsulated Streptokinase |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Dog                             | Number of       | Reperfusion     | Thrombus weight |
|                                 | thrombin        | time (min)      | remaining (mg)  |
|                                 | injections      |                 |                 |
| Free streptokinase              |                 |                 |                 |
| 1                               | 2               | 62              | 5.0             |
| 2                               | 3               | 132             | 16.5            |
| 3                               | 1               | 27              |                 |
| 4                               | 3               | 112             | 18              |
| 5                               | 1               | 55              | 75              |
| Mean±SD                         | 2±1             | 78±43           | 28.6±31.5       |
| Liposomal-encapsulated streptokinase |                 |                 |                 |
| 6                               | 1               | 10              | 2.6             |
| 7                               | 1               | 10              | 0               |
| 8                               | 3               | 72              | 10.5            |
| 9                               | 2               | 17              | 0               |
| 10                              | 3               | 50              | 3.9             |
| Mean±SD                         | 2±1             | 32±28           | 3.4±4.3         |
as 10 minutes. With the assumptions of normal distributions and of equivalent sample populations, this is a statistically significant difference at p<0.05, according to Student’s t test for the difference between means. Not surprisingly, reperfusion times within each group seemed to correlate with the number of thrombin injections required to form a stable clot. The respective (average) reperfusion times for LESK and SK administrations are 10 and 41 minutes with one thrombin injection, 17 and 62 minutes with two thrombin injections, and 61 and 122 minutes with three injections. Thus, the variance above derives largely from the experimental difficulty, observed by us and previous workers, in forming a stable occlusion.

Liposomal enclosure of the plasminogen activator clearly increased the effectiveness of SK in this canine model, because lower total dosages of the encapsulated material were needed to reestablish blood flow. In these preliminary, unoptimized experiments, average total free SK administered equaled 170,500 units compared with 81,900 units of LESK. Furthermore, the sizes of the remnant thrombi, which were isolated from the excised coronary segment, seemed to confirm the accelerated thrombolysis, although the data were incomplete (Table 1). The residual clots were smaller, even though dosages and time of action for LESK were reduced. Most important, encapsulation of SK shortened the time needed for reperfusion by more than one half.

Discussion

Time is of critical importance to acute myocardial infarction patients for two reasons. First, the extent to which the myocardial tissue can be salvaged decreases with time, and second, the rate of recanalization decreases with time because the effectiveness of thrombolysis declines. In general, thrombolytic therapy is believed to be beneficial to myocardium recovery for a period up to 6 hours.2,20,21 Earlier and more recent work in the dog, however, suggests that major tissue damage occurs at shorter time periods. For example, it has been found that 30–40% of the infarcted area is lost at 40 minutes, and 80–90% is lost at 3 hours.10 Though one should be cautious in applying these results to patients,11 recent clinical studies support the contention that myocardium loss is rapid and significant before the 3–6-hour period.22–24 For instance, aggressive intravenous administration of SK by Israeli investigators22 has shown a statistically significant different ejection fraction for a group of patients treated less than 1.5 hours after the onset of pain compared with a group treated between 1.5 and 4 hours. Even when flow is returned after only 1 hour of experimental myocardial ischemia in dogs, results indicate that damage by reperfusion injury is caused through granulocyte occlusion (the no-reflow phenomenon)25 and inflammatory mechanisms that ensue by this time.26–29

Conventional thrombolytic therapy, once started, requires 30 minutes to 1 hour or longer to reestablish flow; this delay can mean considerable tissue loss during and after the treatment period. Liposome encapsulation appears to provide an approach to reduce the time needed for reperfusion.

The mechanism by which liposome encapsulation acts to accelerate thrombolysis in vivo is not clear. As noted above, we suspect that isolating SK in liposomes reduces its inactivation by plasma proteins (as seen in in vitro experiments), retards SK initiation of systemic reactions, and prevents depletion or inactivation of key components of the fibrinolytic system. For example, computer models by our group suggest that present clinical dosages cause rapid formation of SK-plasminogen complex, depletion of free plasminogen available for activation, and, hence, low levels of plasmin. According to the model, the slow release of SK from the liposomes would tend to lower the concentration of the complex while raising that of plasmin. This different profile of fibrinolytic components could shift the mechanism of lysis from one principally of activation of clot-bound plasminogen to one with an increased role for circulating plasmin. Other possibilities are that the liposomes facilitate delivery of SK to or into the clot by concentrating near the clot and/or by preferentially releasing SK as the vesicles experience flow stress as they pass the thrombus. It also cannot be ruled out on the basis of the evidence presented here whether or not the reduced reperfusion time is a result of some action of intact liposomes or even dispersed phospholipids in concert with free SK. Additional work is necessary to determine which, if any, of these mechanisms explains the accelerated thrombolysis by encapsulation.

Lastly, it is unlikely that the results of these initial experiments reflect the greatest possible reduction in reperfusion times. For example, tissue plasminogen activator and urokinase exhibit greater binding affinity for fibrin than SK, as well as an enhanced ability to activate clot-bound plasminogen.4 Consequently, these activators may display more rapid thrombolysis, particularly when protected from inactivation within liposomes. Whether or not even faster reperfusion is realized, it is clear that liposomal encapsulation of SK or other plasminogen activators shows promise in the treatment of acute myocardial infarction.

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