The Influence of the Time Interval between Coronary Artery Occlusion and the Administration of Hyaluronidase on Salvage of Ischemic Myocardium in Dogs

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SUMMARY The purpose of this study was to determine the time interval following coronary artery occlusion during which the administration of hyaluronidase exerts a significant protective effect on injured myocardium. Forty-eight open-chest dogs with coronary artery occlusion were studied. Fourteen were untreated (controls). Hyaluronidase (500 NF units/kg, iv) was administered 20 minutes (12 dogs), 3 hours (8 dogs), 6 hours (8 dogs), or 9 hours (6 dogs) after occlusion. Epicardial electrograms, recorded from 10 to 16 sites on the anterior surface of the left ventricle before, 15 minutes after, and 24 hours after coronary occlusion were analyzed for S-T segment elevation and changes in QRS morphology. Transmural specimens, excised 24 hours after occlusion from the sites at which the electrograms were recorded, were analyzed for creatine phosphokinase (CPK) activity and histological appearance. In all five groups, myocardial CPK depression, histological evidence of the extent of necrosis, and changes in QRS configuration correlated well with one another. In the controls, S-T segment elevation 15 minutes after occlusion (ST\text{max}) correlated well with myocardial CPK depression, histological extent of necrosis, and changes in the QRS complex 24 hours later. When hyaluronidase was given 20 minutes, 3 hours, or 6 hours after coronary occlusion, myocardial salvage was reflected in significantly less myocardial CPK depression for any given ST\text{max}, less histological evidence of infarction, and less extensive changes in QRS configuration than in the untreated dogs, although there was a progressive reduction in tissue salvage as the time interval between occlusion and drug administration lengthened. Hyaluronidase administered 9 hours after occlusion had no demonstrable effect on the development of myocardial necrosis, suggesting that ischemic injury is totally irreversible by this time.

MYOCARDIAL necrosis following coronary artery occlusion can be limited by certain interventions designed to improve the balance between oxygen supply and demand.\textsuperscript{1,2} Numerous interventions—hemodynamic, metabolic, and pharmacological—have been shown to reduce ischemic injury.\textsuperscript{2,3} In most of these studies the intervention under investigation has been applied either immediately before coronary artery occlusion or shortly thereafter (i.e., 30 minutes after occlusion), at a time when most cells still are reversibly injured. A few observations have been made in which interventions have been applied several hours after occlusion, and a beneficial effect has, in general, been observed.\textsuperscript{2,4} However, a systematic study designed to examine the relative efficacy of an intervention administered at various time intervals after coronary artery occlusion has not been performed.

Hyaluronidase has been shown to limit myocardial ne-
crosis when administered 20-30 minutes after coronary artery occlusion. Its effect compares favorably with all other pharmacological interventions that have been investigated. It has been found to exert a significant salutary effect in the rat with coronary artery occlusion, and it appears to be beneficial in patients. For this reason, hyaluronidase was selected to determine the effects of the time interval between coronary artery occlusion and the application of an intervention to salvage myocardium jeopardized by ischemia.

Methods

Forty-eight mongrel dogs of both sexes weighing between 15 and 30 kg were anesthetized with sodium thiamylal (25 mg/kg, iv) and placed on artificial respiration using a Harvard volume respirator. A left thoracotomy was performed in the 5th intercostal space, and the heart was suspended in a pericardial cradle. The left anterior descending coronary artery was dissected free from the adjacent tissue and, when desired, ligated with 00 mersilene suture.

Ten to 16 sites on the anterior surface of the left ventricle were selected for the recording of unipolar electrograms. Each site selected for electrocardiographic recording was recognized by its specific relationship to the branching of the coronary arteries and veins. Sites were chosen arbitrarily from within the ischemic area (both center and periphery) supplied by the occluded vessel as well as from distant regions (and, therefore, presumably nonischemic). The input impedance of the recorder amplifier was 100 MΩ, and the frequency response of the system was ± 0.5 dB from 0.14 to 70 Hz. The impedance of the electrode was maintained constant, as reflected in the reproducibility of the tracings. The electrode employed was a hollow copper cylinder with a base of 15 mm² in which a saline-soaked wick was placed. This electrode was connected to the precordial V lead and held by a cable perpendicular to the electrode, thus minimizing mechanical stress. Because of the large area of the electrode, small variations in location did not change the configuration of the recordings. The electrograms obtained with this system are reproducible following subsequent coronary artery occlusions. Electrocardiographic lead aVF and systemic arterial pressure (Statham P23Db pressure transducers) were recorded continuously for the duration of the experiments on a Brush polygraph.

In all the dogs coronary artery occlusions were maintained for 24 hours. In all groups of dogs the durations of anesthesia and surgery were similar. The dogs were assigned randomly to one of the five groups: 14 served as controls, 12 received hyaluronidase (Alidase, Searle) (500 NF units/kg) intravenously 20 minutes after occlusion, 8 received hyaluronidase 3 hours after occlusion, 8 received it 6 hours after occlusion, and 6 received it 9 hours after occlusion. Epicardial electrograms were recorded immediately before and 15 minutes after occlusion. The thorax then was closed in layers and drained with an underwater tube. Twenty-four hours later the dogs were reanesthetized and placed on artificial respiration, and their chests were reopened. Epicardial electrograms were recorded again from the same sites at which recordings had been made before occlusion and 15 minutes after occlusion. The electrograms recorded 24 hours after occlusion were analyzed for the development of Q waves and the loss of R wave voltage in relation to those obtained prior to occlusion. After the 24-hour electrograms, the dogs were killed, and eight transmural specimens weighing approximately 400 mg were obtained from each dog for analysis of creatine phosphokinase (CPK) activity and for histological examination (two from the area well removed from the occlusion and the other six from the area of ischemia).

Sites at which local conduction delay occurred, as indicated by prolongation of the interval from the onset of the QRS complex to the intraventricular deflection exceeding 40 msec or prolongation of the entire QRS complex beyond 65 msec, were excluded from S-T segment analysis, since in the presence of an intraventricular conduction defect the S-T segment is no longer an accurate index of ischemic injury. Only three sites exhibited such prolongation and, therefore, were not analyzed. In all sites S-T segment elevation was quantified in millivolts by measuring from the T-Q segment to the J point, since the latter is identified as the juncture of QRS complex and the S-T segment. Myocardial CPK analysis was accomplished as previously described.

The specimens for histological examination were fixed in 10% formalin and stained with hematoxylin and eosin. They were examined by one of the authors (M.C.F.), who had no previous knowledge of their origin. Each section was graded according to the percentage of the section deemed to show necrosis: 0 (no evidence of necrosis), 1+ (1-25% necrosis), 2+ (26-50% necrosis), 3+ (51-75% necrosis), or 4+ (>75% necrosis). The following histological features were observed in areas of myocardium considered to be necrotic: (1) increased eosinophilia of the myocardial fibers; (2) loss of cross striations with increased granularity of the cytoplasm; (3) thinning and waviness of the myocardial fibers; (4) presence of contraction bands; (5) pyknosis and disappearance of myocardial cell nuclei; and (6) interstitial infiltration of neutrophilic polymorphonuclear leukocytes.

DATA ANALYSIS

In all groups S-T segment elevation 15 minutes after occlusion (STₜ₁₅) at each epicardial site was compared with (1) myocardial CPK activity in the subjacent myocardium; (2) the extent of infarction by histological examination of the subjacent myocardium; and (3) changes in QRS morphology from before occlusion to 24 hours later. The analysis of the epicardial QRS complex included the following indices described earlier: (a) the development of Q waves (ΔQ) in millivolts; (b) a decrease in R wave voltage (ΔR), also in millivolts; (c) the sum of ΔQ and ΔR; and (d) the percentage fall in R wave voltage (%ΔR). For all statistical analyses performed, each dog was used as an independent statistical unit. Thus, when the relationship between two variables was analyzed, a regression line was first calculated for each individual dog. Then, for each group of dogs the slopes, intercepts, and correlation coefficients were averaged, and a standard error of
this average was calculated. Therefore, for each group of dogs a "mean regression equation" was derived. To determine whether one group of dogs was statistically different from another group, the slopes of the two groups were compared by Student's t-test. A detailed analysis of covariance or variance was not employed; therefore, since multiple comparisons were made, the value of borderline P values is limited.

Results

There was a good correlation among the three parameters of necrosis: depression of myocardial CPK activity, histology, and changes in QRS configuration. First, the development of Q waves (AQ), the fall in R wave voltage (AR), their combination [(AR + AQ) 24h], and the percentage fall in R wave voltage correlated well with CPK activity depression in all five groups of dogs studied (Figs. 1 and 2A). Second, there was a good correlation between the extent of necrosis on histological examination and the changes in QRS morphology (Figs. 1 and 2B). Third, the extent of necrosis demonstrable histologically correlated well with the degree of depression of myocardial CPK activity (Figs. 1 and 2C).

In the 48 dogs used in this study, a total of 96 epicardial sites (two from each dog) were considered normal, since they showed no S-T segment elevation 15 minutes after occlusion (ST15m), normal myocardial CPK activity (average, 34.7 ± 0.4 IU/mg protein), and normal histological appearance (grade 0) 24 hours later. At these sites no new Q waves developed in the 24 hours following occlusion, nor was there a significant fall in R wave voltage (Figs. 1 and 2B).

The effect of the time interval between coronary artery occlusion and the ability of hyaluronidase to salvage ischemic myocardium was similar utilizing all three indices of myocardial damage: myocardial CPK activity (Fig. 3), histological appearance (Fig. 4), and QRS alterations (Fig. 5). The relationship between ST15m and myocardial CPK activity in the control dogs and the four groups that received hyaluronidase is shown in Figure 3. Hyaluronidase administration reduced CPK depression; the magnitude of the effect decreased progressively with longer delays between coronary occlusion and hyaluronidase administration. In the dogs receiving hyaluronidase 9 hours after occlusion, this relationship was no different from that in the control group, demonstrating the ineffectiveness of hyaluronidase with this delay in administration and suggesting that at this time most of the cells are irreversibly injured.

In each of the 48 dogs six histological sections were obtained from sites with significant ST15m. In the 14 control dogs there was a good correlation between ST, 5m and CPK HISTOLOGY. In the 15 mins. ECG ECG NONISCHEMIC CPK HISTOLOGY TREATED 15 mins. ECG ECG CPK HISTOLOGY

FIGURE 1 Examples of epicardial electrograms, myocardial creatine phosphokinase (CPK) values (in IU/mg protein), and histological grades from an untreated (control) dog (left) and a dog treated with hyaluronidase 20 minutes after occlusion (right). In both dogs the nonischemic myocardium exhibited no S-T segment elevation 15 minutes after occlusion. At 24 hours there were no changes in QRS configuration, normal CPK activity, and normal histological appearance (grade 0). In both dogs the border zone showed moderate S-T segment elevation 15 minutes after occlusion (ST15m). At 24 hours the control dog exhibited a significant Q wave and partial loss of R wave voltage. The CPK activity was moderately depressed, and the histological section was graded 3+ (51-75%) necrosis. The hyaluronidase-treated dog had only minimal Q wave development and fall in R wave voltage. The CPK activity was slightly depressed, and the histological section was graded only 1+ (1-25%) necrosis. The center of the ischemic area produced marked ST15m in both dogs. At 24 hours the control dog exhibited a total loss of R wave with a QS complex. The myocardial CPK activity was greatly depressed, and the histological section was graded 4+ (>75%) necrosis. In contrast, the hyaluronidase-treated dog demonstrated significant Q wave development (but smaller than in the control dog) and only partial loss of R wave voltage; the myocardial CPK activity was less depressed than in the control dog, and the histological section was graded 3+ (51-75%) necrosis.
the histological extent of necrosis. An index relating these two variables was calculated as "histological grade/ST_{15m} in mV." This index averaged 0.75 ± 0.05 in the 14 control dogs (Fig. 4). The dogs that received hyaluronidase 20 minutes, 3 hours, or 6 hours after coronary artery occlusion had a significant reduction in the severity of damage evident histologically for any degree of ST_{15m} when compared to controls. Thus, this index averaged 0.39 ± 0.04 (P < 0.0005 compared to control), 0.47 ± 0.09 (P < 0.005 compared to control), and 0.51 ± 0.07 (P < 0.005 compared to control) when hyaluronidase was given 20 minutes, 3 hours, and 6 hours, respectively, after coronary occlusion (Fig. 4). In contrast, the dogs receiving hyaluronidase 9 hours after occlusion showed no change in the extent of necrosis on histological examination when compared to the controls, again indicating that at this time the damage was already irreversible (Fig. 4).
The 14 untreated (control) dogs demonstrated a good correlation between $S_T$ and changes in QRS configuration at the same sites 24 hours later (Fig. 5). The dogs receiving hyaluronidase 20 minutes or 3 hours following occlusion demonstrated significantly less change in QRS morphology at 24 hours for any level of $ST_{15m}$, although this reduction became progressively less as the time of administration of hyaluronidase was prolonged. The progressive increase in the slopes from 20 minutes to 6 hours reflected the decrease in effectiveness as the intervention was delayed. The dogs that received hyaluronidase 9 hours after occlusion were similar to controls, indicating that myocardial necrosis first occurs 20–25 minutes after occlusion and that many myocardial cells die during the first 60 minutes of severe ischemia. It has been shown that myocardial necrosis first occurs 20–25 minutes after occlusion and that many myocardial cells die during the first 60 minutes of severe ischemia.

More recently, Reimer et al. have shown histologically in the dog that papillary muscle necrosis increases progressively as circumflex artery occlusion is extended. However, even as late as 6 hours after occlusion, there is a zone of ischemic but still viable myocardium which is salvageable. Certain interventions have a significant salutary effect on the ischemic myocardium even when they are administered several hours after coronary artery occlusion. The present study systematically analyzes the effects of progressive delays and demonstrates the relative degrees of effectiveness of a potent intervention administered at various times after coronary artery occlusion.

Although the irreversibility of myocardial ischemic injury has been studied extensively, the transition from reversible to irreversible injury remains clouded. The mitochondria demonstrate striking anatomical and metabolic changes as the cells which contain them enter an irreversible phase. It is not clear whether such mitochondria are capable of recovery if ischemia is relieved. The loss of cell volume regulation and increased sarcolemmal permeability have been found to correlate more closely with irreversible injury than has mitochondrial failure. Therefore, the primary event leading to irreversibility may be a sarcolemmal defect which allows excess calcium to enter the injured cells.

Intravenous hyaluronidase has been used previously in the experimental animal and has proved efficacious in reducing myocardial necrosis when administered 20–30 minutes after coronary artery occlusion. In addition, it has been shown by direct measurement to reduce necrosis in the rat which has undergone ligation of the left anterior descending coronary artery. Although the exact mechanism of action of hyaluronidase has not been determined, this enzyme is known to depolymerize the mucopolysaccharides that are abundant in the myocardial interstitium. Hyaluronidase may exert its beneficial effect on the ischemic myocardium by (1) improving the transport of nutrients to the ischemic tissue, (2) enhancing the "washout" of toxic metabolic substances that accumulate during ischemia, and/or (3) increasing collateral blood flow to the ischemic area, probably due to a decrease in myocardial edema. Recent electron microscopic studies have shown that hyaluronidase preserves intracellular glycogen stores in the ischemic tissue and protects the microvasculature, thus improving collateral flow.

In this study hyaluronidase was administered at several different time intervals following coronary artery occlusion in the dog—20 minutes, and 3, 6, and 9 hours. The extent of myocardial necrosis was measured biochemically (myocardial CPK activity), histologically, and electrocardiographically (changes in QRS configuration 24 hours after coronary artery occlusion). All three methods of measuring necrosis were complementary, and the results correlated well with one another (Fig. 2). By correlating S-T segment elevation shortly after occlusion (15 minutes) with these indices of necrosis at 24 hours, it was demonstrated that hyaluronidase administered 20 minutes, 3 hours, or 6 hours after coronary artery occlusion reduced

**Figure 5** The relationship between S-T segment elevation 15 minutes following occlusion ($S_{15m}$) and changes in QRS configuration at 24 hours ($|S_T| + |S_{15m}|$). Group A (occlusion alone) is shown as $|S_T| + |S_{15m}| = (4.08 \pm 0.44)$ ST$_{15m}$ + (6.9 ± 1.2); $r = 0.82 \pm 0.03$, range of slopes, 2.66–6.53. Group B (hyaluronidase given 20 minutes after occlusion) is shown as $|S_T| + |S_{15m}| = (7.73 \pm 0.21)$ ST$_{15m}$ + (7.1 ± 1.1); $r = 0.70 \pm 0.04$, range of slopes, 0.52–2.71. Group C (hyaluronidase administered 3 hours after occlusion) is shown as $|S_T| + |S_{15m}| = (3.0 \pm 1.5)$; $r = 0.85 \pm 0.04$, range of slopes, 1.32–3.85. Group D (hyaluronidase given 6 hours after occlusion) is shown as $|S_T| + |S_{15m}| = (2.61 \pm 0.48)$ ST$_{15m}$ + (3.21 ± 0.48); $r = 0.85 \pm 0.05$, range of slopes, 1.40–4.1. Group E (hyaluronidase given 9 hours after occlusion) is shown as $|S_T| + |S_{15m}| = (4.64 \pm 0.98)$ ST$_{15m}$ + (5.7 ± 2.1); $r = 0.86 \pm 0.06$, range of slopes, 3.87–9.02. Note that for any level of ST$_{15m}$, ($S_T$ × $S_{15m}$) was lower in the dogs given hyaluronidase 20 minutes or 3 hours after occlusion, reflecting less myocardial necrosis. $\star = P < 0.025$, $\star\star = P < 0.0005$ in comparison to controls; $\dagger = P < 0.05$, $\dagger\dagger = P < 0.0025$ in comparison to hyaluronidase at 20 minutes.
significantly the extent of necrosis (Figs. 3–5). Hyaluronidase was most effective when administered 20 minutes after occlusion, and its beneficial effects declined progressively as the interval between coronary artery occlusion and drug administration lengthened. However, even when it was administered 6 hours after occlusion, a significant beneficial effect was demonstrable, although it was less than that observed when it was given within 20 minutes after occlusion. In contrast, hyaluronidase administered 9 hours after occlusion exhibited no discernible beneficial effect. It is possible that a more potent agent or one with a mechanism of action different from that of hyaluronidase may cause significant myocardial salvage at this later time. In addition, salvage of myocardium might have been demonstrable at a later time had a more sensitive method of analysis been used.

All coronary artery occlusions in the present study were maintained for 24 hours, then the dogs were killed. No attempt was made to assess the long-term effects of hyaluronidase on infarct size or survival. However, it should be noted that in the rat, the beneficial effects of hyaluronidase are apparent not only at 48 hours after coronary artery occlusion, but also at 21 days after occlusion, at a time when healing is complete. Therefore, hyaluronidase does not simply delay myocardial necrosis, but rather prevents its occurrence. In addition, the enzyme does not interfere with normal scar formation.10

The extrapolation of the results of this study to patients must be done cautiously, since there are obvious differences between the animal model and acute myocardial infarction in man. However, since the histopathological evolution of infarction in the dog is quite similar to that in man,28 the present investigation supports the hope that those patients reaching the hospital within a few hours after the onset of symptoms may benefit from interventions designed to limit ischemic injury. In this regard, several agents, including hyaluronidase,11-12 propranolol,29 nitroglycerin,30-32 and oxygen inhalation33 have, in pilot studies, proved efficacious in limiting ischemic injury in patients whose therapy is begun several hours after the clinical event.

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