Experimental Lesions of the Coronary Arteries and Heart after Intrapericardial Injection of Proteolytic Enzymes

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Myocardial infarction in experimental animals has been successfully produced by ligating coronary artery branches(1), by injecting obstructing materials into the coronary vessels themselves(2), by external compression(3), and by intravascular electrocoagulation(4). Also, in rats Thomas and Hartroft were able by dietary means to produce coronary thrombosis associated with myocardial necrosis(5). In this laboratory progressive closure of major coronary arteries in the dog was accomplished many years ago by the intravenous injection of allylamine(6). Although allylamine severely damaged the coronary arteries of the treated animals, thrombi and myocardial infarction were decidedly rare. This observation raised major questions as to the types and degrees of injury in the walls of coronary arteries that are critical for their thrombotic occlusion. Also questions were raised as to the pathogenesis of myocardial infarction. That coronary thrombosis and myocardial infarction do occur in man in association with atherosclerosis is common knowledge, but relatively little is known concerning the precise local conditions in which these important lesions arise.

Constantinides(7), and Friedman and Van Den Bovenkamp(8) have found by examination of serially sectioned thrombosed coronary arteries, that in the vast majority a fissure or crack is present in the vessel wall connecting the underlying atheroma and the overlying thrombus. Presumably a thrombus may form at or near an actively autolyzing tissue site (atheroma) in the artery wall. The present experiments had as their objective the creation of an enzymatic counterpart of an autolyzing arterial atheroma and the testing of such a lesion in promoting coronary artery thrombosis. The plan of the experiments was to introduce potent proteolytic enzymes directly into the pericardial sacs of anesthetized dogs at thoracotomy and to study at varying intervals thereafter the action of these sub-

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stances on the coronary arteries and hearts of the experimental animals especially in relation to thrombus formation and to myocardial infarction.

While the objectives of producing a satisfactory model of atheroma and an experimental method for coronary artery thrombosis were only partially realized, the effects of intrapericardially instilled proteolytic enzymes on the coronary vessels and hearts of dogs are not without interest and are reported here.

MATERIALS AND METHODS

The two proteolytic enzymes utilized were collagenase (Nutritional Biochemical Co.) and protease (Pronase, Calbiochemicals, Los Angeles). The collagenase preparation is a protein derived from Cl. histolyticum by ammonium sulfate fractionation, dialysis, and lyphilization. It is supplied as sterile powder and contains some proteinase and peptidase. For intrapericardial injection it was dissolved in 7.5–10.0 ml of physiologic saline. The pH of this solution was 6.45. The protease used (Streptomyces griseus protease) is a sterile lyophilized protein containing 45,000 proteolytic units/g. It was given in 5–10 ml physiologic saline, pH 6.75. Collagenase was given in a single dose of from 25 to 200 mg, the majority of animals receiving 50 mg each. Pronase was given in a single standard dose of 37.5 mg.

Sixteen mongrel dogs of either sex, in good condition, and ranging in weight between 7.0 and 11.3 kg were used for the collagenase experiments and eight similar dogs weighing between 8.0 and 13.0 kg were given protease. Each of these animals was anesthetized by an intravenous injection of sodium pentobarbital (Nembutal) 25–30 mg/kg. With assisted respiration and with sterile technique the right chest was opened at the level of the heart. The pericardium and heart were brought into the operative field by placing sandbags under the left side of the thorax. The sterile collagenase or protease solution was now injected under direct vision beneath the parietal pericardium through a slightly curved 27- or 30-gauge hypodermic needle. Care was taken not to enter the myocardium with the needle tip. Slight trauma was unavoidable because of the motion of the heart, but bleeding was minimal. Injection of the proteolytic enzymes was at the level of the atrioventricular sulcus above the anterior segment of the right ventricle. Immediately upon completing the injection the thorax was closed with continuous silk sutures and the skin with a continuous subcuticular stitch. Residual air was removed from the chest. A sterile gauze dressing was applied to the wound, and the positive-pressure apparatus was removed. The animals breathed spontaneously. All animals were carefully observed postoperatively and those dying were completely autopsied immediately. Animals surviving the experimental procedure were sacrificed at intervals by the intravenous injection of Nembutal and were also examined at once. Control animals, receiving physiologic saline intrapericardially at thoracotomy were sacrificed and examined in the same way as the experimental animals. Many blocks of heart and coronary vessels as well as representative sections of major organs were taken for histological study.
Fixed in buffered 10% formalin, paraffin sections were cut and stained with hematoxylin and eosin, and when indicated with Masson’s trichrome method for connective tissue, Voerhoff’s stain for elastic fibers, and Mallory’s phosphotungstic acid–hematoxylin technique for fibrin. Femoral artery blood pressure measurements were made with the aid of a Hewlett-Packard transistor-recorder.

RESULTS

Collagenase-Injected Animals

Of the 16 dogs injected with collagenase intrapericardially six died within 24 hr and two within 48 hr of operation. The remaining eight recovered. Five of the latter group were sacrificed at 4 days and three at 7 days. The dogs dying in the first 48 hr initially seemed to be recovering from anesthesia, but after several hours became lethargic and prostrated, with low arterial blood pressures. They became progressively more stuporous until death. The dogs surviving rapidly recovered from anesthesia and gave no outward signs of circulatory or respiratory disturbance.

At autopsy the findings in the animals dying within 24–48 hr varied only quantitatively. There was 10–25 ml of fluid blood within the pericardial sac. On the surface of the heart a few thin strands of fresh fibrin were present or occasionally a small localized fibrin patch near the injection site. The myocardium, both of the right auricle and especially of the right ventricle, was massively hemorrhagic. The left auricle, septum and left ventricle were also involved by hemorrhage but not so extensively. The heart muscle itself appeared opaque, with patches of gray discoloration. There was much fresh subendothelial hemorrhage and beginning mural thrombus in the right ventricle. No gross changes in the coronary arteries or cardiac veins could be seen in the animals dying early after intrapericardial collagenase. Two of the dogs in this category had large amounts of unclotted blood in the left pleural cavity, and patchy pulmonary hemorrhages. No gross lesions, other than intense congestion, were present in the other organs. In the collagenase dogs sacrificed at 4 or 7 days the gross lesions were again qualitatively alike. The pericardial sac was now clear of fluid, and only a small patch or two of organizing fibrin was evident on the epicardial surfaces. However, on sectioning the right ventricle scattered gray–yellow zones suggesting necrosis were present in the myocardium in seven of the eight hearts of this group (Fig. 1). In two animals endothelialized mural thrombi were found in the right ventricle (Fig. 2). One involved the endothelium of the pulmonary valve as well. Smaller foci of necrotic myocardium were present in the septum and left ventricle.

Microscopically after collagenase injection there was extensive necrosis of myocardial fibers. In the animals dying within 48 hr after operation and as early as 6 hr, the myocardial lesions were characterized by loss of fiber outline and striations, loss of nuclear staining, and much irregular fragmentation (Fig. 3). Even after 6 hr a copious polymorphonuclear exudate was present between in-
Fig. 1. Dog 8581. Heart 7 days after intrapericardial collagenase. Massive healing infarct.

Fig. 2. Dog 8348. Heart 4 days after intrapericardial collagenase. Mural thrombus in right ventricle.
dividual fibers as well as large zones of hemorrhage. The presence of inflammatory cellular exudate and hemorrhage suggests an active local blood flow. At the longer time intervals of 4 and 7 days repair processes became prominent (Fig. 4). Proliferating fibroblasts invested or surrounded the coagulated, necrotic muscle fibers and less cellular exudate was present. Calcification was observed in the involved zones infrequently. In all cases the pericarditis encountered was mild. The

Fig. 3. Dog 8573. Necrosis of myocardium after intrapericardial collagenase. H and E, ×100.

Fig. 4. Dog 8292. Organizing mural thrombus in right ventricle 4 days after intrapericardial collagenase. H and E, ×100.
inflammatory exudate on the pericardium became progressively organized and involved at most a thin layer of subepicardial myocardial fibers. The impression was gained repeatedly that the myocardial lesions were greatly out of proportion to those of the pericardium.

Twelve of the 16 dogs receiving collagenase intrapericardially had multiple, severe focal lesions of their coronary arteries on microscopic examination. In the dogs dying in the first 2 days many of these vessels exhibited structural dissolution of their walls, varying from focal medial necrosis to nearly complete destruction of the adventitial and medial coats (Figs. 5 and 6). Interestingly, the endothelium lining the involved arteries usually remained intact. Both epicardial and intramural segments of the vessels were damaged. No thrombi were seen in these arteries in spite of the massive damage to their walls, and no rupture of the arteries themselves could be demonstrated. The intramyocardial hemorrhages were thought to have resulted from capillary rupture rather than from breaks in the walls of large arteries. Morphologically intact erythrocytes were present in the lumina of involved vessels suggesting that flow in these vessels may have been continuing. While many arteries involved in the necrotizing, autolytic process were in zones of myocardial necrosis, others were not, demonstrating some selectivity of the proteolytic enzyme for adventitial connective tissue and arterial smooth muscle. In extreme cases the internal elastic membranes of the affected arteries were frayed and fragmented, but this was not the rule. In the collagenase series no thrombi were found in the coronary veins, although their walls often showed segmental degenerative cellular changes. The medial lesions of the coronary arteries were distinctly fibrinoid in their staining reactions, being a homogenous bright red in the Masson preparations and blue–black in the PTAH stains.

Protease-Injected Animals

The gross and microscopic findings in the hearts and coronary vessels of the eight dogs given protease (Pronase) intrapericardially were qualitatively the same as those after collagenase injection, but they were more florid, more uniform, and more consistently present. In this group four dogs died within 24 hr (5, 6, 12, 24 hr) and four were sacrificed between the fourth and seventh days (one at 4 days, one at 6 days, and two at 7 days). In the animals dying within 24 hr there was massive early hemorrhagic necrosis of the right ventricular myocardium without rupture. The gross appearance of the lesions was similar to early myocardial infarct in man. Varying quantities of unclotted blood were present in the pericardial sac, but not enough to suggest cardiac tamponade. Very little fibrin was present on the epicardial surfaces. On section the hemorrhagic necrosis of the right ventricle in each animal extended through the muscle to the endocardium. Even in the animals dying at 5 and 6 hr, mural thrombi had begun to form in the ventricle. Each of the four animals sacrificed from the fourth to the seventh days had large zones of organizing myocardial necrosis grossly visible in the right ventricle. Small, focal scarred zones were present in the left ventricle. Each animal of this group had a gross, endothelialized mural thrombus in the right ventricle. None of the animals had more than a mild
pericarditis. In two of the animals dying early there was blood (approximately 50 ml) in the left pleural cavity and scattered pulmonary hemorrhages. No gross or microscopic lesions were observed in other organs, except for intense congestion.

**Fig. 5.** Dog 8573. Massive fibrinoid necrosis of coronary artery, without thrombus. Coagulative myocardial necrosis. Eighteen hours after intrapericardial collagenase. H and E, ×100.

**Fig. 6.** Dog 8584. Massive necrosis of coronary artery 10 hr after intrapericardial collagenase. H and E, ×100.
Microscopically the myocardial lesions in the protease-injected dogs revealed wide areas of infarct-like necrosis of muscle fibers, even 5–6 hr after surgery. Again, as in the collagenase animals, there was interstitial hemorrhage and a rich polymorphonuclear exudate. The process might be compared histologically, except for increased hemorrhage, to a 2-4-day myocardial infarct in man. In the animals surviving 4-7 days fibrosis was prominent, surrounding coagulated, necrotic muscle fibers, and investing zones of interstitial hemorrhage. There was extensive subendocardial hemorrhage as well and in these areas well-formed mural thrombi of platelets, fibrin, and entrapped cells were attached. Microscopically the pericardium, where involved, exhibited a thin uniform zone of fibrosis with dropout of the outer layers of myocardial fibers. Again the mildness of the pericarditis seemed out of proportion to the severity and extent of the myocardial damage.

In the protease-injected dogs the damage occurring in the coronary arteries and veins was even more florid and uniform than in the collagenase experiments. The most extensive segmental necrosis of many large and small arteries was observed. Arteries of the right ventricle and auricle were predominantly involved. In the animals dying hours after operation these lesions appeared as foci of autolysis of the entire vessel wall, excepting the endothelium. In the animals surviving for several days repair of the vessel wall by fibrosis was evident (Fig. 7). Thrombi were observed in scattered necrotic coronary arteries in three animals. In the same three animals massive thrombosis of multiple cardiac (coronary) veins was evident even grossly. Microscopically the arterial and venous thrombi had the characteristic pattern of intravascular antemortem clots formed in flowing blood (Figs. 8–10). In a few instances the venous thrombi appeared as coagula in the lumens of the vessel selectively on the pericardial side, suggesting that they were caused by the proteolytic enzymes acting directly and rapidly on this segment of the vessel wall. The hearts with coronary artery and vein thrombi were massively necrotic.

In summary each of the eight pronase-injected dogs had massive, infarct-like, myocardial necrosis, each had severe segmental necrosis of many coronary arteries and veins, and six of the eight had gross right ventricular mural thrombi. Three of the group had, in addition, occlusive thrombi of large coronary arteries and veins.

Control Dogs Injected Intrapericardially with Saline

Five dogs were operated upon as were those in the collagenase and protease series, but were given intrapericardially 10 ml each of sterile, physiological saline. They all survived and were sacrificed at the sixth day. No significant lesions of the hearts of these animals were found grossly or microscopically except for a mild organizing pericarditis underlying the injection site.

DISCUSSION

The foregoing experiments indicate that proteolytic enzymes introduced into the pericardial sacs of dogs at thoracotomy damage the coronary arteries and
Fig. 7. Dog 8581. Healing coronary artery with perivascular fibrosis, 7 days after intrapericardial collagenase. H and E, ×100.

Fig. 8. Dog 10562. Necrosis and thrombosis of coronary artery 5 hr after intrapericardial pronase. The surrounding myocardium is also necrotic. H and E, ×100.
Fig. 9. Dog 10562. Coronary vein with destruction of pericardial segment. Early thrombus. Five hours after intrapericardial pronase. H and E, ×100.

Fig. 10. Dog 10514. Pericardial aspect of heart 6 days after intrapericardial pronase. Organizing thrombus in coronary vein. Mild organizing pericarditis. H and E, ×100.
myocardium by direct contact. The most surprising observation was that in spite of almost complete dissolution of the walls of many of the coronary arteries, arterial thrombi occurred in only three of 24 hearts, and then only in scattered vessels in each case. Thrombi in the damaged coronary veins were also rare, but when they occurred many superficial segments were involved. The lack of thrombus formation in damaged vessels was probably not associated with a major systemic coagulation defect as gross right ventricular mural thrombi were repeatedly present. It is possible that the morphologically intact endothelium observed in the majority of severely damaged arteries may have prevented contact of the contents of the autolytic vessel wall with ambient blood and thus have prevented thrombus formation. The observations seem to point away from severe mural damage alone as an overriding critical factor in arterial thrombosis. For thrombi to form, hemodynamic changes, such as reduction in blood flow and lowered blood pressure, may be needed to supplement the effect of this degree of vascular injury. It is difficult to understand why the endothelium remained intact in so many massively damaged vessels. It can be speculated that inhibitors of proteolytic enzymes are present in effective concentrations in the endothelial region of the vessel wall, or that the endothelium, being continuously washed by the luminal blood stream, is protected from the buildup of damaging concentrations of enzyme impinging on it from the adventitial aspect of the vessel.

The second surprising finding was the through and through extent and the infarct-like character of the myocardial lesions encountered frequently with collagenase, and uniformly in the eight animals given protease. These massive lesions, predominantly in the right ventricle and complicated often by underlying intraventricular mural thrombi, were surely not due to obstructive lesions of their coronary arterial or venous circulations, except possibly in the three animals in which thrombosis of coronary arteries and veins were demonstrated. Their preferred location in the right ventricle suggests a direct action of concentrated enzyme at this point. One might have expected a diffuse pericarditis with a band-like layer of superficial myocardial necrosis as the result of enzyme acting at and beneath the epicardial surface. Although this type of mild change was observed, the much more frequent and dramatic lesion was an irregularly penetrating coagulative myocardial necrosis which, if the animal lived, progressed through acute inflammatory and healing stages very similar to those of myocardial infarcts in man. The extent of the lesion suggested that necrotizing concentrations of enzymes had diffused through the thickness of the ventricular wall from the pericardium. It should be emphasized that injection into the myocardium was carefully avoided by keeping the needle-tip under direct vision beneath the semitransparent parietal pericardium during the operative procedure.

The observations reported bear on important questions that have been raised in relation to the pathogenesis of myocardial infarction. While it is generally accepted that infarction occurs in association with thrombosis of the coronary arteries, this is by no means a constant association, as many authors(9–13) have emphasized. In the conditions of the present experiments infarct-like necrosis of
myocardium has resulted from the action of proteolytic enzymes in vivo and except in three instances without demonstrable prior or concomitant obstruction of the coronary arteries. Is it possible that hypoxia in damaging heart muscle cells allows the release from them of proteolytic enzymes in sufficient concentration not only to destroy the cell itself (suicide-bag theory) but also to damage surrounding cells and so extend the infarct? The possibility of such a secondary effect of hypoxia has been suggested by the observations of de Duve(14) on liver cells and even more specifically by those of Brachfeld(15,16) on the activation of lysosomal enzymes in hypoxic heart muscle. Further observations on the effect of exogenous proteolytic enzymes introduced into the pericardium as in the present experiments, or the similar injection of endogenous acid hydrolases extracted from cardiac muscle may allow the separation of the initial effects of hypoxia from those of released proteolytic enzymes. Such experiments would also provide data as to effective quantities of enzymes. The usual experimental models for myocardial injury, involving concomitant acute coronary obstruction, do not have these advantages. Also the present experimental model makes possible the testing of specific enzyme inhibitors for their prophylactic or therapeutic effect on the muscular and vascular necrosis produced in the heart by proteolytic enzymes.

**SUMMARY**

Collagenase and protease (Pronase) instilled into the pericardial sacs of normal dogs at thoracotomy have given rise to severe damage of coronary arteries, and to infarct-like necrosis of the myocardium. In spite of severe mural damage no coronary artery or coronary vein thrombi occurred in the 16 animals given collagenase and in only three of eight dogs given protease. Infarct-like necrosis of the myocardium complicated frequently by intraventricular mural thrombi occurred in 9 of the 16 collagenase-injected animals and in each of eight animals given protease intrapericardially. The intrapericardial injection of proteolytic enzymes is a useful experimental method for studying factors critical for coronary thrombosis and for the pathogenesis of myocardial infarction.

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