Quantitative analysis of contraction band and coagulation necrosis after ischemia and reperfusion in the porcine heart

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ABSTRACT To assess the importance of contraction band necrosis (CBN) in reperfusion, CBN, coagulation necrosis (CN), and infarct size, expressed as CBN + CN, were quantitatively analyzed in 25 porcine hearts without collateral circulation. The left anterior descending coronary artery was ligated for 20, 30, 60, and 120 min and then reperfused for 8 hr (groups 1 to 4, respectively). Five hearts were not reperfused (group 5). The areas of CBN and CN were traced at a magnification of × 100 under an inverted microscope and quantified with use of an image analyzer. There was no change in hemodynamics with either occlusion or reperfusion. Regional myocardial blood flow, measured by the generated hydrogen gas clearance method, decreased to almost zero after occlusion but recovered during reperfusion. Percent of risk area infarcted in groups 1 to 4 was 0 ± 0%, 11 ± 7%, 80 ± 9%, and 96 ± 2%, respectively, and the percent of risk area infarcted in group 4 was the same as that in hearts subjected to permanent occlusion (95 ± 3%). The percent area of CBN was 100 ± 0% in group 2, 68 ± 11% in group 3, 2 ± 1% in group 4, and 2 ± 2% in group 5. In group 3, the inner third of the ischemic left ventricular wall showed CN and the middle and outer third CBN. These findings show that in pig hearts without collateral circulation, the transmural infarct, two-thirds of which is occupied by CBN, is evident even when reperfusion is achieved after 1 hr occlusion. Therefore, protection against CBN might reduce infarct size after reperfusion.


CORONARY THROMBOLYSIS and angioplasty (PTCA) are current treatments for acute myocardial infarction. An increased rate of recanalization in the infarct-related coronary artery and a lower death rate at the acute stage have been reported with their use.1–5 However, it has not been established whether coronary thrombolysis can reduce the size of the infarct.6–8 The observed reduction in infarct size is markedly variable, even among patients in whom coronary thrombolysis is accomplished at the same time after the onset of acute myocardial infarction and in whom recanalization is of the same degree. This could be due to the fact that in patients with acute myocardial infarction, the degree of collateral circulation varies from zero to rich.

There are numerous reports on reperfusion injury after ischemia.9–11 However, most of these studies have been done in a canine preparation, which has a rich collateral circulation and a transmural gradient of regional blood flow in the risk area after occlusion of the coronary artery. Reperfusion after ischemia in the pig heart is a good animal preparation for study of the effects of coronary thrombolysis on acute myocardial infarction in patients without collateral circulation, because the pig heart lacks collateral circulation and the regional myocardial blood flow decreases transmurally to almost zero after ligation of the coronary artery.12–16 In addition, contraction band necrosis as an indicator of early reperfusion has not, to our knowledge, been studied quantitatively. Hence, we quantitatively determined the extent of infarction, contraction band necrosis, and coagulation necrosis in pig hearts reperfused after a period of ischemia. Dynamic changes in the
three study variables after different durations of occlusion were compared.

Methods

Twenty-five farm pigs weighing 25 to 43 kg each were used. These pigs were divided into five groups of five pigs each according to the duration of occlusion of the left anterior descending coronary artery (20, 30, 60, and 120 min of occlusion in groups 1, 2, 3, and 4, respectively). The heart of each pig in these four groups was reperfused for 8 hr. The pig hearts in group 5 were not reperfused.

Procedure. The pigs were sedated with ketamine (2 mg/kg) and anesthetized with sodium pentobarbital (20 mg/kg). Small supplemental doses of sodium pentobarbital and diazepam were administered as required. Lidocaine was given intravenously as a 50 mg bolus immediately before occlusion and reperfusion, and 3 mg/min was given continuously throughout the experiment. Nitroglycerin (5 μg/kg/min) was also administered during the surgical procedure to prevent spasm of the coronary arteries.

A No. 6F polyethylene tube was inserted in the internal jugular vein for administration of drug, and a No. 8F NIH catheter was inserted in the carotid artery for monitoring of aortic pressure. Via a median sternotomy, the heart of each pig was exposed and suspended in a pericardial cradle. A 5 mm segment of the distal one-third of the left anterior descending coronary artery was dissected free from the surrounding tissue. After the baseline measurement of the aortic pressure (Statham P 23Db), the limb lead of the electrocardiogram, and epicardial and endocardial regional blood flow in the risk area (by the generated hydrogen gas clearance method) were obtained, the left anterior descending coronary artery was completely occluded with a Vesseloops (Med General) rubber band. Reperfusion was then established for 8 hr, as described above. Electrocardiographic and blood pressure measurements were made continuously throughout the experiment.

Regional myocardial blood flow was determined at 15 min intervals by the generated hydrogen gas clearance method, as previously described. Briefly, the probe has two platinum electrodes. One electrolyzes the water near the tip, generating a constant volume of hydrogen gas, and the other senses the hydrogen gas, converting the polargraphic current in proportion to its concentration, the concentration being proportional to the blood flow near the probe. In the early phase of the clearance curve, the concentration of hydrogen gas is largely determined by its diffusion rate. The blood flow calculation is performed from the late phase of the clearance curve, which is affected minimally by the diffusion rate. In this experiment, two probes were inserted into the subepicardium and the subendocardium of the ischemic wall.

Histology. The hearts were excised, and small pieces of transmural tissues from the center of ischemic area were removed for electron microscopic studies, as previously reported. The same site of the distal one-third of the left anterior descending coronary artery was again completely ligated with a rubber band. The left and right coronary arteries were cannulated with No. 6F polyethylene tubes, and postmortem coronary arteriography was performed with the use of 5 to 7 ml of a barium-gelatin mixture at a perfusion pressure of 100 mm Hg and perfusion time of 10 min to determine area at risk.

Next, the heart was sliced into 1 cm serial sections in a plane parallel to the atrioventricular groove and x-ray photographs of all slices were taken. The risk area was clearly demarcated by the absence of contrast medium. All slices, including those of the ischemic area, were incubated for 15 to 30 min at 37°C in 0.01M phosphate buffer solution (pH 7.4) containing nitro tetrazolium blue (NTB, Sigma; 50 mg/100 ml). The infarct area was identified macroscopically by the absence of a dark blue color. These slices were then fixed with 10% formalin, embedded in paraffin, cut into slices 4 μm thick, and stained with hematoxylin and eosin and Masson's trichrome.

Microscopically, the infarct area was examined for areas of coagulation necrosis and those of contraction band necrosis (figure 1). Coagulation necrosis is indicated by myocytes of a deep red and multiple granulation in the cytoplasm with the staining method we used. The myofibrils stained with Masson's trichrome showed relaxation and no supercontraction band. Contraction band necrosis was identified as myocytes with irregular and multiple supercontraction bands and with frequently disrupted myofibrils. This was clearly observed in the preparation stained with Masson's trichrome. In addition, a deep red color and multiple granulation were evident. The infarct area observed microscopically was almost the same as the macroscopic infarct area estimated by NTB staining before fixation of each heart. We therefore considered the area of infarct determined microscopically in our analysis.

Quantification of coagulation and contraction band necrosis. Quantification was done by the method of Fujiwara et al. Briefly, a transparent overlay with a thickness of 0.10 mm was placed over the cover glass on the tissue section of the slide glass stained with Masson's trichrome. The preparation was observed under an inverted microscope at magnifications of ×40 to ×400. The area of myocytes with contraction band necrosis and that of coagulation necrosis at a magnification of ×100 or ×200 was carefully traced on transparent film with the use of a stainless-steel needle with a tip width of 10 μm (figure 1). Areas of contraction band necrosis were not present in the tracings of areas with coagulation necrosis. However, minimal contamination with coagulation necrosis was seen in the areas with contraction band necrosis. The tissue preparation with its traced transparent overlay was enlarged to a magnification of ×10 to ×30 on white paper with the use of a photographic enlarger (Fuji 369). The outline of various tissue areas, with fine structures traced on the transparent overlay, and all of the large stained tissue sections on the slide glasses could be clearly traced on the white paper with a fine black pen (figure 1). Quantification of the traced tissue areas was done with an image analyzer (Olympus VIP-21). The infarct area was expressed as a percentage of the risk area (the area perfused by the occluded artery), and the areas of contraction band and coagulation necrosis were expressed as percentages of the infarct area. The values were expressed as round numbers, with fractions of 0.5 or more counted as 1 and those less than 0.5 not counted.

Statistical analysis. Statistical analysis was performed by repeated-measures analysis for hemodynamic and blood flow data. The histologically determined area was analyzed by a one-way analysis of variance and the multiple comparison test. The level of significance was taken as p < .05.

Results

Hemodynamic changes. During occlusion of the distal one-third of the left anterior descending coronary artery, all pigs had ventricular premature beats and in one pig, ventricular tachycardia was followed by ventricular fibrillation. Ventricular tachycardia occurred in eight of the 20 pigs immediately after reperfusion and in two later after reperfusion.

Heart rate and aortic pressure showed no significant changes before or during occlusion or after reperfusion.

Regional blood flow. Before occlusion of the left ante-
rior descending coronary artery, the mean endocardial and epicardial blood flows were 86 ± 19 and 88 ± 19 ml/min/100 g, respectively, in a total of 25 pigs, a nonsignificant difference. During occlusion, both endocardial and epicardial blood flows were reduced to less than 7 ml/min/100 g in each heart. After reperfusion, the regional blood flow returned to the control level in group 1 and 2 pigs. However, in groups 3 and 4 the regional blood flow was less than that before occlusion. The decrease was more prominent in group 4 than in group 3 (table 1).

Infarction in the risk area. Figures 2 to 4 show areas of infarction in the risk areas. The percent infarct area after reperfusion increased significantly with the duration of occlusion: 0 ± 0%, 11 ± 7%, 80 ± 9%, and 96 ± 2% in groups 1, 2, 3, and 4, respectively. The percent infarct area was similar in groups 4 and 5 (95 ± 3% in group 5; figure 3).

The percent infarct area in the inner, middle, and outer thirds of the left ventricular wall in the risk area was 16 ± 8%, 11 ± 10%, and 9 ± 6% in group 2; 91 ± 3%, 88 ± 7%, and 55 ± 18% in group 3; 98 ± 2%, 97 ± 2%, and 95 ± 3% in group 4; and 96 ± 2%, 94 ± 2%, and 94 ± 4% in group 5, respectively (figure 4). In groups 2 and 3, the percent infarct area increased from the outer to the inner third of the left ventricular wall (inner vs outer thirds, p < .05 in group 3) (figure 4).

The percent areas of contraction band and coagulation necrosis in the infarct area. The infarct area was examined to identify areas of contraction band necrosis and those of coagulation necrosis. The contraction band

**FIGURE 1.** The classification of coagulation necrosis and contraction band necrosis and tracings of the areas. A, Coagulation necrosis (Masson’s trichrome stain, original magnification ×400). B, Contraction band necrosis (Masson’s trichrome stain, original magnification ×400). C, The areas of contraction band necrosis (solid lines) traced on the overlay film covering the slide glass with the use of the inverted microscope at an original magnification ×100. D, The final picture traced by the photographic enlarger at an original magnification of ×10 and traced on the white paper. Dotted area indicates coagulation necrosis and shaded area indicates contraction band necrosis. Arrow indicates the same area of contraction band necrosis illustrated in C. Note that coagulation necrosis is located in the inner third of the left ventricular wall, and contraction band necrosis in the middle and outer thirds. Tissue in outer third is partially salvaged. CBN = contraction band necrosis; CN = coagulation necrosis.
necrosis was multifocal, as shown in figure 2. The percent contraction band necrosis was 100 ± 0% in group 2, 68 ± 11% in group 3, and 2 ± 1% in group 4, and it decreased with the duration of occlusion (figure 3). The percent contraction band necrosis in group 4 was similar to that seen in pigs with permanent occlusion (group 5, 2 ± 2%). In group 2, there was little evidence of coagulation necrosis and the infarct area almost always showed contraction band necrosis, mainly in the inner and middle third of the ischemic area (figure 4). In group 3, the percent contraction band necrosis was 26 ± 12% in the inner third, 84 ± 20% in the middle third, and 90 ± 13% in the outer third of the wall (inner vs outer and middle, p < .05). Most of the necrosis was coagulation necrosis in the inner third and contraction band necrosis in the middle and outer thirds. The percent contraction band necrosis in the inner, middle, and outer thirds was 0 ± 0%, 1 ± 1%, and 2 ± 2% in group 4, 1 ± 1%, 1 ± 1%, and 2 ± 3% in group 5, respectively (figure 4). In groups 4 and 5, contraction band necrosis was found only at the edge of the area of infarction, especially beneath the epicardium.

Hemorrhage. Hemorrhage did not occur in group 1. In groups 2 and 3, congestion of blood cells in the microvessels and slight hemorrhage were present in the infarct area, especially in tissues with coagulation necrosis (figure 5). In group 4, moderate-to-severe hemorrhage was diffusely seen in the infarct area of the left ventricle (figure 5). Hemorrhage was always localized within the infarct area. There was no evidence of definite hemorrhage in group 5.

Discussion

The dynamic relationship between duration of occlusion and infarct area, contraction band necrosis, and hemorrhage is shown in figure 2.

The generated hydrogen gas clearance method makes feasible repeated measurements of regional myocardial blood flow. The subendocardial and subepicardial regional blood flow during occlusion was below the resolution of this method (<7 ml/min/100 g) and was transmurally almost zero in all five groups. This evidence confirms previously reported data on pig hearts without a collateral circulation.14-16 Both subendocardial and subepicardial blood flows after reperfusion recovered to the preocclusion control levels in groups 1 and 2, but were approximately 50% of the preocclusion control values in groups 3 and 4. These data reflect the no-reflow phenomenon.23, 24 In the dog heart, with its rich collateral circulation, myocardial blood flow was observed to return to the same level as nonischemic control flow at reperfusion after 1 hr of occlusion.24, 25 The earlier appearance of the no-reflow phenomenon in the pig heart is probably due to the lack of collateral circulation. In the present study the overall hemodynamics were not altered in any group, probably due to the small infarct created by ligation at the distal one-third of the left anterior descending coronary artery. Thus, the difference in the histologic findings among the five groups is independent of the regional blood flow during ischemia and of hemodynamics.

Reperfusion and infarct area. The percent infarct area increased with duration of the occlusion and the level after 120 min of occlusion was the same as that seen in pigs with permanent occlusion (95 ± 3%). In previous studies done to determine whether reperfusion can salvage the ischemic myocardium of dogs, the percent risk area infarcted was 28%, 70%, 72%, and 79% with 40 min, 3 hr, 6 hr, and permanent occlusion, respectively.26 In pigs, the percent infarct area reached 80% and transmural infarction appeared even in pigs undergoing reperfusion after 60 min of occlusion. This difference can be explained by the lack of collateral flow in the pig heart.

In this study, a transmural gradient of myocardial necrosis from the inner third to the outer third of the left ventricular wall was present in pigs undergoing reperfusion after 30 to 60 min of occlusion and became
FIGURE 2. Illustration of dynamic relationship between duration of occlusion and infarct area, contraction band necrosis, and hemorrhage. Diagrams represent typical findings modified from actual tracings. All of the infarct area in group 2 (G2) and most of that in the group 3 (G3) heart is occupied by contraction band necrosis. Most of the infarct areas in groups 4 and 5 (G4 and G5) hearts are occupied by coagulation necrosis. Note that coagulation necrosis in the group 3 heart is located in the inner third of the wall and most of the infarct area is occupied by hemorrhage in the group 4 heart. Dotted line indicates the margin of the risk area, the dotted area indicates coagulation necrosis, the shaded area identifies contraction band necrosis, and "x" represents areas of hemorrhage. A = anterior wall; P = posterior wall; LV = left ventricular cavity; RV = right ventricular cavity.

FIGURE 3. Total percent of risk area infarcted and total percent of infarct area consisting of contraction band necrosis in groups 1 to 5. Data from group 1 were deleted from the right panel, because the infarct area in this group was strictly focal. CBN = contraction band necrosis. Data are mean ± SD. * p < .05 vs groups 3, 4, and 5. ** p < .05 vs groups 4 and 5.
uniform when induced ischemia was more than 120 min in duration. This is compatible with previous findings that myocardial cellular damage in early ischemia progresses from the inner third to the outer third of the pig heart, despite the lack of collateral circulation. In the dog a similar phenomenon was noted, and this transmural gradient of necrosis was attributed to differences in regional blood flow throughout the collateral vessels. However, Rivas et al. speculated that factors other than blood flow were also important. The present findings confirm this hypothesis.

Reperfusion injury. Reperfusion injury is classified as that due to contraction band necrosis and that from hemorrhage. It is well known that reperfusion in the early stage of ischemia leads to the development of contraction band necrosis and reperfusion in the late stage of ischemia causes hemorrhage in areas of coagulation necrosis. Our data are compatible with these observations. Using dog hearts with a rich collateral circulation, Reimer et al. reported that contraction band necrosis occurred diffusely even with reperfusion after 6 hr of occlusion. However, the present study reveals that, in the pig heart, contraction band necrosis appears with reperfusion after 30 min of occlusion.

FIGURE 4. Percent infarct area and percent contraction band necrosis area in the inner, middle, and outer thirds of the left ventricular wall. Data from group 1 were deleted from the bottom panel, because the infarct area in this group was strictly focal. CBN = contraction band necrosis; I = inner one third of the left ventricular wall; M = mid one third of the left ventricular wall; O = outer one third of the left ventricular wall. Data are mean ± SD. * (top panel) p < .05 vs I and M in group 3. * (bottom panel) p < .05 vs M and O in group 3.
Conclusion, becomes diffuse with reperfusion after 60 min of occlusion, and almost disappears with reperfusion after 120 min of occlusion. The discrepancy between the dog and pig heart is explained by the differences in regional blood flow to the risk area after the ligation of coronary artery. Thus, the term “early stage of ischemia” is limited to the period within 1 hr after occlusion in the heart without collateral circulation.

Percent of the area of infarction with contraction band necrosis was 100%, 68%, and 2% (0% to 4%) with reperfusion after 30, 60, and 120 min of occlusion, respectively. The contraction band necrosis was localized in the inner third of the left ventricular wall with the reperfusion after 30 min of occlusion, in the middle and outer thirds with reperfusion after 60 min of occlusion, and in the border zone between the infarct and normal tissue areas with reperfusion after 120 min of occlusion. Distribution of contraction band necrosis with reperfusion after 120 min of occlusion was similar to that with permanent occlusion. Therefore, contraction band necrosis comprising less than 4% of the border zone between infarcted and normal tissue does not indicate reperfusion injury. Such contraction band necrosis may be due to mechanical lesion.

Hemorrhage in the inner third of the ischemic area in groups 2 and 3 was slight, that in group 4 was marked, and in groups 1 and 5 it was rare. Hemorrhage was almost always localized within the boundaries of the infarct. This confirms our previous data on human acute myocardial infarction treated with coronary thrombolysis.30 Microvascular cellular damage in the presence of ischemia slowly follows damage of myocytes.31 This indicates that hemorrhage occurs in tissues already markedly damaged and that it rarely expands the infarct zone.

Clinical implication. Coronary thrombolysis and PTCA are currently the most important treatment for acute myocardial infarction. In the chronic stage, some patients show good left ventricular function and others dyskinetic wall motion, even if good recanalization is achieved at 1 to 2 hr after the onset of acute myocardial infarction. The effectiveness of these procedures is
influenced by the presence or lack of collateral vessels,\textsuperscript{32} which effects regional blood flow to the area of ischemic tissue in each human heart. At present, coronary thrombolysis is usually carried out more than 2 hr after the onset of acute myocardial infarction. This is also the case with thrombolysis using t-PA, the tissue plasminogen activator.\textsuperscript{33, 34} Results of double-blind trials are conflicting with respect to whether or not coronary thrombolysis can limit the infarct area.\textsuperscript{6-8} The present data indicate that transmural infarction may result when coronary thrombolysis is performed 1 hr after the onset of the acute myocardial infarction in patients without collateral vessels. In this situation, 68% of the infarct area is occupied by contraction band necrosis. In addition, we have reported that, in human acute myocardial infarction or with use of controlled reperfusion with a thrombolytic agent,\textsuperscript{35} recanalization is not achieved in 3% of the infarct area) in the heart of the patient in whom recanalization is not achieved during coronary thrombolysis.\textsuperscript{35} Generally, myocytes in the area of contraction band necrosis after reperfusion are considered to be the result of irreversible damage that occurs immediately before reperfusion. However, recent reports on controlled reperfusion with a low-calcium solution suggest that reversible or irreversible cellular damage is not absolute.\textsuperscript{36-38}

In conclusion, to reduce the area of the infarct in the heart without collateral circulation, recanalization should be accomplished within 1 hr after the onset of acute myocardial infarction or with use of controlled reperfusion that will protect against contraction band necrosis should be considered.

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