Identification and quantification of histochemical border zones during the evolution of myocardial infarction in the rat

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SUMMARY Several interventions have been shown to preserve ischaemic myocardium after coronary artery occlusion; yet there is uncertainty concerning the location, extent and very existence of the potentially salvageable myocardium. This study was undertaken to identify and to quantify by planimetry, histochemically different zones of myocardium in evolving infarcts. Serial frozen sections of the left ventricle from 98 rats killed at different time intervals ranging from 5 min to 72 h after coronary artery occlusion were stained for glycogen, neutral lipids and oxidative enzyme activity. From 5 to 20 min after occlusion, the ischaemic area (measured as a percentage of the cross-sectional area at the midventricular level) showed progressive glycogen loss which was, paradoxically, more severe at the periphery than at the center. By 30 min glycogen loss was uniform and complete. Three hours after occlusion 3 abnormal zones were observed: (1) a central zone of severe glycogen loss and severe loss of enzyme activity comprising 21 ± 3% (mean ± SE) of the left ventricular cross-sectional area; (2) a peripheral zone showing severe glycogen loss but only mild loss of enzyme activity comprising 21 ± 4% of the ventricle; and (3) a surrounding lipid-containing, but otherwise normal, zone comprising 9 ± 1% of the ventricle. The zone of mild loss of enzyme activity was smaller but still present 6 h after occlusion, but disappeared by 9 h as the zone of more severe loss of enzyme activity enlarged to equal the size of the glycogen-depleted zone. The lipid-containing zone persisted for 72 h. Thus, 2 histochemical border zones were observed: an outer histochemical border zone which contained lipid but was otherwise normal which was present up to 72 h after coronary occlusion, and an inner histochemical border zone with severe glycogen loss but delayed loss of enzyme activity presented at 3 and 6 h but which disappeared 9 h after coronary artery occlusion. These data support the concept that a myocardial infarction is not a homogeneous, static structure, but rather a dynamic temporally-related phenomenon with different zones that change in size and character as the infarct evolves.

Following coronary artery occlusion in experimental animals, the extent of subsequent myocardial necrosis can be limited by a number of haemodynamic, metabolic, and pharmacological interventions. These interventions are presumed to preserve ischaemic myocardium until a collateral circulation develops that is adequate to sustain cell viability. Thus, it has been suggested that following permanent coronary occlusion there are at least two zones of myocardium within the ischaemic tissue: (1) a zone with severe ischaemia destined to become necrotic irrespective of any intervention; and (2) a ‘border zone’ of less severe ischaemia with damaged yet viable myocardium that can be salvaged by the appropriate intervention. If so, the progression of biochemical abnormalities in the ischaemic myocardium should be non-uniform; that is, the salvageable cells should exhibit either qualitatively different biochemical changes or the same changes proceeding more slowly than those in the non-salvageable cells. Indeed, Hearse et al using multiple sampling techniques have shown gradients of biochemical changes at the margins of evolving infarcts.

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Histochemical techniques, as opposed to biochemical techniques, permit the morphological visualisation and topographic localisation in addition to the determination of the relative concentration of specific substances in tissues. Accordingly, in this investigation, histochemical techniques were used to examine the regional biochemical changes that occur in the myocardium following experimental coronary artery occlusion and to determine whether these biochemical changes proceed at different rates in different regions of the ischaemic and non-ischaemic myocardium. On the basis of our observations, we have characterised and quantitated "histochemical border zones" that were detected during the evolution of myocardial infarction in the rat.

Methods

Myocardial infarction was produced in 98 male Sprague-Dawley rats (Charles River Breeding Laboratories) weighing 250 to 300 g each, by occlusion of the left coronary artery as described previously. Briefly, each rat was anaesthetised with ether, the skin incised along the left sternal border and the heart exteriorised through the 5th intercostal space. The left coronary artery and vein were ligated 1 to 2 mm from their origin and the heart was positioned back into the chest which was then closed. Operative mortality was 25%.

The rats were allowed to recover and at the following time intervals after coronary artery occlusion were reanaesthetised and killed by excision of the heart: 5 min (5 rats), 10 min (4 rats), 20 min (5 rats), 30 min (7 rats), 1 h (6 rats), 2 h (7 rats), 3 h (17 rats), 6 h (14 rats), 9 h (7 rats), 12 h (7 rats), 48 h (16 rats) and 72 h (3 rats). Immediately after excision, a cross-sectional slice of heart was taken at the mid-ventricular level, 4 to 4.5 mm from the apex, and frozen rapidly in 2-methyl-butane which had been cooled in dry ice to -60°C. The tissue was maintained in a frozen state and 10 μm thick serial frozen sections were cut and mounted, and consecutive sections were stained by the following histochemical and histological techniques: (1) periodic-acid-Schiff-diastase (PAS) reaction for glycogen; (2) nicotinamide adenine dinucleotide-diaphorase (NADH-diaphorase) and

FIG 1 Glycogen stain of transverse sections of heart from rats killed 5 (a), 20 (b), and 30 (c) min after coronary occlusion. At 5 and 20 min (a and b) within the ischaemic zone (i-bounded by broken lines) there is central sparing of dark-staining glycogen. Note the normal staining of the septum and right ventricle. At 20 min (b) the central area of delayed glycogen loss is smaller and has lost more glycogen than at 5 min (a). By 30 min (c) glycogen loss is uniform and complete (LV left ventricular cavity) (PAS stain, ×6).
succinic dehydrogenase (SDH) reactions for oxidative mitochondrial enzymes; (3) oil red O (ORO) stain for neutral lipids and (4) hematoxylin and eosin (H&E) stain for standard histological examination.

Each section was examined microscopically to determine whether the intensity of staining was increased (+) or decreased (−) and the degree of change from normal was graded semiquantitatively as follows: 0-control or normal staining; 1-mild change; 2-moderate change, and 3-marked change. Following light microscopic evaluation of the stained sections they were projected onto a small screen at a magnification of 10 × and the areas of staining and non-staining which were clearly distinguishable at this low magnification were measured by planimetry. The following measurements were made from sections of slices from each heart: (1) cross-sectional area of left ventricular myocardium; (2) cross-sectional area of left ventricular myocardium showing glycogen loss; (3) cross-sectional area of left ventricular myocardium showing loss of enzyme activity, and (4) cross-sectional area of left ventricular myocardium showing lipid accumulation. From these measurements the percentage of the cross-sectional area of the left ventricular myocardium involved by each histochemical change was calculated. The purpose of this study was not to measure infarct size per se, but rather the relationship of the different zones identified histochemically. The mid-portion of the interventricular septum and right ventricle, which do not become ischaemic in this model, served as control areas for normal staining. H&E-stained sections from each heart were examined for the classical histological changes of myocardial infarction. Inter and intraobserver

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**FIG 2** Time course of glycogen loss in the various zones of evolving myocardial infarcts after coronary artery occlusion. Note the non-homogeneity of glycogen loss during the first 20 min of ischaemia and the increase in glycogen in the tissue surrounding the area of glycogen loss by 30 min after coronary artery occlusion.

0 Control ○ centre of ischaemic zone
1 Mild * Periphery of ischaemic zone
2 Moderate ■ Surrounding non-ischaemic tissue
3 Marked — centre of ischaemic zone

**FIG 3** Serial transverse sections from the heart of a rat killed 3 h after coronary occlusion. Section (a) is stained for glycogen (PAS) and (b) is stained for NADH-diaphorase activity. In these photomicrographs, areas which are more positive for glycogen and enzyme are darker than the negative areas. Note that in (a) the ischaemic zone (I) of glycogen loss is larger than the zone of loss of enzyme activity shown in (b). The arrows point to some areas of the section where there is glycogen loss but no loss of enzyme activity. There is also diffuse subendocardial and subepicardial sparing of enzyme activity in areas of marked glycogen depletion (LV—left ventricular cavity) (−).
variability in measuring areas was 3 to 4% and intensity of staining was agreed upon by two authors who reviewed all histological sections.

Results

ALTERATIONS IN GLYCOGEN (fig 1)

5 min after occlusion a zone of mild (-1) glycogen depletion was present in the ischaemic left ventricular free wall involving $53 \pm 6\%$ (mean $\pm$ SE) of the left ventricular cross-sectional area ($n = 5$ rats). Within this zone of mild glycogen loss two regions were noted: (1) a peripheral region composed primarily of the subepicardial and lateral margins of the glycogen-depleted zone; and (2) a central region composed of the geometric centre and subendocardial portions of the glycogen-depleted zone. The peripheral region, comprising $65 \pm 3\%$ of the area of glycogen loss, had greater loss than the central region in which glycogen loss was barely detectable. At 10 min glycogen loss had increased in severity and its distribution was still non-uniform. The peripheral $66 \pm 2\%$ of the area of glycogen loss had moderate loss (-2) while the central area had only mild glycogen loss (-1) ($n = 4$ rats). At 20 min, glycogen loss was marked (-3) in the peripheral $82 \pm 2\%$ of the area of glycogen loss, and moderate (-2) in the central area ($n = 5$ rats). Thus, at 20 min, glycogen loss was more extensive and intense than at 5 and 10 min after occlusion. By 30 min glycogen loss was complete (-3) and uniform throughout the area of ischaemia with no difference in the intensity of staining in the central and peripheral portions. Also, at this time, at high magnification (100 x), there was a thin zone of myocardial fibres one-to-three cells thick which surrounded the glycogen-depleted zone that had a mild increase in glycogen content. At 1, 2, 3, 6, 9, 12, 48, and 72 h after occlusion the intensity of glycogen loss (-3) and the size of the area of glycogen loss were not different from that at 30 min (fig 2).

ALTERATIONS IN MITOCHONDRIAL OXIDATIVE ENZYME ACTIVITY

Although the loss of NADH-diaphorase activity was slightly more severe than the loss of SDH activity, both reactions were similar and so only the results of the NADH-diaphorase reaction will be presented. No loss of enzyme activity was detected until 1 h after occlusion at which time there was only a slight suggestion of less intense staining of a portion of the left ventricular free wall ($n = 6$ rats) which could only be detected at high magnification (100 x). At 2 h post-occlusion, mild loss of enzyme activity (-1) was more evident ($n = 7$ rats) and by 3 h non-uniformity of loss of enzyme activity was apparent at low magnification (fig 3b). At 3 h there was a central zone of moderate (-2) loss of enzyme activity comprising $21 \pm 3\%$ of the left ventricle and a peripheral zone of mild (-1) loss of enzyme activity totalling $21 \pm 4\%$ of the left ventricle ($n = 17$ rats). At 6 h the central zone was larger, comprising $33 \pm 4\%$ of the left ventricle and the peripheral zone was smaller, comprising $9 \pm 2\%$ of the left ventricle ($n = 14$ rats). At 9 h the peripheral zone had disappeared completely and only a single zone of uniform,

FIG 4. Sections similar to those of fig 3 but from a rat killed 12 h after coronary occlusion. Section (a) is stained for glycogen and section (b) for NADH-diaphorase activity. Note that in contrast to 3 h after occlusion, at 12 h the area of glycogen loss is identical to the area of loss of enzyme activity (= ischaemic zone; LV = left ventricular cavity) (x 7).
moderate (-2) loss of enzyme activity (n=17 rats) was observed (fig 4). This single zone persisted at 12 h (n=7 rats). At 48 and 72 h post-occlusion there was more marked (-3) loss of enzyme activity within the single enzyme-depleted zone. As loss of enzyme activity progressed in the ischaemic area there was also a loss of the finely granular staining pattern observed in normal myocardium. From 3 h on, the enzyme-depleted zone was surrounded by a thin rim of tissue in which the staining pattern was slightly more intense and coarsely granular than in the remaining normal myocardium (fig 5).

ALTERATIONS IN LIPID

No increase in intracellular lipid droplets was apparent until one hour after occlusion when a faint-staining zone (+1) of the cells with very slight lipid accumulation was evident adjacent to the margin of the glycogen depletion area. This zone was unchanged at 2 h, but at 3 h was more distinct, completely surrounding the glycogen-depleted zone, and comprising 9 ± 1% (n=17 rats) of the left ventricle (fig 6). At 6 h, the intensity of staining in the lipid-accumulating zone had increased noticeably (±2) but the size of the zone remained the same (10 ± 1% of the ventricle (n=14 rats)). At 9 and 12 h after occlusion, the size of the lipid zone was unchanged (12 ± 3% and 9 ± 1%, respectively) but lipid accumulation was more intense (+3). A zone of moderate (+2) lipid accumulation persisted at 48 and 72 h after occlusion (fig 7).

ZONES OF DIFFERENT STAINING AT VARIOUS TIME INTERVALS

At all times up to 2 h after occlusion there was no distinct area of loss of enzyme activity within the area of glycogen depletion. At 1 h, when the area of loss of enzyme activity equalled 0% of the left myocardium the enzyme activity had decreased uniformly (±1) within the area of glycogen depletion. At 3 h, the area of glycogen depletion had increased to 2 ± 1% of the ventricle (n=17 rats) and enzyme activity within the area had decreased uniformly (±1) to equal 9 ± 1% of the remaining normal myocardium (fig 5).

OXIDATIVE ENZYME ACTIVITY IN EVOLVING MYOCARDIAL INFARCTION

FIG 5 Time course of loss of oxidative enzyme activity (NADH-diaphorase and SDH) after coronary artery occlusion. Note the non-uniformity of enzyme loss 3 and 6 h after occlusion. Also, from 3 to 12 h after occlusion there is a mild increase (+1) in enzyme activity in the myocardium surrounding the ischaemic zone.

0 = Control  ○ Centre of ischaemic zone
1 = Mild  * Periphery of ischaemic zone
2 = Moderate  ■ Surrounding non-ischaemic tissue
3 = Marked

FIG 6 Transverse section of heart from a rat killed 3 h after coronary occlusion. Note that in these sections stained for neutral lipids (ORO stain) the area of ischaemic (i) and normal (n) myocardium are separated by a rim of lipid-accumulating cells (arrows). Lipid may accumulate in the ischaemic area around veins and in the subendocardium and subepicardium but is found predominantly at the lateral edges of the infarct (a = 6; B = 34).
ventricle, the area of glycogen depletion equalled 57 ± 7% of the ventricle. At 3 h after occlusion 3 abnormal zones first became evident (figs 3, 8, and 9): (1) a central zone of severe glycogen loss and loss of enzyme activity comprising 21 ± 4% of the left ventricle; (2) a peripheral zone with severe glycogen loss but only very mild loss of enzyme activity comprising 4 ± 4% of the left ventricle (50 ± 7% of the ischaemic, glycogen-depleted zone); and (3) a surrounding lipid-containing zone comprising 9 ± 1% of the left ventricle in which a granular enzyme staining pattern was noted (fig 6).

At 6 h the central zone of severe glycogen loss of enzyme activity was larger, comprising 76 ± 7% of the ischaemic zone (33 ± 4% of the left ventricle). The surrounding lipid-containing zone comprised 10 ± 1% of the left ventricle.

At 9 and 12 h after occlusion the glycogen-depleted zone and zone of loss of enzyme activity were identical: 52 ± 2% vs 52 ± 3% of the left ventricle at 9 h, and 51 ± 3% vs 51 ± 3% at 12 h. At 48 and 72 h there was more severe (-3) loss of residual enzyme activity from the infarcted area, the surrounding lipid-containing zone persisted and there was no change in the glycogen-depleted zone.

**Comparison of histochemical and histological changes**

Before 12 h, H&E-stained sections showed only waviness and thinning of fibres in the ischaemic, glycogen-depleted zone. At 12 h after coronary artery occlusion sections which showed glycogen depletion and loss of enzyme activity by histochemistry showed increased eosinophilia by H&E staining. At 48 and 72 h H&E-stained sections showed that the areas with glycogen loss and loss of enzyme activity were frankly necrotic with increasing eosinophilia, loss of cross-striations, karyolysis and infiltrates of acute inflammatory cells at the margins of the infarct. Planimetry of 48-hour-old infarcts showed that the necrotic zone (by H&E staining) was identical to the area of glycogen loss and loss of enzyme activity (53 ± 4% vs 52 ± 4%, n = 7 rats). Thus, at a time that necrosis can be assessed accurately by H&E staining, the area of glycogen loss and loss of enzyme activity corresponded topographically and quantitatively to the area of necrosis.

**Discussion**

In this study different zones of biochemical changes in damaged myocardium after coronary artery occlusion were characterised and measured utilising histochemical techniques. Glycogen loss per se is an early but entirely reversible biochemical marker of ischaemia which results from a rapid shift from aerobic to anaerobic metabolism, and which occurs much earlier than the morphological features associated with irreversible cell damage. As in other studies glycogen loss was demonstrated early after coronary occlusion; however, in this study for the first time, the temporal evolution and non-uniformity of glycogen loss were characterised and quantitated. Early after coronary occlusion (5, 10, and 20 min) the central portion of the ischaemic zone, where flow is reduced the most, had less glycogen loss than did the periphery, where flow is reduced less. This apparent paradoxical distribution of glycogen may be due to the greater metabolic demand of the peripheral tissue. The centre of the infarct ceases to contract while the periphery is still contractile to some degree. This observation, therefore, may be histochemical evidence that cessation of contraction, by preserving glycogen, has a protective effect on the ischaemic myocardium. Since the central area of an evolving infarct is probably also the most acidotic portion of the ischaemic myocardium, another very likely possibility is that the glycogen sparing in this region is related to the recognised inhibition of glycolysis by a low pH.

Histochemical evidence that loss of enzyme activity occurs later than loss of glycogen is also in accordance with previous studies which indicated that histochemical evidence of loss of enzyme activity represents more severe ischaemic damage. Since previous studies have shown that cells within an area of ischaemia show ultra-struc-
tural signs of irreversible injury before loss of enzyme activity can be demonstrated histochemically, loss of enzyme activity probably occurs sometime after the cell is irreversibly injured. Of note, however, is that loss of enzyme activity within the ischaemic zone is not uniform, with the margins of the ischaemic zone showing delayed loss of activity. The implication is that the cells at the periphery of the infarct remain viable longer and hence, enzyme activity persists longer. Lushnikov, in a histochemical study of myocardial infarction in rabbits, described a peripheral zone of delayed loss of enzyme activity following coronary artery occlusion. However, since he found this zone to be present 12 h to 4 days following occlusion it is doubtful that this zone is analogous to the zone we observed in this study which was present only between 3 and 9 h after occlusion. Cox et al. and Deloche et al. have also described histochemical differences between the centre and periphery of an ischaemic area of myocardium suggesting prolonged viability of the periphery.

Increased lipid has been demonstrated towards the edge of the ischaemic myocardium of experimental animals with coronary artery occlusion. Our studies and those of Page and Polimeni using ultra structural techniques, have demonstrated that most lipid-containing cells at the edge of an infarct are otherwise normal in appearance, except for having prominent "T" bands due to relaxation. Moreover, when the infarct is well established and recognisable in haematoxylin-eosin-stained sections 48 h and 72 h after occlusion, the zone of lipid accumulation persists in the non-infarcted tissue. Since the lipid accumulation observed in this study occurs outside the area of glycogen and enzyme loss it appears that the lipid-containing cells, while metabolically abnormal, may not be severely ischaemic and that the majority of lipid accumulation does not occur in myocardium that usually undergoes necrosis. This zone of viable but metabolically abnormal myocardial cells, which involves approximately 10% of the left ventricle, may be one of the regions prone to becoming necrotic if the myocardium is subjected to unfavourable pharmacological, metabolic, or haemodynamic alternations.

It has been postulated that the regions of ischaemic myocardium that would benefit most from beneficial intervention would be at the periphery in the so-called "border zone". Presumably, in this zone, the process of necrosis would evolve slower and irreversible cellular injury would be delayed. By use of markers of blood flow such as 14 C-antipyrine, microsphere techniques, and thioflavin S, a fluorescent dye, it has been shown that the subendocardial portion of the ischaemic zone and its geometric centre have the lowest flow while the epicardium and the periphery have greater, although still subnormal, flow. Thus, these studies demonstrate a "flow border zone." In experimental studies of interventions that reduce infarct size, histological appearance and myocardial creatine kinase activity indicate that most salvage occurred at the periphery of the ischaemic zone with the centre continuing to show severe necrosis.

In spite of the aforementioned observations, there is no general agreement as to the size, location or very existence of a "border zone" of intermediate flow reduction and intermediate ischaemic injury at the periphery of an ischaemic zone of myocardium. Some studies using microspheres and histological techniques have suggested that there is no "border zone" at all but rather a sharp and sudden change from completely normal to severely ischaemic tissue. Hirzel et al. have reported that 24 h after coronary occlusion in the dog, creatine kinase (CK) activity is uniformly decreased from the lateral edge to the centre of the ischaemic zone. In that study, however, the endocardial layers showed more severe loss of CK activity than the epicardial layers. Reinier et al. and Rasmussen and associates have also demonstrated in the dog that subendocardial ischaemia is more severe than subepicardial ischaemia. In addition, these authors demonstrated that cell death does not occur uniformly within an ischaemic zone but rather in a wave from subendocardium to subepicardium. Thus, their studies support the existence of a subepicardial "border zone."
The present study demonstrates that after coronary occlusion, in the absence of any intervention, there is a zone at the margin of the ischaemic myocardium that is biochemically different from its centre. It is characterised by severe glycogen depletion with only mild loss of enzyme activity which evolves slower than loss of enzyme activity in the centre of the ischaemic zone (figs 8 and 9). Of note is that this zone is not a static structure but a dynamic, temporally-related phenomenon present in the rat at 3 and 6 h but not at 9 h after coronary artery occlusion. Thus, histologic studies or studies of tissue CK activity 24 h after occlusion would not be expected to identify this zone which has already disappeared.

If, as it is hoped, a “border zone” of salvageable myocardium exists in an evolving infarct, it should have the following characteristics: (1) it should be at the periphery where the potential for collateral flow is greatest; (2) until the time that irreversible cell death occurs, it should have less ischaemic injury than nonsalvageable cells; (3) it should be a transient zone that decreases in size with time; and (4) it should be of sufficient size to account for the magnitude of myocardium salvageable by beneficial interventions.

Interestingly, the zone of delayed enzyme loss observed in this study fulfills all these criteria. It must be emphasised that there is no proof that the zone of delay loss of enzyme activity represents the salvageable myocardium; nor is there proof that preservation of oxidative enzyme activity is indicative of cell viability at any given time. It seems reasonable to suggest, however, that those regions which exhibit delayed histochemical abnormalities might be the regions which are surviving longer after an ischaemic insult, and thus may represent the most salvageable portions of the ischaemic myocardium.

Finally, since acute coronary artery occlusion in the rat differs from chronic coronary atherosclerotic disease in man the relevance of these observations to patients with ischaemic heart disease remains to be determined.

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