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Contraction band necrosis at the lateral borders of the area at risk in reperfused infarcts

Observations in a pig model of in situ coronary occlusion

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Abstract The aim of this study was to test the hypothesis that increased mechanical stress at the lateral borders of the area at risk may render this area more susceptible to ischaemia/reperfusion injury in the absence of collateral flow. The spatial distribution of myocardial necrosis within the territory of a transiently occluded left anterior descending coronary artery was investigated in 31 porcine hearts submitted to 48 min of coronary occlusion and 6 h of reperfusion. Immediately before excising the heart, the left anterior descending coronary artery was re-occluded and 10% fluorescein was injected in the left atrium. The area at risk was imaged by ultraviolet illumination of the myocardial slices, and the area of necrosis by incubation in triphenyltetrazolium chloride. The area at risk was divided in four sectors and an index of eccentricity was calculated as the percent of the area of necrosis located in the two lateral sectors of the area at risk. The area of contraction band necrosis was measured in whole heart histological sections. Infarcts were generally small, and were composed almost exclusively of contraction band necrosis. There was a good correlation between the extent of the area of contraction band necrosis and infarct size (r=0.831, P<0.0005). The area of necrosis had a patchy appearance and was predominantly distributed along the lateral borders of the area at risk. This eccentric distribution was more prominent in smaller infarcts, and the eccentricity index was inversely correlated with infarct size (r=–0.471, P=0.007), suggesting that contraction band necrosis occurres first at the interface between control and reperfused myocardium in this model. These results are in agreement with a prominet role of mechanical factors in the genesis of myocardial necrosis during transient coronary occlusion.

Key words Myocardium · Ischaemia · Reperfusion injury · Contraction band necrosis

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Introduction

The spatial distribution of myocardial necrosis within the territory of transiently occluded coronary artery varies, depending mainly on the duration of occlusion. As reperfusion is delayed, necrosis involves a progressively larger fraction of the area at risk. Progression of myocardial necrosis does not take place as an increase in the density of scattered dead myocytes, but as a shift of the border of a well-defined zone of myocardial necrosis where necrotic myocytes from a continuum [11, 18]. This zone of necrosis may however present a complex geometry, and a patchy appearance in histological sections [11]. The wave-front pattern of transmural progression of necrosis was first observed in the dog model, with significant collateral flow [18], and has been also observed in animal species with negligible collateral flow [9]. Whether there is or there is not lateral progression of the necrosis from the centre to the lateral borders of the area at risk, has not been demonstrated clearly. Although the presence of lateral gradients in the concentration of ischaemic by-products and intramyocardial pH [3], across the area at risk would suggest a greater susceptibility to ischaemia in the core than in the periphery of the area at risk, most observations suggest that there is either a very fast lateral progression, or a simultaneous occurrence of necrosis across the lateral extension of the area at risk.

The greater susceptibility of the subendocardial half of the left ventricular wall to ischaemia in the absence of

collateral flow has been explained as a consequence of increased intramural pressure, tension and energy demand in this region [21]. Ischaemia also reduces the mechanical resistance of the sarcolemma [25], and there is increasing evidence that mechanical stress imposed to the sarcolemma during ischaemia/reperfusion may be an important determinant of cell death following transient coronary occlusion [4, 5, 11, 15, 16, 23]. Myocytes at the interface between the area at risk and normal myocardium are exposed to a particularly high mechanical stress during ischaemia and reperfusion. It is reasonable to suggest that increased mechanical stress at the interface between the area at risk and normal myocardium results in an increased susceptibility of the margins of the area at risk to develop necrosis after transient coronary occlusion and reperfusion. This study investigated this hypothesis by analysing the spatial distribution of myocardial necrosis in a pig model, with negligible collateral flow, submitted to a brief transient coronary occlusion resulting in incomplete infarcts composed almost exclusively of contraction band necrosis.

Materials and methods

This study was performed on hearts from 53 Large-White farm pigs (30–55 kg) submitted to 48 min of coronary occlusion and 6 h of reperfusion. These animals formed the control groups of consecutive studies on infarct size limitation, and underwent surgical procedure following strict experimental protocol. Animals were premedicated with 10 mg/kg azaperone i.m. (Stresnil, Janssen), anaesthetized with thiopental 10 mg/kg i.v., intubated and mechanically ventilated with room air (Monaghan 228 Ventilator, Litletown, Colorado) as previously described [11]. An intravenous dose of aspirin (250 mg) was administered in 27 of 53 animals, and 11 received i.v. heparin (160 IU/kg). The right mammary vein and the right femoral artery were catheterized, a mid-line sternotomy was performed and the pericardium opened.

The left anterior descending coronary artery (LAD) was dissected free approximately at the mid-point of its total length and surrounded by an elastic snare. Two pairs of ultrasonic crystals 1 mm in diameter were inserted into the inner third of the left ventricular wall as previously described [11]. One pair was implanted in the myocardium to be made ischaemic and the other pair in the lateral wall of the left ventricle.

After receiving a bolus of intravenous lidocaine (1.5 mg/kg) all animals underwent a 48 min coronary occlusion of the LAD followed by 6 h of reperfusion. Frequent measurements of arterial pH, PaO_2 and $PaCO_2$ were performed to adjust the ventilatory functions to maintain normal blood gases. Aortic blood pressure was continuously monitored with a crystal quartz transducer (Coulbourn T42-205, Coulbourn Instruments Inc., Leihgh Valley, Penn., USA).

The two segment ultrasonic length signals were analyzed by an ultrasonic dimension system (System 6/200, Triton technology inc, San Diego, Calif., USA) and monitored with a HM 205_{-3} oscilloscope (Hameg Instruments, Frankfurt Main, Germany). All these signals, along with lead II of the electrocardiogram, were conditioned in a Coulborn Modular Instrument System (Coulbourn Instruments Inc.) and digitized by means of a digitizing card (Tecfen ISC-16E/CR, R C Electronics Inc., Goleta, Calif., USA), at a sampling rate of 100 Hz per channel. Digital signals were stored in a hard disk and continuously recorded in a thermic pad recorder (MT-9500, Astro-Med, Inc., West Warwick, R.I., USA).

Segment length measurements were performed as previously described [11]. Systolic shortening was calculated as the ratio of systolic shortening to end-diastolic length. Segment measurements were expressed as percent of basal values (values immediately before coronary occlusion), except for systolic bulging, which was expressed as percent of value immediately before reperfusion.

The hearts were excised after 6 h of reperfusion. Immediately before excision, the LAD was reoccluded and 5 ml of 10% fluorescein were injected into the left atrium. The heart was immersed in Ringer solution at 4° C, included in a polyurethane fast-hardening foam (Alcupol S-616/R-11 combined with MDI, Repsol Química, Madrid, Spain) and cut in 5–7 mm slices perpendicular to its long axis. The slices were illuminated, from its basal side, with 356 nm ultraviolet light to delineate the area at risk, and imaged by means of a Sony TR 705E Hi8 video camera. The images were digitized on line into 768 by 576 pixels images by means of a Matrox IP8 digitization card (Matrox Electronic Systems Ltd, Dorval, Quebec, Canada). The slices were then incubated at 37° C in 1% triphenyl tetrazolium chloride, buffered for pH=7.4, for 5–10 min, and imaged again under white light using the same camera and digitization card. A reference scale was also digitized in each experiment.

The zone at risk and the area of necrosis were measured in the digital images by using commercially available software (Image Pro-Plus, Media Cybernetics, Silver Springs, Md., USA), and the mass of myocardium at risk and infarct size were calculated from these measurements and the weight of the slices.

The spatial distribution of necrosis was analysed in the digitized images of the third slice from each heart with the aid of ad hoc software. The area at risk in this slice was divided into four adjacent sectors by means of straight lines originating in the centre of the left ventricular cavity, and the percentage area of necrosis in each of these sectors was measured. An index of eccentricity of the area of necrosis was calculated as the ratio of the extent of the area of necrosis in the two lateral sectors to the extent the area of necrosis in the whole area at risk (the two medial and the two lateral sectors) (Fig. 1). This index could vary between 0 (100% of the area of necrosis contained in the two medial sections) and 2 (100% of the area of necrosis contained in the two lateral sectors). The correspondence between the lateral borders of the area of necrosis and the borders of the area at risk was also analysed in the digitized images from the third slice.

The measurements of the area of necrosis in both sides of the third slice, as measured by triphenyl tetrazolium staining, were averaged and compared with the area of contraction necrosis in an histological section from that slice.

The myocardial slices were weighted and fixed in 10% formaldehyde. The third slice from each heart was processed for histology; 4 μm thick sections including the whole right and left ventricles were obtained using a Polycut microtome (Reichert Jung, Cambridge Instruments, Heidelberg, Germany) and mounted on 10×14 cm glasses. The sections were stained with Masson's trichrome and examined with a Nikon Labphot microscope and a binocular lens (Olympus stereo microscope, SZ, 111) at a magnification of ×100 and ×200. The total area of contraction band necrosis was quantified in each section according to a modification of the method of Fujiwara et al. [6, 15] as previously described [11]. The areas of contraction band necrosis were marked on a transparent overlay 0.1 mm thick placed over the cover glass with a sharp stainless-steel needle. Histological criteria for contraction band necrosis were a deep red colour, the presence of hypercontracture bands and multiple granulations [4, 6, 15]. Colour photographs of the whole section, with the photographic overlay were obtained and digitized, and the ratio of the area of contraction band necrosis to the total area of the slice was calculated.

Eleven of the 53 animals presented transient or permanent reocclusion during the reperfusion period and were excluded. Re-occlusion was identified by abrupt deterioration of systolic shortening during the reperfusion period in the absence of arrhythmias and changes in the control segment and confirmed by visual inspection of the LAD. Ten animals presented very small infarcts, involving <2.5% of the area at risk. Analysis of infarct geometry could not be performed in these very small infarcts and the study group consisted of the remaining 32 animals.

Statistical analysis was performed using commercially available software (SPSS/PC+4.0). Changes in physiological variables within the same animal were assessed by analysis of variance for repeated samples. Group comparisons were performed by means of the *t*-test for independent samples, after checking that variable distribution did not significantly depart from the normal distribution. The relation between normally distributed variables was assessed by linear regression analysis. A critical *P* value of 0.05 was used for all tests. All values are expressed as mean±SEM.

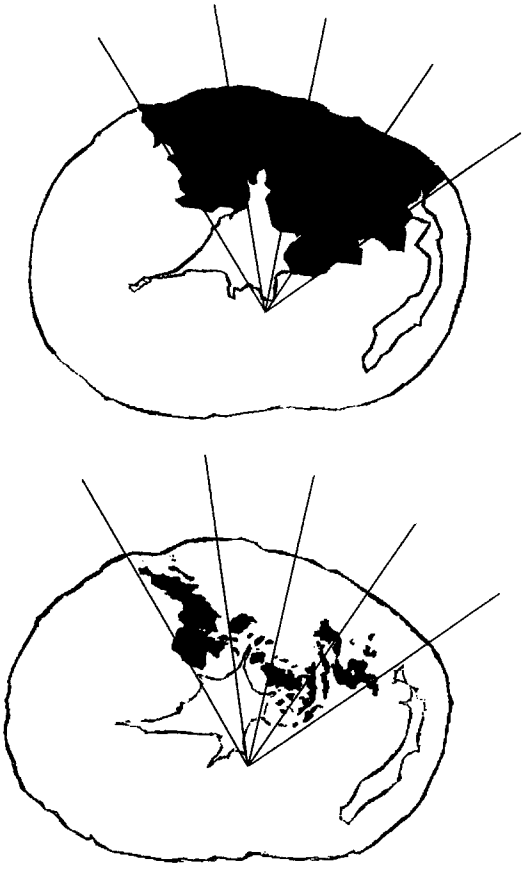

Fig. 1 Digital analysis of the spatial distribution of necrosis in reperfused myocardium. Top: area at risk as assessed by U.V. light illumination, with the four sectors used to calculate the index of eccentricity (see text). Bottom: area of necrosis as assessed by the triphenyl tetrazolium reaction. Note the distribution of the area of necrosis along the interface between the area at risk and adjacent normal myocardium

Results

Haemodynamic data and regional wall function

Haemodynamic and regional function data are summarized in Table 1. Basal heart rate and mean aortic pressure were close to the physiological values. Coronary occlusion produced a rapid abolition of contractile function in the ischaemic segment, with only minor changes in heart rate and mean aortic blood pressure. Systolic shortening remained severely depressed throughout the reperfusion period of all animals, and in most animals only a minor recovery was observed. Ventricular fibrillation occurred in 10 (31.3%) animals during the occlusion period and in 8 (25%) during the first minutes of reperfusion, and was promptly reverted to sinus rhythm by internal DC shock.

Infarct size and infarct histology

In the 32 animals in which infarct geometry could be analysed, the area at risk averaged 10.9±0.5 g, and infarct size 3.7±0.4 g or 33.2±3.0% of the area at risk. At histology, infarcts consisted almost exclusively of areas of contraction band necrosis in all hearts, areas of coagulation band necrosis being virtually absent. No heart showed macroscopic intramyocardial haemorrhage, and microscopic red blood cell extravasation was scant or absent. The area of contraction band necrosis, as assessed by histology, involved 15.7±1.9% of the third slice. The area of myocardial necrosis, as assessed by the triphenyl tetrazolium reaction, involved 10.5±1.8% of the third myocardial slice. There was a good correlation (r=0.831, P<0.0005) between this area, and the area of negative triphenyl tetrazolium staining.

Infarct geometry

The area of necrosis in the third slice showed a patchy aspect in all 32 hearts in which infarct geometry could be analysed. The number of patches of necrosis was 9.7±1.2 by the triphenyl tetrazolium method and 6.9±1.0 by histology. The most frequent number of patches was 4 with

Table 1 Haemodynamic and contractile function data.

	Basal	48 min CO	30 min R	1 h R	4 h R
Heart rate (beats/min)	71.3±2.5	87.4±4.0[a]	88.3±3.7[a]	90.8±3.3[a]	89.3±3.5
Aortic pressure (mm Hg)	82.5±2.9	85.7±2.9	85.8±2.8	87.9±2.8	88.6±3.6
Systolic shortening control segment (%)	20.4±1.1	19.9±1.1	19.1±1.1	19.2±1.2	16.6±1.0
Systolic shortening ischaemic segment (%)	27.3±1.2	1.4±0.4[a]	4.0±0.8[a]	5.4±0.8[a]	3.2±1.0

[a] P<0.05 respect to basal value

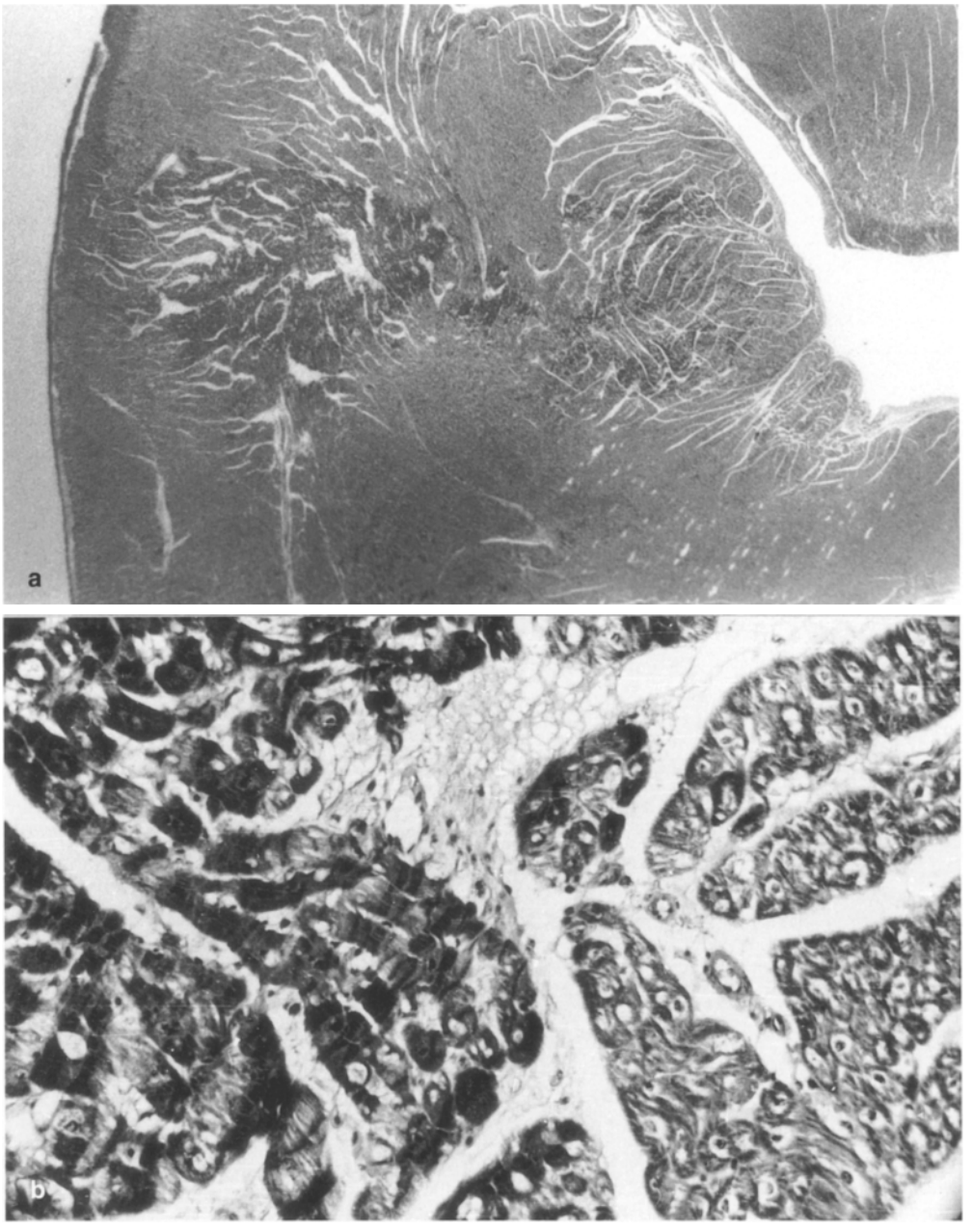

Fig. 2 Histological analysis of the spatial distribution of necrosis. **a)** A zone of myocardial necrosis in an small reperfused infarct. The lateral limit of the necrotic area coincides with the lateral border of the area at risk. Masson's trichrome, 14×. **b)** Closer view of the interface between normal and necrotic myocardium, with contract band formation. Masson's trichrome. 100×

both methods (15.6% and 12.5% of animals respectively).

All but one heart presented areas of necrosis at the lateral borders of the area at risk. In 19 (59.4%) animals the transmural extent was much less in the centre of the area at risk than at its lateral borders. The index of eccentricity averaged 1.15±0.04, and was >1 in 23 hearts (69%), and greater than 1.2 in 10 (31%) hearts. The eccentricity in the spatial distribution of the area of necrosis, with predominant involvement of the lateral limits of the area at risk was more apparent in smaller infarcts, and there was an inverse correlation between the index of eccentricity and infarct size; r=–0.471, P=0.007 (Fig. 3).

Discussion

This study analysed the spatial distribution of myocardial necrosis within the area at risk in a pig model of in situ coronary occlusion and early reperfusion with negligible collateral flow. Myocardial necrosis consisted almost exclusively of areas of contraction band necrosis with irregular contours. In a large proportion of hearts these areas of contraction band necrosis were distributed predominantly along the lateral borders of the area at risk, with only small areas of necrosis in the central zone of the area at risk. This eccentric pattern of post-reperfusion myocardial necrosis was more prominent in smaller infarcts. These observations indicate an increased susceptibility to transient coronary occlusion at the lateral

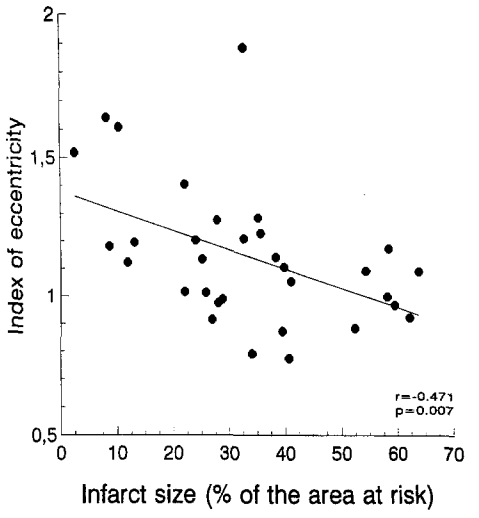

Fig. 3 Correlation between the index of eccentricity and infarct size

borders of the area at risk, the site were mechanical stress is maximal, and are in agreement with an important role of physical stress in the genesis of myocyte death secondary to ischaemia/reperfusion injury.

Severe myocardial ischaemia produces complete cessation of contractile activity within a few beats of its onset [11, 23]. When ischaemia lasts more than a few minutes contractility does not recover immediately after reperfusion, but hours to days later. According to the law of Laplace, left ventricular wall tension during systole is increased at the area at risk when compared with contracting myocardium, due to increased cavity radius and reduced wall thickness in the ischaemic area [2, 12]. Wall tension is particularly high at the lateral borders of the area at risk where cavity radius is maximal. The myocytes at the interface between normal and ischaemic myocardium are exposed to two additional sources of mechanical stress: the swing movement of the dyskinetic segment pivoting on that interface, outward during systole and inward during diastole, and the lateral friction between quiescent ischaemic myocytes and adjacent non-ischaemic, contracting myocytes. The prediction of an increased tension at the interface between the area at risk and normal myocardium is supported by observations from necropsy studies, showing that free wall left ventricular rupture complicating acute transmural infarctions occurs at this site in more than 50% of cases [1, 13, 17].

The transmural progression of myocardial necrosis within the area at risk as a function of occlusion time was first described by Jennings and Reimer in the dog model [18]. These authors described a simultaneous appearance of subendocardial necrosis at the lateral borders and at the core of the area at risk. The exact relationship between the lateral extension of reperfused infarcts and the area at risk has not been investigated in depth due, in part, to the difficulties in identifying the lateral borders of this area accurately in histological sections. The complex geometry and patchy appearance of incomplete, reperfused infarcts are further complicating factors. Some local factors would predict an improved tolerance of the borders of the area at risk to transient coronary occlusion. Catabolite accumulation and acidosis induced by coronary occlusion has been found to be less prominent in this region of the area at risk, probably due to passive diffusion towards or from adjacent normal myocardium [3]. Electrophysiological studies have demonstrated diffusion of potassium ions from reperfused to normal myocardium during reperfusion [2]. The observation that despite reduced catabolite accumulation tolerance to transient coronary occlusion is reduced at the borders of the area at risk further stresses the importance of mechanical factors in the genesis of myocardial necrosis during ischaemia/reperfusion.

The prodominantly subendocardial involvement in reperfused incomplete infarcts has been observed consistently in experimental studies with different animal models, as well as in human necropsy studies [11, 18, 22]. In the presence of collateral circulation, the poorer tolerance to transient coronary occlusion in the inner myocardial layers can be explained by the higher intramural pressure in this region of the left ventricular wall, which results in a preferential distribution of collateral flow towards the epicardium [19]. In the absence of collaterals, the poorer tolerance of subendocardial layers to transient coronary occlusion has been explained by increased energetic demand in this area resulting from the increased tension in this region [21]. However, the complete cessation of contractile activity during the first seconds of zero flow ischaemia makes severely ischaemic myocardium less sensitive to factors increasing ventricular work.

There is increasing evidence that mechanical stress may have a direct, deleterious effect on myocytes during ischaemia and reperfusion, unrelated to increased energetic demand. Experiments involving incubation of cardiac myocytes in hypotonic media have demonstrated that ischaemia reduces the mechanical resistance of the sarcolemma to mechanical stress imposed by swelling [25, 26]. Although the mechanism of this effect is not well-known, some observations suggest that proteolytic damage to the union of sarcolemma with the cellular scaffold may play an important role [7, 26]. More recent studies have shown that excessive contraction and osmotic cell swelling during reperfusion may kill viable myocytes [11, 16, 23]. Transient contractile inhibition during the first minutes after restoration of oxygen supply has been found to prevent hypercontracture of isolated myocytes submitted to anoxia/reoxygenation and to reduce contraction band necrosis and infarct size after in situ transient coronary occlusion [11, 23]. Reperfusion with highly hyperosmotic solutions matching intracellular osmolality, has also resulted in reduced myocardial injury and infarct size [12]. Mechanical stress imposed to the sarcolemma by excessive contraction and swelling is amplified in in situ cardiomyocytes by cell-to-cell physical interaction through tight intercellular junctions [5, 8, 9]. Reperfusion induced hypercontracture results invariably in sarcolemmal disruption and enzyme loss in

in situ cardiac myocytes, but isolated, unrestrained cardiomyocytes may maintain an intact sarcolemma and be metabolically competent after hypercontracture [24]. It has been suggested that physical interaction could contribute to cell-to-cell progression of myocardial necrosis within the area at risk [9]. In this context, the increased susceptibility to transient coronary occlusion at the lateral borders of the area at risk observed in the present study can be explained as the consequence of increased mechanical stress imposed to the sarcolemma of myocytes in this area during ischaemia and reperfusion.

Certain of these phenomena may be explained in other ways. Studies with magnetic resonance imaging indicate a minor but definite increase in myocardial water content at the lateral borders of the ischaemic bed during coronary occlusion [12]. However, the severity of this myocardial oedema was much less than that observed during reperfusion and less than that supposedly required to disrupt the sarcolemma of viable myocytes. Leukocytes have been proven to be able to contribute to cell injury during myocardial reperfusion [14]. Although leukocyte infiltration distributes mainly across the lateral borders of non-reperfused infarcts, the distribution of these cells is homogeneous across the area at risk in reperfused infarcts [20, unpublished observations].

The results in this study could represent an artefact, that is, the detection of contraction band necrosis at the lateral borders of large infarcts could represent coagulation necrosis. Triphenyl tetrazolium staining could be falsely positive in the core of the infarct due to the no reflow phenomenon and slower washout of NADH in this area, while histology could have failed to detect coagulation necrosis at the core of the infarct after 6 h of reperfusion. However, this possibility seems extremely unlikely. First, areas of severe microvascular damage and no reflow are usually absent or minimal after 48 min of coronary occlusion in the pig model [10]. Second, animals with reocclusion, which could have lead to extensive coagulation necrosis, were excluded. Moreover, the triphenyl tetrazolium reaction consistently identifies transmural massive infarcts secondary to re-occlusion in this pig model 6 h after reperfusion.Finally, in this pig model submitted to 48 min coronary occlusion, 30 min of reperfusion followed by re-occlusion suffice to induce extensive areas of contraction band necrosis [10]. This study was performed in an animal model with negligible collateral flow. In this model, infarct size increases abruptly with duration of coronary occlusion, infarcts being virtually absent when reperfusion is performed before 30 min and almost complete when it is performed after 90 min of ischaemia [10]. Collateral circulation is scanty in a large number of patients developing acute myocardial infarction as a first manifestation of coronary artery disease. In the present study infarcts were generally small and consisted almost exclusively of contraction and necrosis. These specific experimental conditions could have favoured the detection of the eccentric pattern of myocardial necrosis. Longer occlusion times result in this model in larger, more compact infarcts, where the involvement at the lateral borders of the area at risk is no longer striking. The presence of significant collateral flow could have however, have a protective effect on the lateral borders of the area at risk which might overcome the negative effects of increased tension in this area.

The present results describe a previously unrecognized pattern of eccentric distribution of myocardial necrosis within the area at risk in hearts with small infarcts following a relatively brief coronary occlusion in the absence of collateral flow. Although these results do not allow us to identify the mechanisms leading to the occurrence of this pattern, the increased susceptibility of an area where passive mechanical stress is maximal during ischaemia and reperfusion is in agreement with an important role of mechanical factors in the genesis of myocardial necrosis during transient coronary occlusion. These factors may became prominent determinants of infarct geometry in the absence of collateral flow.

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