Infarct-related artery occlusion, tissue markers of ischaemia, and increased apoptosis in the peri-infarct viable myocardium

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Aims Unfavourable cardiac remodelling often complicates acute myocardial infarction (AMI) as a result of increased cardiomyocyte apoptosis. It is currently unclear whether ongoing or recurrent ischaemia is an independent determinant for increased apoptosis in peri-infarct viable myocardium.

Methods and results In order to assess the link between infarct-related artery (IRA) occlusion, ischaemia, and apoptosis, 30 subjects dying 7–120 days after AMI (16 with IRA occlusion and 14 with patent IRA) and five control subjects were selected at autopsy. Cardiomyocytes were defined as apoptotic if co-expressing TUNEL and activated caspase-3. Expression of both hypoxia-inducible factor-1 and cyclo-oxygenase-2 was assessed in the peri-infarct myocardium and considered as tissue markers of ischaemia. Evidence of ischaemia was significantly more frequent in cases with IRA occlusion (53%) than in cases with patent IRA (15%) or control hearts (0%, \( P = 0.026 \)). The finding of IRA occlusion and markers of ischaemia identified cases with higher apoptotic rates (ARs) in the peri-infarct viable myocardium \[12.2\% (8.2 – 14.0), P < 0.001 \text{ vs. others}\], whereas IRA occlusion without ischaemia was associated with lower AR, not significantly different from patent IRA \[3.0\% (1.0 – 7.9) \text{ vs. } 2.2\% (1.0 – 5.8), \text{ respectively, } P = 0.42 \].

Conclusion Ischaemia in the peri-infarct viable myocardium is present in over 50% of subjects dying late after AMI with IRA occlusion, and it is associated with increased apoptosis. Relief of ischaemia after AMI may prove of benefit in preventing apoptosis and its consequences.

KEYWORDS
Apoptosis; Ischaemia; Myocardial infarction; Remodelling; Cyclo-oxygenase-2; Hypoxia-inducible factor-1

Introduction
Acute myocardial infarction (AMI) is associated with high, early, and late mortality, but prompt reperfusion of the ischaemic myocardium reduces mortality by favouring myocardial salvage.1 A sizeable proportion of patients, however, do not receive reperfusion or may experience re-occlusion of the infarct-related artery (IRA) shortly after the index event. We have recently reported extremely high apoptotic rates (ARs) in hearts of patients with permanent IRA occlusion, suggesting a role of persistent or recurrent ischaemia in promoting apoptosis.2 Apoptosis represents indeed a modality of cell death different from necrosis being characterized by a highly regulated energy-dependent cascade of events, which leads to primary mitochondrial and nuclear alterations, death, and phagocytosis by surrounding cells without eliciting an acute inflammatory reaction. It may be apparently silent while causing subtle progressive cell loss leading to heart failure and death, and may be responsible for the time-dependent deterioration observed in viable hibernating myocardium after AMI.3,4 The pathophysiology underlying increased apoptosis and adverse remodelling is still unclear. The aim of this study was to mechanistically investigate the potential association between IRA occlusion, ischaemia, apoptosis of the peri-infarct myocardium, and cardiac remodelling.
Methods

Selection of cases

Thirty consecutive subjects were selected at post-mortem examination according to the following inclusion criteria: (1) AMI during the previous year; (2) death occurring at least 4 days after AMI, and (3) no evidence of re-infarction at clinical and pathological assessment. All patients were hospitalized prior to death. Cause of death was trauma in four cases, whereas congestive heart failure and multiple comorbidities were present in the others [respiratory failure (17 cases), gastrointestinal bleeding (seven cases), renal failure (six cases), stroke (three cases), pulmonary embolism (two cases)]. They were divided in two groups: permanent IRA occlusion (16 cases) and patent IRA (14 cases). Five consecutive subjects without a history of cardiac disease, who had died from traumatic non-cardiac causes [hanging (one case), gunshot wounds (one case), extensive trauma (three cases)] undergoing autopsy at our institution were also included in the study as controls.

Clinical and pathological evaluation

Clinical data were obtained from clinical records. Symptomatic heart failure was defined on the basis of clinical findings according to ACC/AHA heart failure guidelines (stages C/D and NYHA class IV). Clinical and demographic characteristics of the subjects are shown in Table 1. Autopsy was performed within 30 h after death. IRA occlusion was defined as absence of residual lumen at pathology due to atheroma and/or thrombosis in the artery supplying the infarcted myocardium as identified at pathology. Infarct size was determined at gross pathology and quantified on a three grade scale by two independent pathologists (R.B. and F.S.) as previously described. Cardiac diameters were calculated at the atrioventricular section and LV free wall thickness was measured at the median third of the unaffected free wall. Tissue specimens were obtained at peri-infarct sites and in unaffected regions of the left ventricle remote from the infarcted area supplied by a patent coronary artery. Peri-infarct area was defined as the zone bordering the infarct only where viable myocardium was prevalent and reparative fibrosis was only marginal. Specimens from unaffected right ventricles were obtained in five cases. Control hearts were sampled in the anterior and inferior LV wall. Specimens were fixed in paraformaldehyde and then processed as previously described. Morphological analysis was performed at light microscopy.

Immunohistochemistry for TUNEL, caspase-3, and actin

In situ end labelling of DNA fragmentation (TUNEL) was performed using the Apoptag kit (Oncor, Gaithersburg, MD, USA), according to the supplier’s instructions. One hundred fields (250/μm²) per section were analysed (12.5 mm²). Several series of TUNEL-stained sections were subsequently stained for different markers. Sections were incubated with antibodies against muscle actin to identify cardiomyocytes (mouse monoclonal anti-human actin HHF35 from DAKO; dilution 1:50), and activated caspase-3 [cleaved caspase-3 (Asp 175) antibody from Cell Signaling Technology, Beverly, MA, USA; dilution 1:50]. Suitable negative and positive controls for

Table 1  Characteristics of the patients in the two groups

<table>
<thead>
<tr>
<th></th>
<th>Occluded IRA</th>
<th>Patent IRA</th>
<th>Controls</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>16</td>
<td>14</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>74 (68–80)</td>
<td>77 (68–88)</td>
<td>59 (40–78)</td>
<td>0.15</td>
</tr>
<tr>
<td>Male sex</td>
<td>12 (75%)</td>
<td>8 (57%)</td>
<td>3 (67%)</td>
<td>0.77</td>
</tr>
<tr>
<td>Recent MI (&lt;30 days)</td>
<td>11 (68%)</td>
<td>9 (64%)</td>
<td>—</td>
<td>0.90</td>
</tr>
<tr>
<td>Time to death post-MI (days)</td>
<td>14 (10–50)</td>
<td>20 (9–60)</td>
<td>—</td>
<td>0.66</td>
</tr>
<tr>
<td>Fibrinolysis</td>
<td>5 (31%)</td>
<td>7 (50%)</td>
<td>—</td>
<td>0.50</td>
</tr>
<tr>
<td>Transmural infarction</td>
<td>15 (94%)</td>
<td>12 (86%)</td>
<td>—</td>
<td>0.90</td>
</tr>
<tr>
<td>Infarct extent</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small (&lt;10%)</td>
<td>2</td>
<td>5</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Moderate (10–20%)</td>
<td>6</td>
<td>8</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Extensive (&gt;30%)</td>
<td>8</td>
<td>1</td>
<td>—</td>
<td>0.032</td>
</tr>
<tr>
<td>Anterior AMI</td>
<td>6 (38%)</td>
<td>7 (50%)</td>
<td>—</td>
<td>0.75</td>
</tr>
<tr>
<td>Previous additional AMI</td>
<td>9 (56%)</td>
<td>4 (29%)</td>
<td>—</td>
<td>0.25</td>
</tr>
<tr>
<td>Pathological characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>510 (470–578)</td>
<td>542 (466–602)</td>
<td>—</td>
<td>0.26</td>
</tr>
<tr>
<td>Transverse diameter (mm)</td>
<td>125 (121–136)</td>
<td>128 (120–135)</td>
<td>—</td>
<td>0.69</td>
</tr>
<tr>
<td>Longitudinal diameter (mm)</td>
<td>104 (100–115)</td>
<td>104 (96–112)</td>
<td>—</td>
<td>0.33</td>
</tr>
<tr>
<td>LV free wall thickness (mm)</td>
<td>13 (11–16)</td>
<td>15 (12–18)</td>
<td>—</td>
<td>0.39</td>
</tr>
<tr>
<td>Diameter-to-wall thickness ratio</td>
<td>9.8 (7.9–11.6)</td>
<td>8.5 (7.2–11.0)</td>
<td>—</td>
<td>0.050</td>
</tr>
<tr>
<td>Right ventricular enlargement</td>
<td>8 (50%)</td>
<td>4 (29%)</td>
<td>—</td>
<td>0.41</td>
</tr>
<tr>
<td>Multivessel CAD</td>
<td>8 (50%)</td>
<td>7 (50%)</td>
<td>—</td>
<td>0.71</td>
</tr>
<tr>
<td>Associated conditions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart failure</td>
<td>10 (62%)</td>
<td>7 (50%)</td>
<td>—</td>
<td>0.75</td>
</tr>
<tr>
<td>Diabetes</td>
<td>4 (25%)</td>
<td>4 (29%)</td>
<td>0 (0%)</td>
<td>0.41</td>
</tr>
<tr>
<td>Trauma</td>
<td>1 (6%)</td>
<td>3 (21%)</td>
<td>3 (67%)</td>
<td>0.032*</td>
</tr>
<tr>
<td>Respiratory failure (including sepsis)</td>
<td>12 (75%)</td>
<td>9 (64%)</td>
<td>3 (67%)</td>
<td>0.74</td>
</tr>
<tr>
<td>Gastrointestinal bleed</td>
<td>3 (19%)</td>
<td>4 (29%)</td>
<td>0 (0%)</td>
<td>0.38</td>
</tr>
<tr>
<td>Renal failure</td>
<td>4 (25%)</td>
<td>2 (14%)</td>
<td>1 (20%)</td>
<td>0.32</td>
</tr>
<tr>
<td>Stroke</td>
<td>2 (12%)</td>
<td>1 (7%)</td>
<td>0 (0%)</td>
<td>0.66</td>
</tr>
<tr>
<td>Pulmonary embolism</td>
<td>0</td>
<td>2 (14%)</td>
<td>0 (0%)</td>
<td>0.70</td>
</tr>
</tbody>
</table>

*Quantitative results are expressed as median and interquartile range.

*P = 0.032 among the three groups, P = 0.50 for occluded vs. patent IRA groups.
TUNEL and activated caspase-3 were performed, as defined elsewhere. Briefly, controls for TUNEL were performed as indicated by the supplier (using a female rodent mammary gland after weaning of rat pups for positive control, and sham stainings leaving out active TdT but including proteinase K digestion to control for non-specific incorporation of nucleotides or non-specific binding of enzyme conjugate). A human lymph node was used as a control for activated caspase-3. The ‘stringent’ TUNEL assay (leaving out proteinase K digestion) was also performed in a subset of 20 cases, showing a very high correlation with our standard approach ($R = +0.90$, $P < 0.001$). Finally, negative controls indicating the non-interference of TUNEL and secondary antibodies were performed. Co-localizations for TUNEL/actin and for TUNEL/caspase-3 were performed. Cardiomyocytes were defined as apoptotic if co-localization of TUNEL/caspase-3 was evident. TUNEL-negative/caspase-3-positive cells were not considered to be apoptotic as caspase-3 activation may represent a reversible step.

Correction for potential false-positive results (PCNA and SC-35)
Cardiomyocytes co-expressing TUNEL-pozitivity and markers of DNA synthesis (PCNA) and/or of transcription activity (RNA splicing factor SC-35) were not included in the cell count, as they were considered potential false-positive results. Expression was assessed as previously described. Nevertheless, as PCNA may also represent potentially replicating cells, we measured the number of PCNA-positive cardiomyocytes in cases with and without IRA occlusion.

Apoptotic rate
The AR was expressed as the ratio of cardiomyocytes co-expressing TUNEL/caspase-3 on total number of cardiomyocytes per field, calculated on 100 random fields per case. In order to avoid potential confounders deriving from the selection of fields with abnormal cardiomyocyte density, fields for the AR count were selected in the zone bordering the infarct only where viable myocardium was prevalent and reparative fibrosis only marginal, considering only those fields (40×) were more than 30 cardiomyocytes were present. Actin-negative cells were not included in cell count. Immunohistochemistry assays and AR counts were performed by two blinded pathologists (A.B. and F.B.).

Markers of ischaemia
As markers of ischaemia, we assessed nuclear hypoxia-inducible factor 1-alpha (HIF-1alpha) expression and cyclo-oxygenase-2 (COX-2) cytoplasmic expression. HIF-1alpha is an instantaneous and transient marker of ischaemia. Its expression was evaluated using mouse antihuman HIF-1alpha antibody (IgG2a, Novus Biological, Littleton, CO, USA) at 1:100 dilution, according to the supplier’s directions. Co-staining for both HIF-1 and actin was also performed. COX-2 is induced in cardiomyocytes in response to ischaemia and appears as a cytoplasmic protein 6–12 h after hypoxia induction, thus representing a subacute marker of ischaemia. Specific staining for COX-2 was assessed using a primary antibody (goat polyclonal sc-1745, Santa Cruz Biotechnology, CA, USA) at the dilution of 1:100. Negative controls for HIF-1 and COX-2 leaving out the primary antibodies were performed. Results for each of these markers, were described by two independent, blinded pathologists (S.S. and F.V.) on a dichotomous (positive/negative) basis, and subsequently positive results were graded in mild (þ), intense (þþ) and very intense (þþþ) staining according to the intensity and extension of the staining, therefore a three-step scale of expression was developed. However, in order to enhance specificity for ischaemia, samples were considered ‘positive for ischaemia’ only if they expressed both HIF-1 and COX-2 in the peri-infarct viable myocardium. COX-2 expression in the tissue was confirmed with reverse transcription-PCR (RT-PCR). Fresh tissue specimens were obtained at peri-infarct sites in five patients with persistent IRA occlusion, five with patent IRA, and five control hearts. Total cellular RNAs were prepared from each of these specimens using a Trizol (Life Technologies, Inc.) extraction technique. COX-2 transcript levels were measured using a RT-PCR assay; β-actin transcript served as internal control. cDNAs were synthesized using 1 μg of total RNA from each sample. PCR amplification was then performed. The primer sequences and PCR product sizes were as follows: (a) COX-2 sense (5’-CAGGACTCTACCCATGTGTGAG-3’) and COX-2 antisense (5’-CTTGCTGATGGAAAGCCTGCT-3’) 756 kb; (b) β-actin sense (5’-TGTGCTGATGGAAAGCCTGCT-3’) and β-actin antisense (5’-CTGTGTTGGAAGGCTGAT-3’), 436 kb. PCR conditions consisted of 35 cycles of 94°C for 1 min, 59°C for 1 min, and 72°C for 1.5 min for a 25 μL reaction mixture. Amplified cDNAs were electrophoresed on 1% agarose gels and visualized by ethidium bromide staining. All experiments were performed in duplicate. A qualitative analysis was performed.

Statistical analysis
Given the limitation to enrolment typical of any autopic studies, and assuming a 60% prevalence of IRA occlusion, a 50% rate of ischaemia, a mean difference of 5% (SD of 5%) in AR, and aiming at alpha value 0.01 and power of 90%, a sample size of 30 was chosen. Statistical analysis was performed with SPSS 10.1 for Windows (SPSS, Chicago, IL, USA). Quantitative results are expressed as median (interquartile range), because of potential deviations from assumptions of normality. The uncorrected $\chi^2$ test or the Fisher exact test were used for categorical variables, when appropriate. Continuous variables were analysed with Mann–Whitney $U$, Wilcoxon rank-sum, Kruskal–Wallis, or Spearman correlation tests. Two-tailed statistical significance was at the 0.05 level. Bonferroni’s correction was used to control Type I error when comparison between two of the four groups were made. Multivariable analysis was performed by means of linear regression. Three models for multivariable analysis were used. Model 1 included only the variables associated with increased AR with significant or near-significant P-values ($P < 0.1$) at univariate analysis. Model 2 included the former variables plus all those variables significantly associated with IRA occlusion. A third model was obtained by forcing into the multivariable analysis model all the 19 available variables listed in Table 1. Finally, agreement between dichotomous variables (i.e. COX-2 and HIF tissue expression) was tested by means of Cohen kappa (with 95% confidence intervals).

Results
Demographic, clinical, and pathological characteristics
No differences in demographic characteristics were found comparing subjects with vs. those without permanent IRA occlusion (Table 1). In particular, causes of death were similarly distributed in the two groups. Subjects dying after AMI with permanent IRA occlusion had significantly greater infarct extent than cases with patent IRA ($P = 0.032$) and also showed a less favourable cardiac remodelling than those with open IRA (i.e. transverse diameter-to-free wall thickness ($P = 0.050$, Table 1).

Cardiomyocyte apoptosis
Apoptosis was significantly higher in peri-infarct and remote regions in cases with AMI than in control hearts (Table 2). Figure 1 shows examples of gross and microscopy examination of the hearts.
IRA occlusion was associated with increased AR in cardio-
myocytes in the peri-infarct regions [8.5% (5.5–13.0) vs. 2.0% (1.0–6.0), \( P < 0.001 \)]. Statistical analysis was performed to identify additional clinical and pathological vari-
ables associated with increased apoptosis. Heart failure and male gender were associated with significantly higher AR (\( P = 0.006 \) and 0.050, respectively). Transverse diameter-
to-left ventricle free wall thickness ratio and left ventricle free wall thickness were significantly correlated with AR (\( R = 0.35 \) and \( P = 0.019 \)), with higher AR being present in enlarged hearts with thinner walls (adverse remodelling). When creating a model for multivariable analysis using these five variables (Model 1), the statistical significance for IRA occlu-
sion was maintained (\( B = 74, SE = 29, P = 0.002 \)). Moreover, a significant association between IRA occlusion and increased AR was validated also using an additional multi-
variable analysis model which included the former five vari-
ables plus infarct extent (Model 2) (\( B = 88, SE = 28, P = 0.004 \)), and in order to exclude potential biases due to differences in infarct size, we compared apoptosis in occluded vs. open IRA in the subgroup of cases with moder-
ate size infarcts (14 cases), showing significantly greater apoptosis in this subgroup (\( P = 0.005 \)). Even when forcing into the multivariable analysis model all the 19 available variables listed in Table 1 (Model 3), IRA occlusion was still the most significant independent predictor of increased cardiomyocyte apoptosis (\( B = 134, SE = 45, P = 0.014 \)). Notably, the number of PCNA-positive cardiomyocytes in peri-infarct regions was similar in cases with and without IRA occlusion [0.6% (0.05–0.9) vs. 0.5% (0.01–1.1), respectively, \( P = 0.91 \)]. Moreover, no differences were encountered comparing SC-35 positive cells in the two groups.

IRA status, apoptosis, and ischaemia

Evidence of ischaemia, as documented by combined HIF-1 and COX-2 expression, was significantly more frequent in cases with permanent IRA occlusion (53%) than in cases with patent IRA (15%) or in patients without cardiac disease (control hearts, 0%, \( P = 0.026 \)). The height of the boxes indicate the prevalence of ischaemia in terms of percentage of cases.

IRA status and apoptosis

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Evidence of ischaemia, as documented by combined HIF-1 and COX-2 expression, was significantly more frequent in cases with permanent IRA occlusion (53%) than in cases with patent IRA (15%) or in control hearts (0%) (\( P = 0.026 \)). HIF-1 and COX-2 expression in cardiomyocytes was concordant in 73% of cases [kappa 0.47 (95%CI 0.15–0.78)]. The combined presence of IRA occlusion and tissue markers of ischaemia identified cases with extremely high AR [12.2% (8.2–14.0), \( P < 0.001 \) vs. others], whereas
IRA occlusion without ischaemia was associated with lower AR, indeed not significantly different from cases with patent IRA [3.0% (1.0–7.9) vs. 2.2% (1.0–5.8), respectively, $P = 0.42$] (Figure 3). When assessing the multivariable analysis the independent values of IRA occlusion and evidence of ischaemia, both were predictors of increased apoptosis ($P = 0.010$ and $P = 0.040$, respectively). Similar results were obtained even when considering only the intense staining for HIF-1 and COX-2 as positive for ischaemia (in order to avoid the potential confounding factor of mild positive results). The AR, the prevalence of ischaemia, and the PCNA rate were independent of time from AMI to death (data not shown).

Individual markers of ischaemia

The assessment of different markers of ischaemia provided additional data. The isolated expression of HIF-1 (and not of COX-2) (early ischaemia) was not associated with increased ARs when compared with cases without expression of both markers (no ischaemia) [1.9% (0.5–7.8) vs. 1.8 (0.6–6.9), respectively, $P = 0.82$]. Conversely, the presence of COX-2, a subacute marker of ischaemia with or without concomitant expression of HIF-1, was associated with significantly higher AR, while being similar between the two groups [8.9% (6.0–13.0) and 8.5% (5.0–13.1), respectively, $P = 0.72$], therefore showing a prevalent role of subacute markers of ischaemia as the most important predictor of increased apoptosis. Intense COX-2 expression was found in 75% of COX-2 positive cases, and it was predictive of significantly higher AR [12.3% (9.0–14.1) vs. 6.1% (5.3–8.9) for mild COX-2 expression and vs. 1.8% (0.6–7.2) for COX-2 negative cases, $P < 0.001$]. COX-2 mRNA expression in the peri-infarct myocardium of patients with permanent IRA occlusion was confirmed using RT-PCR (Figure 1). Notably, neither HIF-1alpha nor COX-2 expression was correlated with the intensity of the inflammatory infiltrate assessed as the number of cells expressing the DR receptor and in particular the number of T lymphocytes expressing the DR (data not shown).

Discussion

The present study demonstrates that persistent IRA occlusion is associated with evidence of ischaemia and increased apoptosis independently of all major clinical and pathological variables. It also demonstrates that the association of IRA occlusion and tissue markers of ischaemia is the strongest predictor of increased cardiomyocyte apoptosis, even late after the index event. Ischaemia induces apoptosis both in vitro and in vivo. We previously reported increased apoptosis associated with permanent IRA occlusion in a smaller number of patients. The current study confirms and expands this association in a larger series of cases and controls, and provides evidence for a pathophysiological link between IRA occlusion, ischaemia (as shown by tissue markers), and apoptosis.

Clinical relevance of persistent IRA occlusion

Despite relevant advances in treatment for AMI in the last decade, a large amount of patients continue to have persistent IRA occlusion after AMI. Approximately 50% of patients after thrombolysis and 10% after primary PTCA have total coronary occlusion or suboptimal coronary flow after treatment. In addition, the presence of ischaemia identifies a group of patients with unfavourable prognosis following AMI. Several observational studies suggest that IRA patency after AMI may provide benefits in terms of mortality, which extend beyond acute myocardial salvage. In the present study, more than 50% of cases had IRA occlusion at the time of death. As expected, they showed larger infarcts than patients with patent IRA. Moreover, importantly, IRA occlusion was associated with significantly higher apoptosis in cardiomyocytes in the surviving peri-infarct regions, and with more unfavourable remodelling, as expressed by a greater diameter-to-wall thickness ratio. Notably, in experimental studies, apoptosis determines cardiomyocyte loss leading to heart failure and its inhibition prevents unfavourable remodelling.

Peri-infarct ischaemia and apoptosis

IRA occlusion is not always associated with ischaemia of the peri-infarct regions. In recent studies, 33–83% of patients with total IRA occlusion after AMI had evidence of ischaemia at stress echocardiography. In the present study, we found histopathological evidence of ischaemia in about half of cases with IRA occlusion using co-expression of two tissue markers of ischaemia. HIF-1 and COX-2 are indeed not expressed in healthy myocardium. HIF-1 nuclear translocation occurs rapidly after oxygen deprivation and mediates hypoxic cell death occurring ~12 h after HIF-1 nuclear translocation. COX-2 mRNA expression occurs 2–4 h after induction of hypoxia in experimental models, whereas COX-2 protein expression in cardiomyocytes peaks at 24 h in cardiomyocytes. It is expressed in recent AMI and in ischaemic cardiomyopathy. Ischaemic-dysfunctional myocardium after AMI is usually referred as hibernating. Hibernation identifies a condition of down-regulated myocardium metabolism and function, probably due to repetitive ischaemia, that may be reversed by coronary revascularization. Our findings and the result of previous experimental studies support the concept that in the presence of an occluded IRA, apoptosis may prevail as the propensity to develop reversible ischaemia increases. In this setting, the earlier the myocardium is revascularized, the higher is the number of cardiomyocytes which can be
rescued from apoptosis.\textsuperscript{19–21,32} The finding of an extremely high AR in the peri-infarct area may explain why hibernating myocardium exhibits time-dependent deterioration and the viable myocardium is substituted by a non-viable scar.\textsuperscript{4}

Apoptosis and regenerating cardiomyocytes

Although the concept of cardiomyocyte loss due to apoptosis early and late after AMI is largely accepted, the role of regenerating cardiomyocytes is still questioned. Beltrami \textit{et al.}\textsuperscript{33} have shown the presence of regenerating cardiomyocytes after AMI (mitotic index of 0.08\%). In our study, the number of potentially regenerating cells was much smaller than the number of apoptotic myocytes. In addition, we did not observe any mitotic features in the examined hearts despite the presence of myocytes expressing PCNA, a marker of DNA synthesis. Of note, the percentage of PCNA positive cells was similar in cases with and without IRA occlusion. Therefore, the impact of regenerating myocytes is unlikely to affect the findings of different myocyte loss in cases with and without IRA occlusion.

Limitations

Drawbacks of the current study derive from its retrospective design and post-mortem analyses. Selection biases have at least in part influenced our results, because of the inclusion of cases with extremely unfavourable clinical course in the study. Peri-mortem phenomena altering the tissue response to ischaemia and enhancing AR in apoptotic-prone regions bordering the infarct cannot thus be excluded.\textsuperscript{34,35} Moreover, the amount of apoptosis in our study and in others\textsuperscript{36–39} might be overestimated by influence of several factors (such as selection bias, sampling in a small area of myocardium at risk, altered myocyte density in the peri-infarct region).\textsuperscript{3}

Conclusions

Persistent IRA occlusion is associated with higher prevalence of ischaemia and with increased apoptosis independently of all major clinical and pathological variables. Moreover, the association of IRA occlusion and tissue markers of ischaemia is the strongest predictor of increased cardiomyocyte apoptosis in humans, suggesting a pathophysiological link between IRA occlusion, ischaemia, and apoptosis. The comparison of AR in cases with and without IRA occlusion and peri-infarct ischaemia in humans suggests that relief of ischaemia after AMI through medical or interventional treatments may ultimately prove of benefit in preventing apoptosis and adverse post-infarction remodelling, thus reducing the overall burden of ischaemic cardiomyopathy.\textsuperscript{30}

These findings claim for clinical studies aimed at evaluating short- and long-term effects of revascularization in the subset of patients who exhibit myocardial viability and spontaneous or inducible ischaemia after AMI. If proven to be true, the benefits due to the several-fold decrease in myocardocyte loss by apoptosis may be comparable with the supposed effects of myocardial regeneration therapy. Additional and specifically designed experimental and clinical studies are needed in order to prove or disprove such hypotheses.

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